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AVALIAÇÃO DA TOXICIDADE E DAS ATIVIDADES ANTI-INFLAMATÓRIA E ANALGÉSICA DO EXTRATO ETANÓLICO de *Gomphrena celosioides* MART. (Amaranthaceae) EM ROEDORES

LUIS FERNANDO BENITEZ MACORINI

Dourados - MS
Ano 2021

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Orientadora: Profª. Drª. Arielle Cristina Arena
Co-orientadora: Profª. Drª. Candida Aparecida Leite Kassuya

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O ser humano sem Deus
não pode compreender
a si mesmo; como, também,
não poderá realizar-se sem Deus
(São João Paulo II)

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SÍMBOLOS

AINES	Anti-inflamatório não esteroidal
AMPc	Adenosina monofosfato cíclico
ANOVA	Análise de Variância
C57BL/6	C57 Black 6
CAA	Célula apresentadora de antígeno
CAM	Molécula de adesão endotelial
CFA	Freund's complete adjuvant
COX	Ciclooxigenase
DEXA	Dexametasona
EDTA	Ácido etilenodiamino tetra-acético
EECG	Extrato Etanólico de *Gomphrena celosioides*
ESI-MS	Espectrometria de massas com ionização por electrospray
ESL-1	Ligante de E-selectina
IASP	Associação internacional para estudo de dor
ICAM	Molécula de adesão intercelular
IL	Interleucina
INDO	Indometacina
LFA-1	Antígeno associado a função linfocitária-1
LOX	Lipoxigenase
LPS	Lipopolissacarídeo
MAD-CAM-1	Moléculas de adesão de mucosa
NFkB	Fator nuclear kappa B
NGF	Fator de crescimento nervoso
NO	Oxido nítrico
NSAIDs	Anti-inflamatório não esteroidal
OECD	Organização para cooperação econômica e desenvolvimento
PBS	Tampão fosfato-salino
PPARs	Receptor ativado por proliferadores de peroxissoma
PSGL-1	Ligantes glicoproteínas P-selectina
TLR	Receptor toll-like
TNF	Fator de necrose tumoral

VCAM	Molécula de adesão vascular

AVALIAÇÃO DA TOXICIDADE E DAS ATIVIDADES ANTI-INFLAMATÓRIA E ANALGÉSICA DO EXTRATO ETANÓLICO de *Gomphrena celosioides* MART. (Amaranthaceae) EM ROEDORES

RESUMO

Introdução: *Gomphrena celosioides*, é conhecida popularmente no Brasil como "Perpétua Brava" e utilizada para o tratamento de diversas doenças, tais como, hepáticas, dermatológicas, dismenorréia, infecções, dores, entre outras. Estudo químico do extrato etanólico das folhas de *G. celosioides* mostrou a presença de ácido málico, ácido cafeico, ácido ferúlico, catequina, ácido vanílico, irisona B, dimetoxiflavona, cafeoil-glicose. Além disso algumas atividades biológicas da planta, incluindo atividade anti-inflamatória, diurética, antimicrobiana, gastroprotetiva, antioxidante e imunomodulação já foram descritas na literatura. Neste contexto, o objetivo desse estudo foi avaliar o potencial anti-inflamatório, antiartrítico, analgésico e a toxicidade do extrato etanólico das folhas de *Gomphrena celosioides* (EEGC) em roedores. **Material e Métodos:** Para avaliação do Edema de pata, hiperalgesia mecânica e alodinia ao frio os camundongos C57Bl6 foram tratados via oral com EEGC (300, 700 ou 1000 mg/kg), salina (grupo controle) ou dexametasona (1 mg/kg) e após 30 minutos foram induzidos com carragenina. O modelo de pleurisia em camundongo Swiss fêmeas foi induzido por administração de 1 ml de carragenina intrapleural após 30 minutos de tratamento com EEGC (300, 700 ou 1000 mg/kg), salina (grupo controle) ou dexametasona (1 mg/kg). Para indução de artrite, foi utilizado modelo inflamatório induzido por zymosan em região intra articular de camundongos Swiss tratados 30 minutos antes com EEGC 300 mg/kg, salina (grupo controle) ou dexametasona 1 (mg/kg). Para avaliação da peritonite, camundongos Swiss machos foram divididos em: grupo naive, controle, EEGC 300 mg/kg e dexametasona (1 mg/kg). Após 30 minutos de tratamento, a peritonite foi induzida por Zymosan. Rolamento e adesão de leucócitos em endotelo mesentérico foi avaliado após indução por carragenina e tratamento com EEGC 300 mg/kg e indometacina (5 mg/kg). O modelo persistente para avaliação anti-inflamatória e analgésica foi realizado com Freund's Complete Adjuvant (CFA) em camundongos C57BL6 tratados com EEGC 100 mg/kg, controle salina e dexametasona (1 mg/kg) por 22 dias. Para a avaliação da toxicidade foi utilizado modelo agudo com ratos Wistar seguindo guideline OECD e a toxicidade subaguda foi avaliado utilizando camundongos Swiss, avaliados durante 28 dias, verificando-se parâmetros hipocráticos, comportamentais, histológicos, bioquímicos e hematológicos. **Resultados**: EEGC apresentou

