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CAMPUS MINISTRO REIS VELOSO

CENTRO DE CIÊNCIAS DA SAÚDE

CURSO DE MEDICINA

MARIA EDUARDA MAURIZ RODRIGUES

AVALIAÇÃO DA ATIVIDADE ANTITUMORAL E DA ATIVIDADE PROTETORA DO CLORIDRATO DE EPIISOPILOTURINA NOS EFEITOS COLATERAIS DO 5-FLUOROURACIL EM UM MODELO DE CARCINOMA COLORRETAL MURINO (CT26.WT)

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Trabalho de Conclusão de Curso apresentado ao curso de Medicina da Universidade Federal do Delta do Parnaíba - UFDPar, como pré-requisito para obtenção do título de Bacharel em Medicina.

Orientador: Prof. Dr. José Delano Barreto Marinho Filho

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ATA DE DEFESA DO TRABALHO DE CONCLUSÃO DE CURSO

Ao oitavo dia do mês de fevereiro de 2024, às 08 horas, em sessão pública na sala PPG BIOTEC, na presença da Banca Examinadora presidida pelo Prof. Dr. José Delano Barreto Marinho Filho e composta pelos examinadores: (1) Profa. Dra. Leiz Maria Costa Véras e (2) Profa. Dra. Nayze Lucena Sangreman Aldeman. A aluna Maria Eduarda Mauriz Rodrigues apresentou o Trabalho de Conclusão de Curso em Bacharelado em Medicina da UFDPar intitulado "Avaliação da atividade antitumoral e da atividade protetora do Cloridrato de Epiisopiloturina nos efeitos colaterais do 5-fluorouracil em um modelo de carcinoma colorretal murino (CT26.WT)" como requisito curricular indispensável à integralização do curso. A Banca Examinadora após reunião em sessão reservada deliberou e decidiu pela Aprovação do referido Trabalho de Conclusão de Curso, divulgando o resultado formalmente a aluna e aos demais presentes, e eu na qualidade de presidente da Banca lavrei a presente ata que será assinada por mim, pelos demais componentes da Banca Examinadora e pela aluna orientada.

Orientador
Presidente da Banca

Leiz Maria Costa Véras
Examinador 1

Nayze Lucena Sangreman Aldeman
Examinador 2

Maria Eduarda Mauriz Rodrigues
Orientanda

Dedico este trabalho aos meus pais, ao meu irmão e à minha sobrinha que logo conhecerá o mundo.

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar, mas o mar seria menor se lhe faltasse uma gota”.

Madre Teresa de Calcutá

RESUMO

O câncer constitui-se como uma das principais causas de morbidade e mortalidade no mundo. Dentre as terapêuticas disponíveis, os quimioterápicos apresentam baixa especificidade provocando lesão em tecidos hígidos e acarretando efeitos colaterais indesejáveis. A fim de lidar com essa problemática, novos esquemas terapêuticos são fundamentais. Nesse sentido, o Cloridrato de Epiisopiloturina na sua forma salinizada (EPI-HCL), um alcaloide extraído da *Pilocarpus microphyllus*, foi avaliado quanto ao seu potencial antitumoral e redutor da toxicidade colônica provocada pelo 5-Fluorouracil (5-FU) *in vitro* e *in vivo*. Para avaliar interferência no efeito quimioterápico *in vivo*, camundongos BALB/c, fêmeas, inoculados com células de linhagem CT26.WT, foram tratados por via intraperitoneal durante 10 dias com 5-FU 20mg/kg em dias alternados, EPI-HCL nas concentrações de 25 e 50mg/kg/dia e com a associação de 5-FU 20mg/kg/2dia + EPI-HCL 50mg/kg/dia. O EPI-HCL não apresentou citotoxicidade, nem sinergismo com o 5-FU contras as linhagens de câncer colorretal CT26.WT e HCT-116 no ensaio de MTT. No estudo *in vivo,* o alcaloide não provocou inibição tumoral isolado e não influenciou o percentual de inibição do quimioterápico. A análise macroscópica dos órgãos revelou que o tratamento associado foi capaz de impedir a redução do peso do fígado e do baço induzida pelo 5-FU, além do aumento do peso do cólon e da relação peso/comprimento colônico que sugere a presença de edema. Os animais do grupo 5-FU apresentaram aumento da profundidade das criptas intestinais no cólon, que foi evitada com a administração conjunta do EPI-HCL. Além disso, camundongos BALB/c, saudáveis, sem indução tumoral, tratados com EPI-HCL 50mg/kg/dia, não evidenciaram sinais de toxicidade. Observou-se apenas uma redução do peso do fígado comparado com o grupo saudável salina. Esses resultados destacam o potencial do EPI-HCL como parte do esquema terapêutico com 5-FU, visto que não apresentou toxicidade e reduziu efeitos colaterais sem interferir na atividade antitumoral do quimioterápico.

Palavras-chave: Cloridrato de Epiisopiloturina. Antitumoral. 5-Fluorouracil. Efeitos Colaterais. Cólon.

ABSTRACT

Cancer is one of the main causes of morbidity and mortality in the world. Among the available therapies, chemotherapy drugs have low specificity, causing damage to healthy tissues and causing undesirable side effects. In order to deal with this problem, new therapeutic regimens are essential. In this sense, Epiisopiloturine Hydrochloride in its saline form (EPI-HCL), an alkaloid extracted from Pilocarpus microphyllus, was evaluated for its antitumor potential and reduction of colonic toxicity caused by 5-Fluorouracil (5-FU) in vitro and in alive. To evaluate interference with the chemotherapeutic effect in vivo, female BALB/c mice, inoculated with CT26.WT lineage cells, were treated intraperitoneally for 10 days with 5-FU 20mg/kg every other day, EPI-HCL at concentrations of 25 and 50mg/kg/day and with the combination of 5-FU 20mg/kg/2day + EPI-HCL 50mg/kg/day. EPI-HCL did not show cytotoxicity or synergism with 5-FU against colorectal cancer lines CT26.WT and HCT-116 in the MTT assay. In the in vivo study, the alkaloid did not cause isolated tumor inhibition and did not influence the percentage of inhibition of the chemotherapy agent. Macroscopic analysis of the organs revealed that the associated treatment was able to prevent the reduction in liver and spleen weight induced by 5-FU, in addition to the increase in colon weight and the colonic weight/length ratio, which suggests the presence of edema. Animals in the 5-FU group showed an increase in the depth of intestinal crypts in the colon, which was avoided with the joint administration of EPI-HCL. Furthermore, healthy BALB/c mice, without tumor induction, treated with EPI-HCL 50mg/kg/day, did not show signs of toxicity. Only a reduction in liver weight was observed compared to the healthy saline group. These results highlight the potential of EPI-HCL as part of the therapeutic regimen with 5-FU, as it did not present toxicity and reduced side effects without interfering with the antitumor activity of the chemotherapy drug.

Keywords: Epiisopiloturin hydrochloride. Antitumor. 5-Fluorouracil. Side effects. Colon.

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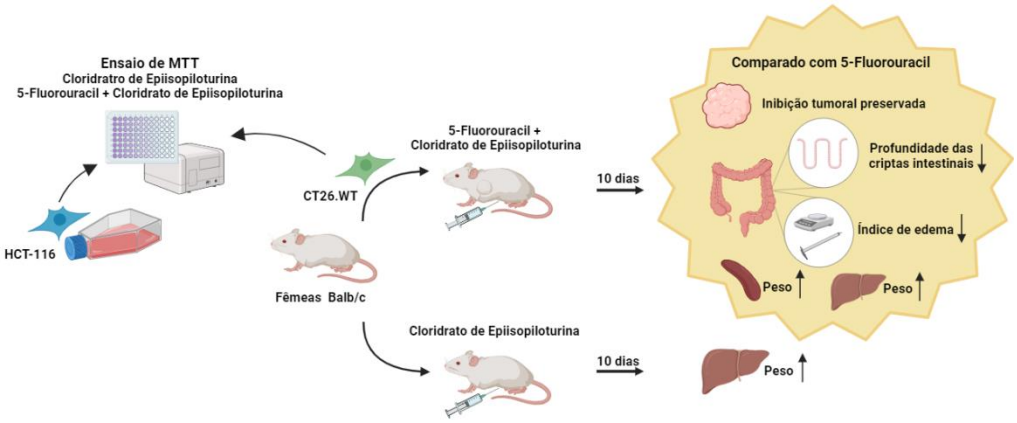

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AVALIAÇÃO DA ATIVIDADE ANTITUMORAL E DA ATIVIDADE PROTETORA DO CLORIDRATO DE EPIISOPILOTURINA NOS EFEITOS COLATERAIS DO 5-FLUOROURACIL EM UM MODELO DE CARCINOMA COLORRETAL MURINO (CT26.WT)

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Resumo

O câncer representa uma das principais causas de mortalidade no mundo. Dentre as terapêuticas disponíveis, os quimioterápicos apresentam baixa especificidade provocando efeitos colaterais indesejáveis. A fim de lidar com essa problemática, novos tratamentos são fundamentais. Nesse sentido, o Cloridrato de Epiisopiloturina (EPI-HCL), um alcaloide extraído da *Pilocarpus microphyllus*, foi avaliado quanto ao seu potencial antitumoral e redutor da toxicidade colônica provocada pelo 5-Fluorouracil (5-FU) *in vitro* e *in vivo*. O EPI-HCL não apresentou citotoxicidade, nem sinergismo com o 5-FU contras as linhagens de câncer colorretal CT26.WT e HCT-116 no ensaio de MTT. Para avaliar interferência no efeito quimioterápico *in vivo*, camundongos BALB/c, fêmeas, inoculados com células de linhagem CT26.WT, foram tratados por via intraperitoneal durante 10 dias com 5-FU 20mg/kg em dias alternados, EPI-HCL nas concentrações de 25 e 50mg/kg/dia e com a associação de 5-FU 20mg/kg/2dias + EPI-HCL 50mg/kg/dia. O alcaloide não provocou inibição tumoral e não influenciou o percentual de inibição do quimioterápico. A macroscopia dos órgãos revelou que o tratamento associado impediu a redução hepática e esplênica induzida pelo 5-FU, além do aumento do peso do cólon e do índice sugestivo de edema. Os animais do grupo 5-FU

apresentaram aumento da profundidade das criptas colônicas, que foi evitado com a associação do EPI-HCL. Além disso, camundongos BALB/c, sem indução tumoral, tratados com EPI-HCL 50mg/kg/dia, não evidenciaram sinais de toxicidade. Observou-se apenas uma redução hepática comparado com o grupo saudável salina. Esses resultados destacam o potencial do EPI-HCL como parte do esquema terapêutico com 5-FU, visto que não apresentou toxicidade e reduziu efeitos colaterais sem interferir na atividade antitumoral do quimioterápico.