redução do Edema de Pata em todas as doses nas 3 horas avaliadas e ainda foi possível observar redução da hiperalgesia mecânica na terceira hora em todas as doses, bem como uma redução da alodinia ao frio, com 3 horas na dose de 700 mg/kg de EEGC e com 4 horas em 700 e 1000 mg/kg de EEGC em modelo induzido por carragenina. Após indução de pleurisia, foi possível observar uma redução da migração leucocitária nas doses de 700 e 1000 mg/kg de EEGC, porém não houve redução do extravasamento proteico em cavidade pleural. Foi observado redução da migração leucocitária e da hiperalgesia mecânica em modelo de artrite induzido por Zymosan, após tratamento com EEGC 300 mg/kg. O EEGC também reduziu a migração leucocitária em peritonite induzida por Zymosan na dose de 300 mg/kg de EEGC, porém não houve diferença na dosagem de óxido nítrico (NO) do lavado peritoneal. Após avaliação da redução de leucócitos em cavidade peritoneal, foi avaliado o rolamento e adesão dos leucócitos em endotélio mesentérico e observado redução, tanto do rolamento, quanto da adesão após indução com carragenina e tratamento com EEGC 300 mg/kg. Em modelo de inflamação e dor persistente induzido por CFA, animais tratados com EEGC 100 mg/kg apresentaram redução do edema de pata no 22º dia, alodinia ao frio em 6, 11 e 16 dias, e redução da hiperalgesia mecânica em todos os dias avaliados. A análise toxicológica aguda após administração de 2000 mg/kg de EEGC em ratos Wistar não demonstrou alteração nos parâmetros avaliados, assim como também não apresentou alterações em parâmetros avaliados da toxidade subaguda nas doses de 75, 150 e 300 mg/kg de EEGC. **Conclusão**: O EEGC demonstrou potencial anti-inflamatório, antiartrítico e analgésico em diferentes modelos de indução agudo e persistente, com diminuição do rolamento e adesão endotelial de leucócitos. Além disso, o extrato não apresentou toxicidade em modelo agudo e subagudo.

Palavras-chave: Inflamação; dor neuropática; dor aguda; interleucinas; toxicidade aguda; toxicidade sub-aguda; migração leucocitária; diapedese.

EVALUATION OF TOXICITY AND ANTI-INFLAMMATORY AND ANALGESIC ACTIVITIES OF THE ETHANOLIC EXTRACT of *Gomphrena celosioides* MART. (Amaranthaceae) in rodents