Palavras-chave: Cloridrato de Epiisopiloturina; Antitumoral; 5-Fluorouracil; Efeitos Colaterais; Cólon

Abstract

Cancer represents one of the main causes of mortality in the world. Among the available therapies, chemotherapy drugs have low specificity, causing undesirable side effects. In order to deal with this problem, new treatments are essential. In this sense, Epiisopiloturine Hydrochloride (EPI-HCL), an alkaloid extracted from Pilocarpus microphyllus, was evaluated for its antitumor potential and reduction of colonic toxicity caused by 5-Fluorouracil (5-FU) in vitro and in vivo. EPI-HCL did not show cytotoxicity or synergism with 5-FU against colorectal cancer lines CT26.WT and HCT-116 in the MTT assay. To evaluate interference with the in vivo chemotherapeutic effect, female BALB/c mice, inoculated with CT26.WT lineage cells, were treated intraperitoneally for 10 days with 5-FU 20 mg/kg every other day, EPI-HCL at concentrations of 25 and 50mg/kg/day and with the combination of 5-FU 20mg/kg/2days + EPI-HCL 50mg/kg/day. The alkaloid did not cause tumor inhibition and did not influence the percentage of inhibition of the chemotherapy agent. Macroscopy of the organs revealed that the associated treatment prevented the hepatic and splenic reduction induced by 5-FU, in addition to the increase in colon weight and index suggestive of edema. The animals in the 5-FU group showed an increase in the depth of the colonic crypts, which was avoided with the association of EPI-HCL. Furthermore, BALB/c mice, without tumor induction, treated with EPI-HCL 50mg/kg/day, did not show signs of toxicity. Only a reduced liver function was observed compared to the healthy saline group. These results highlight the potential of EPI-HCL as part of the therapeutic regimen with 5-FU, as it did not present toxicity and reduced side effects without interfering with the antitumor activity of the chemotherapy drug.

Keywords: Epiisopiloturin hydrochloride; Antitumor; 5-Fluorouracil; Side effects; Colon

Declarações

Os autores desse trabalho declaram não possuir conflitos de interesses que comprometam o estudo e agradecem às seguintes instituições pelo seu valioso apoio: Instituto Nacional de Ciência e Tecnologia – INCT BioNat, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Fundação de Amparo à Pesquisa do Estado do Piauí (FAPEPI).

1.1 Introdução

O câncer colorretal (CCR) é o terceiro câncer mais incidente no mundo, excluindo câncer de pele não melanoma e o segundo em termos de mortalidade, sendo responsável por quase 1 milhão de óbitos no ano de 2020 (WHO 2023). O tratamento do CCR baseia-se no estadiamento pelo sistema TNM e no status basal do paciente (Buccafusca et al. 2019). Em torno de 10% dos casos são diagnosticados em fase localmente avançada sem metástases à distância, comprometendo útero e anexos, bexiga e intestino delgado (Horta et al. 2009). Nesse estádio, o tratamento primário é a ressecção tumoral que pode ou não ser associada à quimioterapia adjuvante conforme o plano terapêutico (Buccafusca et al. 2019). Além disso, pacientes com CCR metastático irressecável se beneficiam da quimioterapia paliativa com melhora significativa das taxas de sobrevida e do bem-estar (Biller, Schrag 2021).

Dentre os esquemas quimioterápicos tradicionais, destacam-se as associações com o 5-Fluorouracil (5-FU). De acordo com Morris et al (2023), ele compõe os principais esquemas antineoplásicos: FOLFOX (5-FU, ácido folínico e oxaliplatina), FOLFIRI (5-FU, ácido folínico e irinotecano) e o FOLFOXIRI (5-FU, ácido folínico, oxaliplatina e irinotecano). Contudo, em contrapartida a oferecer um efeito sistêmico contra as células tumorais, a quimioterapia causa toxicidade inespecífica ao inibir concomitantemente células hígidas de proliferação rápida (Smith, Lavery, Turkington 2020). Células das criptas do epitélio intestinal pela sua alta capacidade proliferativa são mais suscetíveis ao dano, provocando entre seus efeitos colaterais o quadro de mucosite intestinal (Sougiannis et al. 2021). Outros efeitos incluem anemia, leucopenia e trombocitopenia (Smith, Lavery, Turkington 2020).

A mucosite intestinal pode ser definida como uma inflamação das mucosas que revestem o trato digestivo e que leva a alterações estruturais, funcionais e imunológicas. A sua sintomatologia abrange quadros de diarreia, vômito, náusea, dor abdominal e desidratação em níveis proporcionais a dose quimioterápica administrada, trazendo um grande impacto na qualidade de vida e na resposta ao tratamento do paciente (Kadoyama et al. 2012; Sougiannis et al. 2021). Nesse sentido, buscam-se novas estratégias terapêuticas com menos efeitos colaterais ou novos esquemas capazes de reduzi-los. Dentre os novos compostos, os alcaloides demonstram um grande potencial pela sua atividade anti-inflamatória, antineoplásica e com baixa toxicidade (Olofinsan, Abrahamse, George 2023; Kalmarzi et al. 2019).

Pilocarpus microphyllus é uma planta nativa do norte e nordeste do Brasil, conhecida como Jaborandi e explorada comercialmente para a produção da pilocarpina utilizada no tratamento de glaucoma, Fig. 1 (Pinheiro 2002). O Cloridrato de Epiisopiloturina (EPI-HCL) é uma forma salinizada do alcaloide imidazólico mais abundante encontrado na biomassa descartada durante a extração da pilocarpina a partir das folhas do Jaborandi, caracterizado pela presença de um anel imidazólico ligado a um ciclopentano heterocíclico (lactona) através de uma ligação C-C, Fig. 1 (Mafud et al. 2017). Não existem dados na literatura sobre a atividade antineoplásica do EPI-HCL. No entanto, ele demonstrou atividade gastroprotetora contra o dano induzido por naproxeno na mucosa gástrica de camundongos através da redução de citocinas pró-inflamatórias, do estresse oxidativo e aumento do fluxo sanguíneo local (Nicolau et al. 2017). Além disso, Barbosa et al. (2022) evidenciou redução da mucosite no intestino delgado induzida por 5-FU com a administração de Epiisopiloturina, sugerindo um papel de inibição da ciclooxigenase-2 (COX-2).

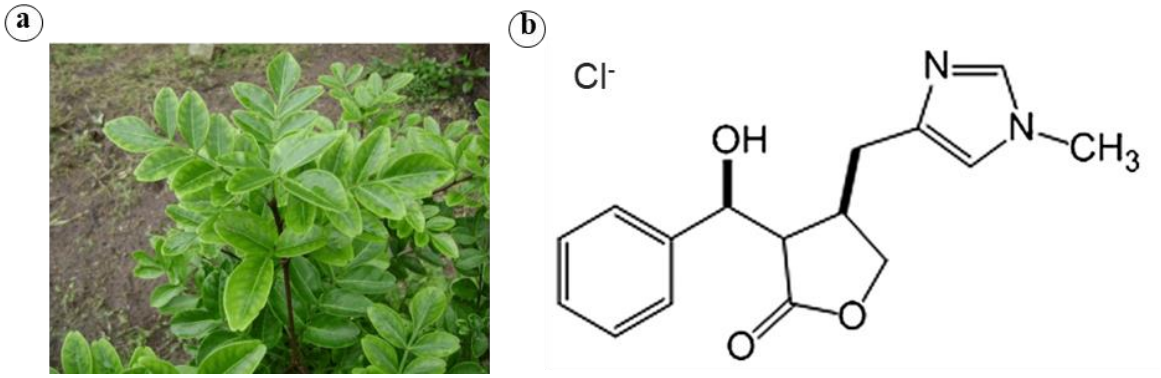

Fig. 1 Cloridrato de Epiisopiloturina. **a.** Folhas do Jaborandi. Foto: David Fernandes Lima. **b.** Estrutura molecular do EPI-HCL

Portanto, esse trabalho teve como objetivo avaliar o potencial antitumoral *in vitro* e *in vivo* do EPI-HCL, bem como seu efeito na toxicidade sistêmica e colônica decorrente do tratamento quimioterápico com 5-FU em um modelo tumoral.

1.2 Material e métodos

1.2.1 Químicos e reagentes

Os meios DMEN - High glucose e RPMI-1640 foram obtidos da indústria Sigma-Aldrich (St. Louis, MO, EUA). O antibiótico penicilina/estreptomicina foi obtido da Capricorn Scientific GmbH (Ebsdorfergrund, Alemanha) e o soro bovino fetal (SBF) proveniente da Cultilab Materais Para Cultura de Células LTDA (Campinas, SP, Brasil). A solução de MTT (brometo de 3-[4,5-dimetiltiazol-2-il]-2,5-difenil tetrazólio) e o DMSO (dimetilsufóxido) foram produzidos na Êxodo Científica Química Fina Indústria e Comércio LTDA (Sumaré, SP, Brasil). O 5-FU foi proveniente da Cayman Chemical Company (Ann Arbor, Michigan, EUA).

1.2.2 Extração e purificação do alcaloide

O EPI-HCL foi cedido pelo Laboratório de Cromatografia da Universidade Federal do Delta do Parnaíba (UFDPar), com cadastro nº A95905B no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN). O alcaloide foi purificado a partir da biomassa gerada durante a extração da pilocarpina das folhas da *Pilocarpus microphyllus* pela Anidro do Brasil Extrações SA®, entidade do grupo Centroflora, localizada em Parnaíba (PI), Brasil. O EPI-HCL apresenta estrutura neutra em pH 10.2, permitindo a formação do sal em uma solução ácida com etanol e acido clorídrico através da precipitação de cristais solúveis coletados por evaporação lenta ao longo de 2 semanas à 4ºC. A confirmação da massa molecular foi realizada por Espectrometria de Massas (AmaZon SL, Bruker Daltonics, Bremen, Alemanha) com valores equivalentes da literatura (Mafud et al. 2017). A purificação foi concluída conforme descrito por Veras et al. (2013).

1.2.3 Cultivo das linhagens celulares

As linhagens de carcinoma colorretal humano (HCT-116) e carcinoma de cólon murino (CT26.WT) foram cultivadas *in vitro* em frascos de cultura de células com seus respectivos meios, DMEN e RPMI-1640, suplementados com 10% de SBF e 1% de antibiótico (penicilina/estreptomicina). As células foram manuseadas em ambiente estéril de

câmara de fluxo laminar vertical (Pachane, modelo PCR T4 Eco, classe II) e mantidas em incubadora a 37°C com atmosfera de 5% de CO_2 (Thermo Scientific, modelo 8000 WJ Series). A manutenção foi realizada sempre que as células alcançavam aproximadamente 80% de confluência.

1.2.4 Ensaio de MTT

As células cultivadas das linhagens HCT-116 e CT26.WT foram distribuídas em placas de 96 poços, nas respectivas concentrações de 6 x 10^4 células/mL e 8 x 10^4 células/mL, e incubadas em estufa a 37ºC com 5% de CO_2 durante 24 horas. Após esse período, as células receberam o tratamento com EPI-HCL isolado na concentração máxima de 350µM e com EPI-HCL na concentração fixa de 70µM associado a 5-FU em diluição seriada de 50µM a 0,78µM. O 5-FU em diluição seriada de 50µM a 0,78µM também foi utilizado como controle positivo. Em seguida, as células foram incubadas durante mais 69 horas.

Para conclusão do ensaio de citotoxicidade, cada cavidade das multiplacas recebeu 150 µL da solução de MTT a 10% em meio RPMI-1640 ou DMEN e foi levada a incubadora durante 3 horas. Após o período de incubação o sobrenadante foi retirado e desprezado. O precipitado foi ressuspendido em 150 µL de DMSO para a quantificação do sal reduzido. As absorbâncias foram obtidas por espectrofotômetro de placa, no comprimento de onda de 595nm. Utilizando o *software* GraphPad Prism versão 8.0, a viabilidade celular foi avaliada a partir do controle (células não tratadas) com dados em triplicata para o cálculo da CI_{50} (concentração inibitória média) e do intervalo de confiança de 95% ($IC_{95\%}$).

1.2.5 Delineamento experimental in vivo

Foram utilizados 68 camundongos BALB/c fêmeas, saudáveis, provenientes do Biotério da UFDPar, *Campus* Ministro Reis Velloso, Parnaíba, Piauí, sendo aprovados pelo seu Comitê de Ética no Uso de Animais (CEUA) sob registro 002/22. Durante todo o experimento os animais foram mantidos em gaiolas plásticas de polipropileno com água e ração comercial ofertadas *ad libitum*, em ambiente controlado com ciclo claro-escuro de 12 horas e temperatura de aproximadamente 22 °C no biotério da UFDPar. Após 24 horas do último tratamento, os animais foram eutanasiados com uma overdose anestésica e tiveram o sangue coletado por punção intracardíaca.

A indução tumoral ocorreu com a inoculação de 1 x 10^6 células de linhagem CT26.WT por via subcutânea na axila esquerda de 50 camundongos, com idade entre 2 e 3 meses, que foram acompanhados diariamente até a identificação de tumor palpável. Em seguida, foram divididos aleatoriamente em cinco grupos (n = 10) e tratados por via intraperitoneal (i.p) durante 10 dias. Utilizaram-se os seguintes esquemas: grupo tumor salina tratado diariamente com soro fisiológico 0,9% (TS), grupo controle positivo (5-FU) tratado com 5-FU 20mg/kg/2d, grupo tumor EPI-HCL 50mg/kg/d (TEPI-50), grupo tumor EPI-HCL 25mg/kg/d (TEPI-25) e grupo Associação tratado com 5-FU 20mg/kg/2d + EPI-HCL 50mg/kg/d (ASSOC).

Dezoito camundongos saudáveis sem indução tumoral, com idade entre 4 e 7 meses, foram divididos em dois grupos (n = 9): saudável salina tratado com soro fisiológico 0,9% (SS) e grupo saudável EPI-HCL 50mg/kg/d (SEPI-50), ambos tratados diariamente por via i.p durante 10 dias.

O 5-FU foi preparado em PBS conforme orientações do fabricante e o EPI-HCL reconstituído em solução salina.

Os animais tiveram seu peso monitorado em dias alternados e o crescimento tumoral mensurado diariamente utilizando um paquímetro mecânico, onde o volume foi obtido pela fórmula de Steel: volume do tumor = $(D \times d^2)/2$, onde D fornece a medida do maior diâmetro (mm) e d do menor diâmetro (mm).

1.2.6 Análise hematológica

As células hematológicas foram contadas e analisadas por esfregaço sanguíneo corado com kit Panótico Rápido (Laborclin) e através do contador automatizado HumaCount 60^{TS} (Human).

1.2.7 Macroscopia dos órgãos

O baço, fígado, rins, cólon e tumor foram coletados, pesados, verificados quanto à presença de lesões óbvias e armazenados em solução tampão de formol 10%. Além disso, retirou-se o intestino grosso e posteriormente o cólon, registrando seus comprimentos e permitindo o cálculo do índice peso/comprimento do cólon a fim inferir a presença de edema (Chen et al. 2017).

1.2.8 Análise morfométrica do cólon

Os cólons armazenados em formol a 10% foram parafinizados e seccionados em micrótomo, desparafinizados em xilol por 15 minutos, desidratados em soluções crescentes de álcool etílico até 70%, lavados com água destilada e corados com hematoxilina e eosina (HE) para análise em microscópio de luz Carl-Zeiss-Promenade 10, aumento de 20x. Utilizando o *software* ZEN core, foram medidas de 10 a 20 criptas por grupo, com exibição de toda a arquitetura da glândula e com distância mínima entre elas de 200µm, adaptado de Justino et al. (2015).

1.2.9 Análises estatísticas

Os dados foram analisados no *software* GraphPad Prism versão 8.0, a partir da média ± erro padrão da média (EPM). Para verificação da ocorrência de diferenças estatísticas entre os grupos, os dados foram comparados por análise de variância *(ANOVA*) e a significância estabelecida usando o teste de comparações múltiplas de Dunnett. Valores de $p < 0.05$ foram considerados estatisticamente significativos.

1.3 Resultados e discussão

1.3.1 Citotoxicidade do Cloridrato de Epiisopiloturina (EPI-HCL)

O EPI-HCL nas linhagens de câncer colorretal humano e murino testadas não provocou alteração da viabilidade celular após 72h de exposição na concentração de 350µM. Também não foi observado modificações do valor de CI_{50} do 5-FU tratado em associação com o EPI-HCL (CI_{50} = 3,9µM) ou isolado (CI_{50} = 3,39µM) na linhagem HCT-116 após 72h de exposição. No entanto, na linhagem CT26.WT, a associação do EPI-HCL com o

quimioterápico provocou um discreto aumento da CI_{50} do 5-FU de 0,03µM ($IC_{95\%}$ = 0,006 a 0,15µM) para 0,17µM. Este discretamente acima do intervalo de confiança (Tabela 1).

Nota-se que o EPI-HCL não exerce efeito citotóxico contra as linhagens neoplásicas avaliadas. Resultados similares estão descritos na literatura em linhagens não tumorais, conforme visto por Rocha et al. (2019), que não identificou citotoxicidade da Epiisopiloturina em concentrações variadas de 3,5 a 350nM contra neutrófilos e por Veras et al. (2012), que durante estudo do potencial antiparasitário do alcaloide no combate a infecção por Schistosoma mansoni demonstrou que não há atividade citotóxica contra macrófagos peritoneais de camundongos e fibroblastos renais de macaco-verde africano nas concentrações de 300 a 500µg/mL.