ABSTRACT

Introduction: *Gomphrena celosioides*, is popularly known in Brazil as "Perpétua Brava" and used for the treatment of several diseases: liver, dermatological, dysmenorrhea, infections, pain, among others. Chemical study of the ethanolic extract of the leaves showed the presence of malic acid, caffeic acid, ferulic acid, catechin, vanillic acid, irisone B, dimethoxyflavone, caffeyl-glucose, the biological activities of the plant have already been described as anti-inflammatory, diuretic, antimicrobial, gastroprotection, antioxidant and immunomodulators. In this context, the objective of the research was to evaluate the anti-inflammatory, anti arthritic, analgesic and toxicological potential of the ethanolic extract of Gomphrena celosioides (EEGC). **Materials and Method:** Paw edema, mechanical hyperalgesia and cold allodine were induced in male Swiss mice with carrageenan after oral treatment of EEGC at doses of 300, 700 and 1000 mg/kg, control and 1 mg/kg dexamethasone. The female Swiss mouse pleurisy model was induced after administration of 1 mL of intrapleural carrageenan and the animals treated with EEGC at doses of 300, 700 and 1000 mg/kg, control and dexamethasone (1 mg/kg). For arthritis induction, C57Bl6 mice were induced with intra-articular zymosan and after 30 minutes treated with EEGC 300 mg / kg, control and dexamethasone 1 (mg/kg). For the evaluation of peritonitis, male Swiss mice were divided into a naive group, control, EEGC 300 mg/kg and dexamethasone (1 mg/kg). 30 minutes after treatment, peritonitis was induced by zymosan. Leukocyte rolling and adhesion in mesenteric endothelium was evaluated after carrageenan induction and treatment with EEGC 300 mg/kg and Indomethacin (5 mg/kg). The persistent model for anti-inflammatory and analgesic evaluation was performed with Freund's Complete Adjuvant (CFA) in C57BL6 mice treated with EEGC 100 mg/kg, saline control and dexamethasone 1 mg/kg for 22 days. The toxicological evaluation was determined by an acute model using Wistar rats following OECD 2008a guideline and subacute was performed with Swiss mice evaluated for 28 days, evaluating hypocratic, behavioral, histological, biochemical and hematological parameters. **Results**: EEGC showed a reduction in Paw Edema in all doses in the 3 hours evaluated, it was still possible to observe a reduction in

mechanical hyperalgesia in the third hour in all evaluated doses and a reduction in cold allodine with 3 hours in the dose of 700 mg / kg and with 4 hours in 700 and 1000 mg/kg in a model induced by carrageenan. After induction of pleurisy, it was possible to observe a reduction in leukocyte migration at doses of 700 and 1000 mg/kg, but there was no reduction in protein leakage in the pleural cavity. A reduction in leukocyte migration and mechanical hyperalgesia was observed in a model of arthritis induced by Zymosan after treatment with EEGC 300 mg/kg. EEGC also reduced leukocyte migration in Zymosan-induced peritonitis at a dose of 300 mg/kg, but there was no difference in the nitric oxide (NO) dosage. After assessing the reduction of leukocytes in the peritoneal cavity, the leukocyte rolling and adhesion in mesenteric endothelium was evaluated and a reduction in both rolling and adhesion was observed after induction with carrageenan and treatment with 300 mg/kg EEGC. In a persistent CFA model, animals treated with EEGC 100 mg/kg reduced paw edema on the 22nd day, cold allodynia on 6, 11 and 16 days and reduced mechanical hyperalgesia on all evaluated days. The acute toxicological analysis after administration of 2000 mg/kg in Wistar rats, there was no change in the parameters evaluated as well as no changes in the parameters evaluated for subacute toxicity at doses of 75, 150 and 300 mg / kg. **Conclusion**: EEGC demonstrated anti-inflammatory, antiarthritic and analgesic potential in different models of acute and persistent induction, with decreased bearing and leukocyte endothelial adhesion. Still, it did not present toxicity in an acute and sub-acute evaluation model.

***Keywords*:** Inflammation; neuropathic pain; acute pain; interleukins; acute toxicity; sub-acute toxicity; leukocyte migration; diapedesis.

SUMÁRIO

INTRODUÇÃO

A etnofarmacologia traz o uso de produtos naturais como importantes fontes terapêuticas utilizadas em diversas culturas, transformando-a em uma importante ferramenta na descoberta de novas drogas, instigando a busca por medidas profiláticas eficazes para o tratamento de diversas doenças ou desordens fisiológicas (COSTA et al., 2010).

As plantas medicinais enquadram-se nesse grupo de produtos naturais com potencial farmacológico, possuindo importantes compostos químicos em suas diversas regiões anatômicas, como folhas, cascas, raízes e frutos. Dentre estes compostos, destacam-se classes dos flavonoides, alcaloides, taninos, saponinas e esteroides, que já demonstraram diversos efeitos biológicos, como atividade anti-hipertensiva, anti-inflamatória, antitumoral, gastroprotetora, antimicrobiana, além de efeitos citotóxicos (FILHO; YUNE, 1998; QUEIROZ et al., 2003; MELO, 2021).