	CI_{50} (µM) Intervalo de confiança 95%		
Linhagens celulares	EPI-HCL	5-FU	EPI-HCL + 5-FU
HCT-116	> 350	3,39 (2,55 a 4,40)	3,9 (2,58 a 5,67)
CT26.WT	> 350	0,03 (0,006 a 0,15)	0,17 (0,02 a 0,64)

Tabela 1 CI_{50} e $IC_{95\%}$ obtidas no Ensaio de MTT. EPI-HCL em diluição seriada a partir de 350µM; 5-FU em diluição seriada de 50µM a 0,78µM; EPI-HCL na concentração de 70µM + 5-FU em diluição seriada de 50µM a 0,78µM

Importante destacar que este é o primeiro estudo do EPI-HCL em linhagens tumorais e de associação com a quimioterapia. A associação foi pensada baseada no trabalho de Carvalho (2018) que verificou sinergismo com Anfotericina B contra agentes da cromoblastomicose. Embora o EPI-HCL não tenha demonstrado atividade *in vitro*, seguiu-se para o estudo em animais devido a sua atividade biológica já descrita na literatura. Trabalhos como de Nicolau et al. (2017) que demonstram o efeito anti-inflamatório do EPI-HCL na mucosa gástrica de camundongos contra o dano induzido por naproxeno e de Oliveira (2022) que verificou o efeito protetor da Epiisopiloturina na hepatoxicidade decorrente do uso de paracetamol, nos faz pensar que trata-se de um agente com grande potencial protetor contra as lesões teciduais, isto é, contra os efeitos colaterais da quimioterapia. Essa proposta foi verificada por Barbosa et al. (2022) na mucosite do intestino delgado provocada por altas doses de 5-FU. Todavia, agentes capazes de reduzir os efeitos colaterais dos antineoplásicos não raramente prejudicam a sua atividade antitumoral (Menegazzi, Masiello, Novelli 2021; Amjadi et al. 2019). Logo, um modelo capaz de avaliar outros parâmetros de toxicidade e especialmente a ação do alcaloide na inibição tumoral do 5-FU é extremamente relevante.

1.3.2 Atividade antitumoral

Os grupos TEPI-25 e TEPI-50, tratados com EPI-HCL nas respectivas doses de 25 e 50mg/kg/dia não exibiram diferença significativa no volume tumoral ao longo do experimento, evidenciando que o alcaloide não apresenta efeito antineoplásico no modelo testado, Fig. 2.

O estudo também demonstrou que o EPI-HCL não interfere potencializando ou reduzindo a inibição tumoral provocada pelo 5-FU. O grupo associação obteve um peso tumoral médio de 1,52 ± 0,32g e o 5-FU de 1,30 ± 0,25g, atingindo um percentual de inibição quando comparados com o TS de 52,3% e 59,1%, respectivamente (Tabela 2). Ressalta-se que o grupo associação não demonstrou diferença significativa ao ser comparado com o 5-FU, indicando que não houve influência na atividade antitumoral do quimioterápico. Esse resultado é visualizado ao longo de todo o experimento ao compararmos o volume tumoral (Fig. 2a) e a macroscopia dos tumores (Fig. 2b).

Dessa forma, os resultados se assemelham com o perfil de citotoxicidade do alcaloide no ensaio *in vitro* e abrem espaço para sua utilização contra os efeitos colaterais da quimioterapia.

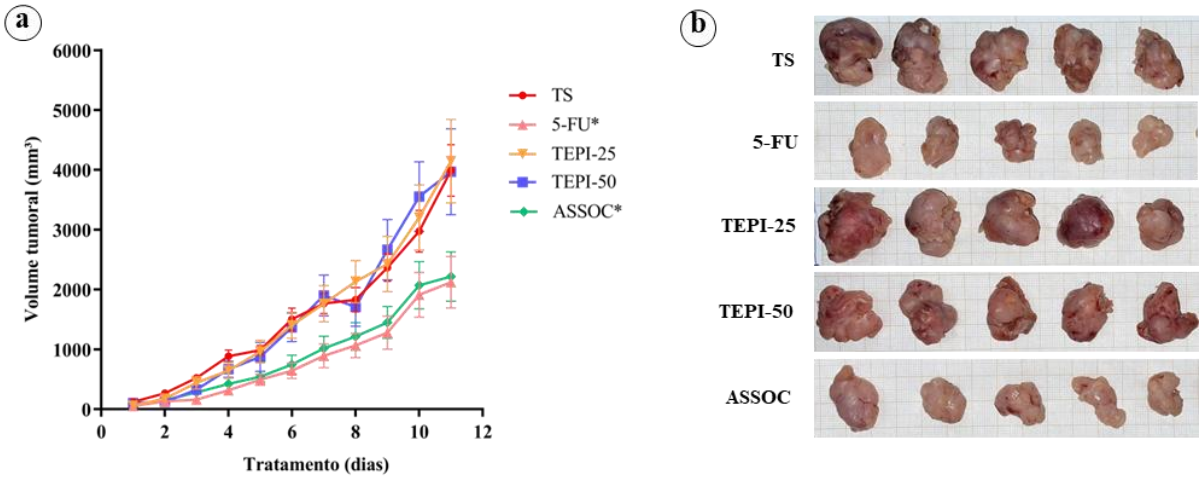

Fig. 2 Crescimento tumoral. **a.** Curva de crescimento tumoral até a eutanásia. **b.** Macroscopia dos tumores. TS = tumor salina 0,9%; 5-FU 20mg/kg/2d; TEPI-25 = tumor EPI-HCL 25mg/kg/d; TEPI-50 = tumor EPI-HCL 50mg/kg/d; ASSOC = associação de EPI-HCL 50mg/kg/d + 5-FU 20mg/kg/2d. Todos os dados são mostrados com média + EPM. Grupos comparados com o TS com *p < 0.05 e com 5-FU com $^{\#}$p < 0.05 utilizando o ANOVA unidirecional seguido pelo teste de comparações múltiplas de Dunnett

Grupos	Dose (mg/kg)	Peso dos camundongos (g)		Peso tumoral (g)	Inibição tumoral (%)
		Inicial	Final		
TS	-	21.19 ± 0.20	23.86 ± 0.41	3.19 ± 0.36	-
5-FU	20	20.90 ± 0.46	22.94 ± 0.55	1.30 ± 0.25*	- 59,1
TEPI	25	20.31 ± 0.52$^{\&}$	23.39 ± 0.67	3.21 ± 0.49$^{\#}$	+ 0,62
TEPI	50	21.85 ± 0.81	24.46 ± 0.67	2.94 ± 0.44	- 7,84
5-FU + EPI-HCL	20 + 50	19.40 ± 0.41$^{\&}$	21.47 ± 0.42	1.52 ± 0.32	- 52,3
SS	-	23.45 ± 0.65*$^{\#}$	23.22 ± 0.75	-	-
SEPI	50	23.36 ± 0.74*$^{\#}$	22.24 ± 0.68	-	-

Tabela 2 Peso dos grupos ao início e fim do tratamento, peso e inibição tumoral na eutanásia. TS = tumor salina 0,9%; 5-FU 20mg/kg/2d; TEPI-25 = tumor EPI-HCL 25mg/kg/d; TEPI-50 = tumor EPI-HCL 50mg/kg/d; 5-FU + EPI-HCL = associação de EPI-HCL 50mg/kg/d + 5-FU 20mg/kg/2d; SS = saudável salina 0,9%; SEPI-50 = saudável EPI-HCL 50mg/kg/d. Todos os dados são mostrados com média + EPM. Inibição tumoral calculada a

partir do grupo TS, indicado com "+" ganho em relação ao TS e "-" inibição. Grupos comparados com o TS com *p < 0.05, com 5-FU com $^{\#}p < 0.05$ e com SS com $^{\&}p < 0.05$ utilizando o ANOVA unidirecional seguido pelo teste de comparações múltiplas de Dunnett

1.3.3 Toxicidade sistêmica dos tratamentos

Não foi observada redução do peso dos animais em nenhum grupo após os tratamentos (Tabela 2). Com relação ao peso dos órgãos, os animais do grupo 5-FU apresentaram uma diminuição significativa no peso do fígado (Fig. 3a) e do baço (Fig. 3b) quando comparados com o TS, nosso controle negativo.

A redução do peso hepático está intimamente relacionada ao dano decorrente da metabolização do quimioterápico. Sabe-se que até 80% do 5-FU administrado é catabolizado no fígado pela dihidropirimidina desidrogenase, favorecendo a ocorrência de hepatotoxicidade (Longley, Antonuzzo, Cherny 2003). Estudos em animais, como o de Montenegro et al. (2008) demonstram a redução do peso do fígado associado a degeneração hidrópica, esteatose microvesicular e infiltrado inflamatório diante da administração de 5-FU 50mg/m^2 durante 7 dias em camundongos Swiss.