Dentre as plantas medicinais com relevância farmacológica, destaca-se a *Gomphrena celosioides*, pertencente à família Amaranthaceae, com mais de 140 espécies distribuídas na América, sendo 46 registradas já no Brasil (SANGARE et al., 2012; VIEIRA et al., 1994). A *G. celosioides* é conhecida popularmente no Brasil como "Perpétua Brava" e é utilizada para o tratamento de diversas doenças hepáticas, dermatológicas, dismenorréia, infecções, para combater dores, entre outras (SANGARE et al., 2012). Pesquisas realizadas com extratos de *G. celosioides* demonstraram atividades biológicas, como ação anti-inflamatória, diurética, antimicrobiana, gastroproteção, antioxidante e imunomoduladores (VIEIRA et al., 1994; OLUWABUNMI; OLUWABUNMI, 2015; GHONIME et al., 2015; EKUNDAYO, 2010).

Estudos químicos da planta já demonstraram a presença de compostos como hidrocarbonetos, álcoois, esteróides, terpenos, ecdisteroides, flavonóides, saponinas, aminoacidos, butacianina, açucares redutores e cetoses, o que justificaria suas propriedades biológicas (SALVADOR, 2012). Vale ainda destacar que pesquisa realizada por De Paula Vasconcelos et al. (2017), identificou no extrato etanólico das folhas da planta utilizada para esse trabalho, compostos como, ácido málico, ácido cafeico, ácido ferúlico, catequina, ácido vanílico, irisone B, dimetoxiflavona e cafeoil-glicose. Além desses compostos citados, Dosumu et al. (2014), isolaram o composto aurantiamida do extrato hexano das partes aéreas de *G. celosioides*, que possui atividades biológicas como anti-inflamatória, antinoceptivas, imunomodulatorias e inibidor seletivo de catepsinas (ISSHIKI et al., 2001; PAN et al., 2015; YANG et al., 2015).

Neste contexto, a *Gomphrena celosioides*, demonstra grande potencial biológico e instiga a busca por ações anti-inflamatórias, analgésicas e bem como a avaliação da toxicidade.

2 REVISÃO DE LITERATURA

2.1 Plantas medicinais

Plantas medicinais são utilizadas desde os primórdios da vida em sociedade, na busca de alternativas para tratamento de diversas doenças (QUEIROZ, 2003). Segundo a organização mundial da saúde (OMS), cerca de 80% da população mundial faz ou já fez a utilização de plantas no objetivo de buscar algum alívio para desordens ou doenças que a acometem.

As plantas medicinais são consideradas usinas químicas e naturalmente formam diversos compostos químicos como flavonóides, alcalóides, terpenos, saponinas, aminoácidos, peptídeos com grande potencial biológico, sendo muitos já transformados em medicamentos e seus produtos podem ser encontrados nas prateleiras de drogarias espalhadas pelo mundo (QUEIROZ, 2003).

Em adição, a etnofarmacologia auxilia a ciência, trazendo o conhecimento do uso popular de produtos naturais como importantes fontes terapêuticas utilizadas em diversas culturas, transformando-a em uma importante ferramenta na descoberta de novas drogas, principalmente devido à necessidade de medidas terapêuticas eficazes (COSTA et al., 2010; ADJANOHOUN et al., 1989). Como exemplo, pesquisa realizada por Salehi et al. (2019) traz uma discussão referente a diversas plantas que demonstraram grande potencial no combate a diabetes como possível tratamento complementar aos convencionais já descritos. Além disso, diversas plantas com propriedades anti-inflamatórias e analgésicas também já foram descritas na literatura, das quais muitas não têm como mecanismo alvo processos inibitórios das enzimas ciclo-oxigenase 1 e 2 (COX-1 e COX-2, respectivamente), o que diminui os efeitos colaterais presentes em tratamentos com anti-inflamatórios não esteroidais (AINES) convencionais (LOPES et al. 2019; NAKAMORI et al., 2019).