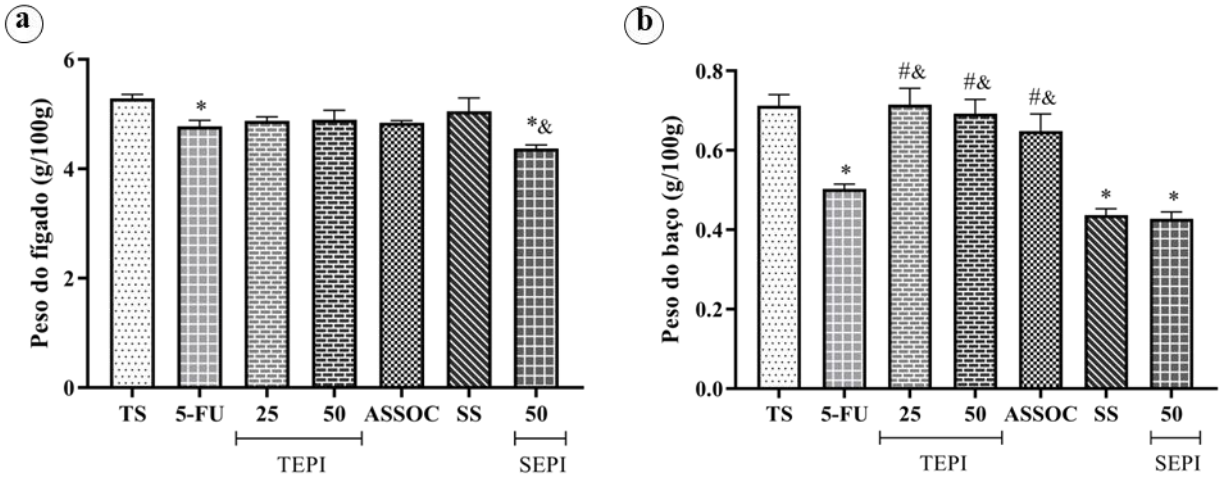

Fig. 3 Peso dos órgãos após 10 dias de tratamento. Valores normalizados pelo peso do animal, todos os dados são mostrados com média + EPM. **a.** Peso do fígado **b.** Peso do baço. TS = tumor salina 0,9%; 5-FU 20mg/kg/2d; TEPI-25 = tumor EPI-HCL 25mg/kg/d; TEPI-50 = tumor EPI-HCL 50mg/kg/d; ASSOC = associação de EPI-HCL 50mg/kg/d + 5-FU 20mg/kg/2d; SS = saudável salina 0,9%; SEPI-50 = saudável EPI-HCL 50mg/kg/d. Grupos comparados com o TS com *p < 0.05, com 5-FU com $^{\#}p < 0.05$ e com SS com $^{\&}p < 0.05$ utilizando o ANOVA unidirecional seguido pelo teste de comparações múltiplas de Dunnett

A respeito da redução esplênica, compreende-se que o baço é um órgão linfo-reticular que atua no controle da população eritrocitária, como reservatório de plaquetas e possui um papel integral de resposta imune através da linfopoiese e da concentração de células da imunidade inata (Lewis, Williams, Eisenbarth 2019). A sua redução induzida pelo 5-FU é descrita na literatura frequentemente associada à atrofia do órgão e leucopenia, indicando a atividade imunossupressora do quimioterápico, conforme visto por Amaral et al. (2020) após aplicação de 5-FU 25mg/kg/d por 7 dias em camundongos Swiss.

No entanto, a associação do EPI-HCL evitou essas modificações (Fig. 3). Dentre os efeitos colaterais da quimioterapia, a hepatotoxicidade e a supressão imune, além dos efeitos gastrointestinais, representam eventos potencialmente graves que diante do risco ao paciente podem evoluir com a necessidade de redução de dose, interrupção e cessação do tratamento (Smith, Lavery, Turkington 2020). A capacidade de impedir a redução do peso hepático e esplênico que estão descritos na literatura como sinais de lesão a esses órgãos pelo 5-FU, indica o grande potencial de associação do EPI-HCL com os quimioterápicos. Além disso, a Epiisopiloturina já demonstrou a capacidade de reduzir o dano hepático decorrente do uso de paracetamol em camundongos, sugerindo um envolvimento da via autofágica (Oliveira 2022).

A cerca dos animais saudáveis (Fig. 3), o grupo SEPI-50 apresentou redução do peso do fígado comparado ao grupo SS. Contudo, em um estudo de toxicidade com a Epiisopiloturina em camundongos Swiss, não foi observada alteração histológica hepática, renal, pulmonar ou cerebral na dose única de 530mg/kg, evidenciou-se apenas uma depressão focal da polpa vermelha e estabeleceu-se a dose letal capaz de matar 50% dos animais em 8000mg/kg (Guimarães et al. 2015). Não houve diferença significativa no peso médio dos rins em nenhum grupo do experimento.

Nas análises hematológicas do modelo tumoral (Tabela 3), os grupos tratados com EPI-HCL isolado (TEPI-25 e TEPI-50) apresentaram um aumento nos padrões hematimétricos (RBC e HGB). A diminuição no número de hemácias, que pode atingir níveis anêmicos, é um efeito colateral da quimioterapia tradicional (Pujade-Lauraine, Gascón 2004). Entretanto, não foi observada nenhuma alteração com a administração do 5-FU, possivelmente devido à dose utilizada, pois, se encontra presente em outros trabalhos, como de Coronado-Cerda et al. (2016) que verificou a anemia e leucopenia induzidas com 5-FU 75mg/kg, dose única, em camundongos BALB/c.

Grupos	Dose (mg/kg)	Concentração de células sanguíneas			
		RBC (milhões/mm³)	HGB (g/dL)	WBC (mm³)	PLT (mm^3)
TS	-	8.83 ± 0.16	13.08 ± 0.19	7.29 ± 0.53	383.7 ± 72.94
5-FU	20	8.77 ± 0.07	13.08 ± 0.15	4.69 ± 0.69	448.5 ± 37.0
TEPI	25	9.78 ± 0.12*#	14.68 ± 0.21*#	7.79 ± 0.74	427.6 ± 44.1
TEPI	50	9.85 ± 0.32	14.63 ± 0.47*#	8.97 ± 0.65#	413.3 ± 6.36
5-FU + EPI-HCL	20 + 50	9.49 ± 0.39	14.30 ± 0.43	6.80 ± 0.93	460.0 ± 42.1
SS	-	9.98 ± 0.38*#	14.50 ± 0.40	6.66 ± 0.70	356.3 ± 18.1
SEPI	50	10.38 ± 0.02*#	15.07 ± 0.03*#	6.19 ± 0.98	626.0 ± 279.3

Tabela 3 Concentração de células sanguíneas. RBC (hemácias), HGB (hemoglobina), WBC (células brancas), PLT (plaquetas). TS = tumor salina 0,9%; 5-FU 20mg/kg/2d; TEPI-25 = tumor EPI-HCL 25mg/kg/d; TEPI-50 = tumor EPI-HCL 50mg/kg/d; 5-FU + EPI-HCL = associação de EPI-HCL 50mg/kg/d + 5-FU 20mg/kg/2d; SS = saudável salina 0,9%; SEPI-50 = saudável EPI-HCL 50mg/kg/d. Todos os dados são mostrados com média + EPM. Grupos comparados com o TS com *$p < 0.05$, com 5-FU com $^{\#}p < 0.05$ e com SS com $^{\&}p < 0.05$ utilizando o ANOVA unidirecional seguido pelo teste de comparações múltiplas de Dunnett

Nos animais saudáveis (Tabela 3), não houve aumento na contagem de eritrócitos e na concentração de hemoglobina com a admiministração do EPI-HCL (RBC = 10.38 milhões/mm³; HGB = 15.07g/dL) comparado ao SS (RBC = 9.98 milhões/mm³; HGB = 14.50g/dL), sugerindo que o aumento nos grupos do modelo tumoral ocorreu como uma

resposta induzida pelo tumor. Os demais parâmetros não apresentaram alterações significativas.

1.3.4 Efeito protetor no cólon

Os animais do grupo 5-FU apresentaram aumento do peso do cólon (Fig. 4c.) e da razão peso/comprimento colônico (Fig. 4d). Em contrapartida, o grupo tratado com a associação de EPI-HCL + 5-FU foi capaz de evitar essas alterações.

De maneira geral, o encurtamento do cólon, aumento do peso e a presença de edema são parâmetros úteis para avaliar lesão intestinal (Ali et al. 2019). A redução do seu comprimento está associada a manifestações mais graves de mucosite, incluindo diarreia e redução do peso médio dos grupos (Li et al. 2017). No nosso estudo, não foi observado encurtamento do cólon, diarreia ou emagrecimento (Fig. 4a, Tabela 2) provavelmente devido à dose e ao esquema de 5-FU (20mg/kg) administrado em dias alternados. Ali et al. (2019) verificou que com a administração de 5-FU 50mg/kg/dia, i.p, por 3 dias consecutivos, há redução do peso dos animais, encurtamento do cólon e perda da arquitetura tecidual no intestino grosso de camundongos BALB/c.

Contudo, ainda conseguimos observar o aumento do peso e da proporção peso/comprimento colônico induzidas pelo quimioterápico quando comparados ao controle negativo (TS) e que o grupo associado com EPI-HCL impediu essas alterações (Fig. 4c,d). De acordo com Chen et al. (2017), um índice peso/comprimento aumentado nos permite inferir indiretamente a presença de edema. Na macroscopia (Fig. 4e) nota-se a presença de tumefação e vasocongestão na parede do cólon no grupo tratado com 5-FU e ausente no ASSOC.

Na análise morfométrica, identificou-se que o 5-FU provocou um aumento da profundidade das criptas intestinais e que o tratamento associado com EPI-HCL impediu totalmente essa alteração (Fig. 5h). Visualiza-se na Fig. 5d (seta vermelha), o aumento das criptas e a normalização do parâmetro no grupo ASSOC (Fig. 5e, seta preta). Dito isso, o aumento das criptas intestinais induzido pelo 5-FU associado de desordem na arquitetura tecidual está relatado na literatura em linhagem BALB/c tratada com 5-FU 50mg/kg/d por 3 dias (Zeeshan et al. 2021). No entanto, modelos voltados para a mucosite intestinal que fazem uso de altas doses de 5-FU (300mg/kg) verificam a destruição tecidual associada tanto ao encurtamento das criptas e vilosidades no intestino delgado e grosso, conforme Wei et al. (2020), como ao aumento da profundidade das criptas no intestino delgado (Hu et al. 2020).

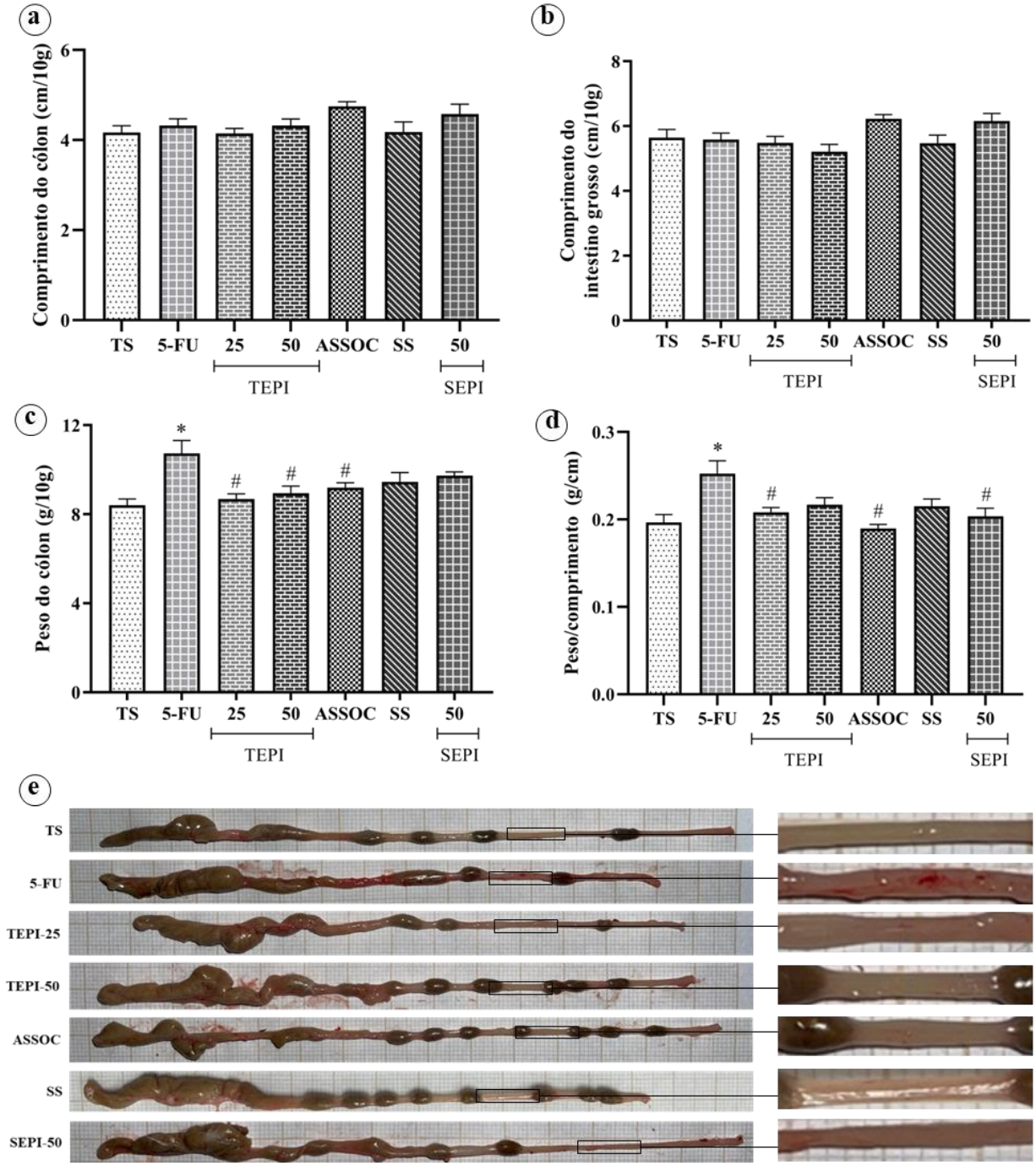

Fig. 4 Avaliação macroscópica do cólon após 10 dias de tratamento. Valores normalizados pelo peso do animal. **a.** Comprimento do cólon. **b.** Comprimento do intestino grosso (cólon + ceco). **c.** Peso do cólon. **d.** Índice de edema do cólon (peso/comprimento). **e.** Macroscopia do intestino grosso, recorte em aumento de 2,5x evidenciando a presença de tumefação e vasocongestão na parede do cólon do grupo 5-FU. TS = tumor salina 0,9%; 5-FU 20mg/kg/2d; TEPI-25 = tumor EPI-HCL 25mg/kg/d; TEPI-50 = tumor EPI-HCL 50mg/kg/d; ASSOC = associação de EPI-HCL 50mg/kg/d + 5-FU 20mg/kg/2d; SS = saudável salina 0,9%; SEPI-50 = saudável EPI-HCL 50mg/kg/d. Grupos comparados com o TS com $^{*}p < 0.05$, com 5-FU com $^{\#}p < 0.05$ e com SS com $^{\&}p < 0.05$ utilizando o ANOVA unidirecional seguido pelo teste de comparações múltiplas de Dunnett

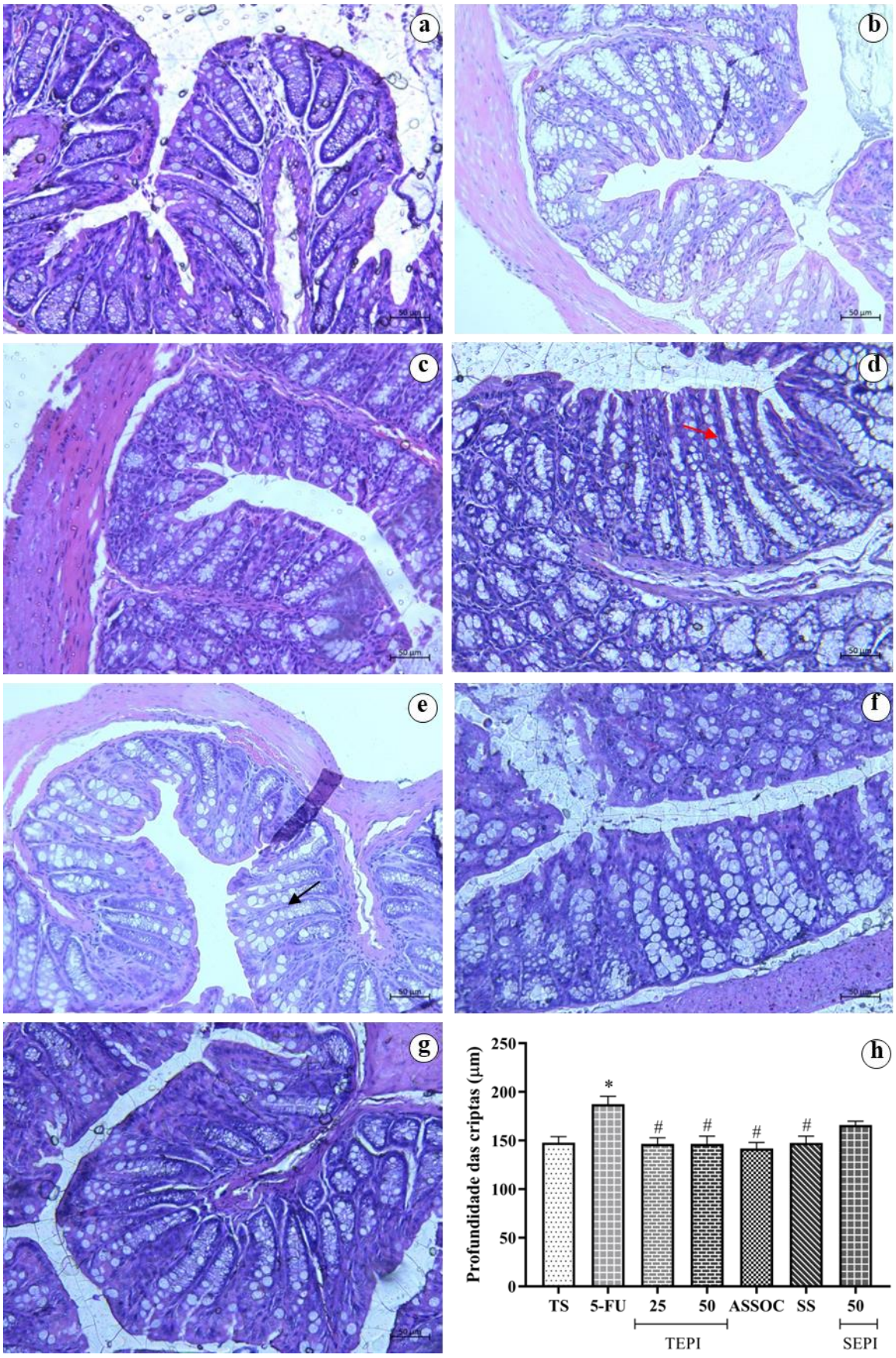

Fig. 5 Morfometria do cólon. Fotomicrografia (20x) do cólon dos grupos **a.** SS, **b.** SEPI-50, **c.** TS, **d.** 5-FU, **e.** ASSOC, **f.** TEPI-25 e **g.** TEPI-50 evidenciando as criptas intestinais. O tratamento associado com EPI-HCL (**e.**

seta preta) impediu o aumento da profundidade das criptas induzido pelo 5-FU (**d.** seta vermelha**)**. **h.** Profundidade das criptas intestinais. TS = tumor salina 0,9%; 5-FU 20mg/kg/2d; TEPI-25 = tumor EPI-HCL 25mg/kg/d; TEPI-50 = tumor EPI-HCL 50mg/kg/d; ASSOC = associação de EPI-HCL 50mg/kg/d + 5-FU 20mg/kg/2d; SS = saudável salina 0,9%; SEPI-50 = saudável EPI-HCL 50mg/kg/d. Grupos comparados com o TS com *$p < 0.05$, com 5-FU com $^{\#}p < 0.05$ e com SS com $^{\&}p < 0.05$ utilizando o ANOVA unidirecional seguido pelo teste de comparações múltiplas de Dunnett

No nosso estudo, optou-se por uma dose baixa de quimioterápico com o objetivo de permitir o desenvolvimento parcial do tumor a fim de avaliar a atividade antineoplásica do alcaloide e a interferência deste na inibição tumoral do 5-FU. Dessa forma, não se induziu uma franca mucosite intestinal. Entretanto, as alterações macroscópicas e morfométricas apontam para a presença de lesão tecidual em um estado de tolerabilidade dos camundongos, a ser confirmado com a posterior análise histopatológica.

A mucosite intestinal costuma ser descrita em cinco fases evolutivas: início, regulação positiva com ativação de mensageiros, amplificação de sinal e ulceração com inflamação e cicatrização. A lesão inicial leva a produção de espécies reativas de oxigênio e a ativação do fator nuclear kappa-B (NF-κB), resultando na regulação positiva de aproximadamente 200 mensageiros, entre os mais notáveis o fator de necrose tumoral α, interleucina (IL) 6 e IL1β, bem como a COX-2 quando a amplificação do sinal é atingida. Além disso, o processo inflamatório e ulceração amplia a proliferação bacteriana que estimula receptores toll-like e leva à ativação de NF-κB através de múltiplas vias, agravando ainda mais a inflamação através do aumento de citocinas inflamatórias (Sougiannis et al. 2021).

A Epiisopiloturina demonstrou ação inibitória contra as principais enzimas e produtos envolvidos na inflamação, conforme Carvalho et al. (2018) que identificou redução do peso úmido do cólon, de IL1β e redução da expressão de COX-2 e óxido nítrico-sintase com a administração de Epiisopiloturina 1mg/kg, i.p, em um modelo de colite induzida por ácido trinitrobenzenossulfônico. De forma semelhante, Barbosa et al. (2022) demonstrou o efeito protetor na mucosite do intestino delgado decorrente do uso de 5-FU 450mg/kg, i.p, dose única, evidenciando a recuperação da arquitetura tecidual e a redução da expressão de COX-2 com a administração de Epiisopiloturina 10mg/kg, i.p, por 5 dias em camundongos Swiss.

A incidência de diarreia em pacientes com CCR tratados com terapia infusional de 5-FU atinge médias superiores a 30%, sendo que a diarreia grave compromete mais de 10% dos doentes e se torna o sintoma com maior impacto na qualidade de vida (Jones et al. 2006; Bossi et al. 2018). Portanto, trabalhos como de Barbosa et al. (2022) verificando a redução da mucosite no intestino delgado, associado do nosso que demonstra a ausência de interferência na atividade antitumoral e o efeito protetor no cólon destacam o potencial do EPI-HCL para a elaboração de esquemas com menos efeitos colaterais.

Nesse sentido, demonstramos com esse estudo os danos induzidos pelo 5-FU na linhagem de camundongos BALB/c em uma dose-alvo para modelo antitumoral, além da capacidade do EPI-HCL de reduzir a toxicidade sistêmica e proteger o cólon, sem interferir no efeito antineoplásico do 5-FU.

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2 ANEXO A: DECLARAÇÃO DE CADASTRO NO SISGEN

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão

Cadastro nº A95905B

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**A95905B**
Usuário:	**LEIZ MARIA COSTA VÉRAS**
CPF/CNPJ:	**841.003.203-10**
Objeto do Acesso:	**Patrimônio Genético/CTA**
Finalidade do Acesso:	**Pesquisa**

Espécie

Pilocarpus microphyllus

Fonte do CTA

CTA de origem identificável diretamente com provedor

Provedor

Centroflora

Título da Atividade:	**Avaliação da atividade antitumoral de alcaloides de Pilocarpus microphyllus**

Equipe

LEIZ MARIA COSTA VÉRAS	**Universidade Federal do Piauí**
José Delano Barreto Marinho Filho	**UFDPAR**
Ana Jérsia Araújo	**UFDPAR**
Maria Eduarda Mauriz Rodrigues	**UFDPAR**
William Cataldo Teixeira	**UFDPAR**

Data do Cadastro:	**28/04/2022 16:51:46**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **15:40** de **23/01/2024**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN

3 ANEXO B: CARTA DE APROVAÇÃO - CEUA/UFDPAR

UNIVERSIDADE FEDERAL DO DELTA DO PARNAÍBA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Universidade Federal do Delta do Parnaíba - UFDPar
Comissão de Ética no Uso de Animais – CEUA
Av. São Sebastião, 2819, Parnaíba, Piauí, Brasil; CEP: 64202-020.
E-mail: ceua.ufdpar@gmail.com

CERTIFICADO

Certificamos que a proposta intitulada **"Avaliação do efeito antitumoral da combinação de alcaloides naturais epiisopilosina e epiisopiloturina com os quimioterápicos 5-fluorouracil ou irinotecano em camundongos Balb/c transplantados com carcinoma CT26.WT."**, registrada sob protocolo Nº **002/22**, de a responsabilidade do **Prof. Dr. José Delano Barreto Marinho Filho** do **Curso de Medicina** da **Universidade Federal do Delta do Parnaíba/UFDPar,** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de Pesquisa Científica e/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 foi ***APROVADO*** pela Comissão de Ética no Uso de Animais da Universidade Federal do Delta do Parnaíba (CEUA/UFDPar), em Reunião na presente data **11/02/2022**.

Finalidade	**( ) Ensino (X) Pesquisa Científica**
Vigência da Autorização	março/ 2022 a março/ 2024
Espécie/Linhagem/raça:	Mus musculus / variedade Balb/c
Nº de Animais:	204
Peso/ Idade:	25-30 g, 1 mês
Sexo:	Fêmeas
Origem:	Biotério da Universidade Federal do Delta do Parnaíba - UFDPar

Parnaíba, 11 de fevereiro de 2022.

Jefferson Soares de Oliveira

Prof. Dr. Jefferson Soares de Oliveira
Coordenador da CEUA/UFDPar

Para verificar a autenticidade deste documento acesse o site https://ufpi.br/comite-de-etica-no-uso-de-animais-ceua-parnaiba-ufdpar e procure os dados do projeto no arquivo disponível em "Acesse aqui a lista de Projetos Aprovados pela **CEUA/UFDPar***" ou entre em contato com a CEUA-UFDPar.*

4 ANEXO C: NORMAS DE SUBMISSÃO DA REVISTA BRASILEIRA DE FARMACOGNOSIA

Brazilian Journal of Pharmacognosy

Manuscript Submission
Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Online Submission
Please follow the hyperlink "Submit manuscript" and upload all of your manuscript files following the instructions given on the screen.

Source Files
Please ensure you provide all relevant editable source files at every submission and revision. Failing to submit a complete set of editable source files will result in your article not being considered for review. For your manuscript text please always submit in common word processing formats such as .docx or LaTeX.

Types of articles
The Brazilian Journal of Pharmacognosy accepts for publication original scientific work, reviews and communication articles written only in English.

• Original papers: Original papers are research articles describing original experimental results. The manuscript should be arranged in the following order: Graphical abstract, Title, Abstract, Keywords, Introduction, Material and methods, Results, Discussion, Authors' contributions, Acknowledgements, References, Figures with Legends, Tables, Structural Formulae and Supplemental files (if applicable). Results and Discussion sections may appear as a combined 'Results and Discussion' section. The normal length of the main text of an Original Paper (excluding references, tables, figures and figure legends) is approximately 3,000 words. Longer manuscripts may be accepted only in exceptional and well justified cases.

•Short communications: This section will cover mainly the isolation of known compounds from new neotropical sources, or complementary results of on-going work. The text should be arranged as follows: Graphical abstract, Title, Abstract of 200 words, Keywords, Introductory Remarks, Material and Methods with brief experimental details without subheadings, Results and Discussion as one body of text without headlines, Acknowledgements, Authorship, References (up to 20 citations) and Figures and/or Tables (up to 3). The text should not exceed 2,000 words.

•Reviews: Authors are invited to submit a review article that provides concise and critical updates on a subject, and with around 100 references. The main purpose of reviews is to provide a concise, accurate introduction to the subject matter and inform the reader critically of the latest developments in the field. Review articles should be designed to give an interesting insight into a hot topic in Pharmacognosy, focusing on the key developments that have shaped a field rather than giving a very comprehensive overview of a very specific topic. They should be concise and include details of the search strategy used, such as time frame, search terms, used databases. A review should be an article that produces knowledge and not just a survey of the existing literature. The review must be a response to an initial question. Reviews of a particular herbal drug will be considered if they contain the newest issue and a perspective on future directions.

Authors are strongly recommended to prepare a manuscript using a A4-sized paper, double-spaced, with Times New Roman size-12 font, fully justified, with margins of 2 cm.

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure the following:

• One author has been designated as the corresponding author with contact details: Institutional e-mail address; full postal address;

• ORCID ID for all authors;

• Authors' contributions;

• All authors, with their respective email addresses, should be entered into the system.

• All necessary files have been uploaded: Graphical abstract, Manuscript; Include keywords; All figures with Legends; All tables (including titles, description, footnotes); and Supplemental files (if applicable).

• All figure and table citations in the text match the files provided;

• Manuscript has been 'spell checked' and 'grammar checked;

• All references mentioned in the Reference List are cited in the text, and vice versa;

• Permission has been obtained for use of copyrighted material from other sources (including the Internet);

• Relevant declarations of interest have been made;

• Journal policies detailed in this guide have been reviewed.

Additional information

• All plant, microorganism and marine organism materials used in the described research should be supported by an indication of the site (including GPS coordinates, if possible) and country of origin, the name of the person identifying the biological material and the location of the voucher specimen.

• Authors should be prepared to provide documentary evidence that approval for collection was afforded from an appropriate authority in the country of collection and, if applicable, to follow the rules concerning the biodiversity rights.

• The journal will not accept responsibility for research works that do not comply with the legislation of the country of residence of the author.

• We strongly recommend that authors avoid stating that the popular or traditional use of a certain herb was confirmed by pre-clinical, in vitro assays or in vivo tests using animals.

• The Revista Brasileira de Farmacognosia-Brazilian Journal of Pharmacognosy strongly encourages the submission of original works in which the experimental procedures were conducted taking into consideration green chemistry principles, such as by employing green solvents and environmental resource saving experimental designs in any step of the investigation.

• Evaluations using animal models to provide evidences for the pharmacological efficacy of plants do not fall into the areas of interest of the journal when polyphenol-rich plant extracts are involved and, therefore, making the results predictable.

• For the pharmacological studies to explore the therapeutic roles of polyphenols, such as antioxidant activity, antibacterial, antiviral, hepatoprotective, anti-inflammatory, antipyretic, anti-obesity, cardioprotective, neuroprotective, anti-hypertension, free radicals scavenger, and central nervous system stimulators, the journal will give preference to articles that make use of molecular tools over animal models for the characterization of the mechanism(s) of action of isolated pure compounds.

• For the pharmacobotanical studies, the use of DNA barcodes combined with chemical profile analysis (TLC and HPLC) would be the required approach to solve the problems of quality control of medicinal plants.

• The use of HPLC or UHPLC coupled to hybrid state-of-the-art mass spectrometers are becoming a key tool for the rapid and accurate analysis and dereplication of substances in complex plant matrices to rapidly estimate their pharmacological potential, making unnecessary the use of in vivo models to validate it where polyphenols are the major constituents.

• The Revista Brasileira de Farmacognosia-Brazilian Journal of Pharmacognosy explicitly encourages the submission of chemically characterized extracts by GC (volatile oils), HPLC and/or NMR.

• It is a mandatory requirement for authors to include copies of NMR spectra for all new compounds and tested bioactive compounds from a natural source in the Supporting Information.

• For works describing the structural elucidation of novel natural products, it is mandatory to include evidences for the absolute configuration.

The following immediate rejection criteria apply
i. the manuscript does not fall into the areas of interest of the journal;

ii. manuscripts not formatted in accordance with the standards of the journal, e.g., papers requiring English proof-reading can be refused without further editorial inspection.

iii. the manuscript results are preliminary, e.g., chemical analysis using different reagents for the identification of classes and types of secondary metabolites; pharmacobotanical studies without the use of DNA barcodes combined with chemical profile analysis;

iv. results not presenting chemically characterized extracts;

v. experimental work based on preliminary biological and pharmacological analysis for extracts without the identification of the active constituent(s) or dereplication of major constituents by GC (volatile compounds), HPLC or NMR.

vi. manuscripts reporting activity data without comparison with a reference, without a positive control/appropriate control or not based on adequate statistics;

vii. the biological source (e.g. plant, microorganism, marine organism etc.) is not clearly identified, authenticated and documented;

viii. experimental work on antioxidant activity of crude extracts without isolation, identification and content estimation of the active compounds; phenolic compounds are widely spread in nature and fully recognized as antioxidants or scavengers;

ix. experimental work on antimicrobial activity with crude extracts without isolation and identification of the active compounds, with large MIC values (μg/ml) for antimicrobial activity (≥250 μg/ml for plant extracts and ≥50 μg/ml for pure compounds) and without appropriate identification of culture collections/strain designation codes;

x. experimental work on volatile oils with only one sample of a single plant specimen with a single chromatographic analysis and without appropriate statistical analyses; without oil yield (%) and characterization and component quantification not undertaken using GC-MS-FID. Analyses of the retention indices of the components not calculated using n-alkane homologous series together with analyses of some of the isolated natural components. Biological activity of essential oil without chemical characterization;

xi. too preliminary data using in vitro or in vivo assays will not be acceptable if (i) no information on the type of activity is given; (ii) single dose or very high concentrations (must show dose–response studies); (iii) repetition of a simple bioassay (usually one assay with replicates); (iv) lack of appropriate controls (solvents; positive or negative substances according to the study); (v) no IC50 values (if applicable); (vi) predictable bioactivity is described (e.g., therapeutic roles of polyphenols);

xii. pharmacological efficacy of plants in animal models when the results involved polyphenol-rich plant extracts;

xiii. use of only the brine shrimp assay (Artemia salina) to access the toxicity of extracts;

xiv. isolation and bioassay of well-known compounds with small or no relationship to the activity, or to the medicinal use of the plant without clear justification;

xv. manuscripts reporting pharmacological or biological activities of crude extracts or phytopharmaceuticals without chemical and technical standardization. Standardization of the plant extracts is considered to be the complete description of manufacturing parameters such as granulometry, solvent-plant ratio, time of extraction, solvent composition etc., together with marker quantification and chromatographic fingerprint analyses by HPLC.

xvi. structural elucidation of novel natural products without complete spectroscopic and spectrometric analyses (NMR and HRMS) and evidences for the absolute configuration.

Graphical abstract

A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise and simple pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. This graphical abstract should capture the reader's attention and, in conjunction with the manuscript title, should give the reader a quick visual impression of the essence of the manuscript without providing specific results. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 x times; 1328 pixels (h & w) or proportionally more. The image should be readable at a size of 5 x 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. *BJP does not accept Graphical abstract using images of animals.*

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Please provide 4 to 6 keywords which can be used for indexing purposes.

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Text Formatting

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Headings

Please use no more than three levels of displayed headings.

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References

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Cite references in the text by name and year in parentheses. Some examples:

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- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

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- Journal article
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  Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:
  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329
- Article by DOI
  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086
- Book
  South J, Blass B (2001) The future of modern genomics. Blackwell, London
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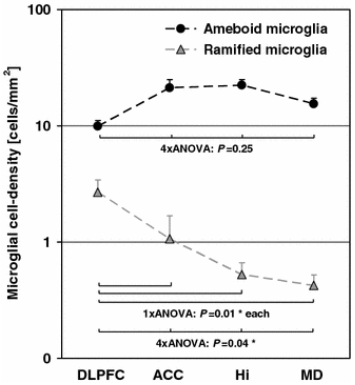

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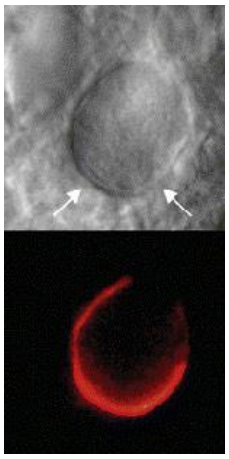

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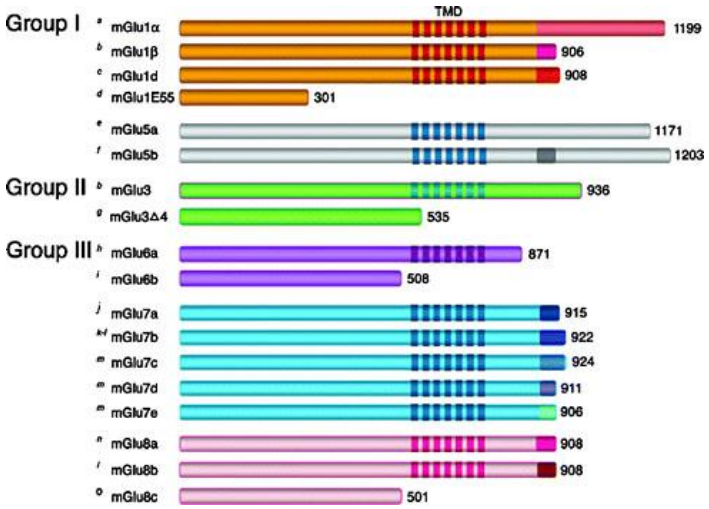

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Patients signed informed consent regarding publishing their data and photographs.
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