Entre as plantas medicinais com relevância terapêutica, destacam-se aquelas pertencentes à família Amaranthaceae, que apresenta cerca de 140 gêneros e mais de 2000 espécies amplamente distribuídas em locais com clímas tropical e subtropical, sendo que no Brasil foram descritos cerca de 20 gêneros e 100 espécies. Os representantes desta família apresentam-se com características de arbustos, subarbustos, ervas daninhas e/ou trepadeiras, podendo ser encontradas em diversos tipos de terrenos, destacando ainda sua importante presença no cerrado, incluindo o Sul Matogrossense (SIQUEIRA, 2002; SOUZA; LORENZI, 2005; VIEIRA et al., 1994). Dentre as plantas que compõem essa família, destaca-se o gênero *Gomphrena*, com mais de 120 espécies

descritas, sendo que só no Brasil, já foram identificadas 46 espécies e dessas, cerca de 19 presentes no cerrado, incluindo a *Gomphrena celosioides* (FANK-DE-CARVALHO; GRACIANO-RIBEIRO, 2005; WAGNER et al., 1999). Além disso, importantes atividades biológicas já foram descritas sobre plantas do gênero relatado, tais como propriedades antimicrobianas, anti-inflamatórios, diuréticas, efeito gastroprotetor, ação antioxidante, citotóxica, antitumoral, entre outras (DE MOURA et al., 2004; PROMRAKSA et al., 2019; POMILIO et al., 1992; POMILIO et al., 1994; SILVA et al., 2012).

2.2 *Gomphrena celosioides*

A *G. celosioides* está amplamente distribuída nos continentes da América do Sul, Asia, África e Oceania. Tem característica de erva daninha de vida curta possuindo caule ereto de 10 a 20 cm de comprimento com poucos ramos, apresentando folhas oblongo-lanceoladas ou oblongo-obovadas (WAGNER et al., 1999). No Brasil ela é conhecida popularmente como "Perpétua Brava" e descrita como erva nociva, sendo pouco consumida por animais silvestres (FANK-DE-CARVALHO; GRACIANO-RIBEIRO, 2005). Esta planta vem sendo utilizada na medicina folclórica popular para tratamentos alternativos para combater dores, distúrbios gástricos, doenças da pele, dermatites, antimicrobiano, antitumoral, condições alérgicas, hemorróidas, disminorréia, malária (NANDINI et al., 2018), doenças respiratórias, febre, tosse, doenças cardíacas (OLIVEIRA, 2008), urolitíase (PRACHI et al., 2009), refluxo, diarréia de bebês (MILLION, 2017), diurético e bronquite infecciosa (OLUWABUNMI; ABIOLA T., 2015).

Estudos realizados com diversos tipos de extração da planta apresentaram várias propriedades biológicas, mostrando grande potencial. Dentre estas propriedades, de Paula Vasconcelos et al. (2018) demonstraram atividade diurética, redutora da pressão arterial e proteção a remodelação cardíaca do extrato etanólico das folhas *G. celosióides* em modelo utilizando ratos *Wistar*.

As avaliações da composição química da planta já identificaram a presença de hidrocarbonos, alcool, esteróides, terpenos, ecdisteroides, flavonóides, saponinas, aminoacidos, redutores de açúcares e butacianina (DOSUMU et al., 2010; DOSUMU et al., 2014). Em especial, o extrato etanólico de *G. celosióides,* objeto desse estudo, apresentou em sua composição o ácido málico, ácido cafeico, ácido ferúlico, catequina, ácido vanílico, irisone B, dimetoxiflavona e cafeoil-glicose (DE PAULA VASCONCELOS et al., 2017). Além destes compostos, já foram isolados Aurantiamida a partir de extrato hexânico e 3-(4-hidroxifenil) metilpropanoato do extrato

metanólico da planta (DOSUMU et al., 2010; DOSUMU et al., 2014). Aurantiamida é caracterizada como um dipeptídeo com potencial inibidor da ativação de mastócitos, por inibição do Fator de Necrose Tumoral (TNF-α) e Interleucina 2 (IL-2) em processos inflamatórios (SASHIDHARA et al., 2009). Em sequência, pesquisa realizada por Isshiki et al. (2001), identificaram a aurantiamida como um inibidor seletivo de catepsina. Essa enzima é caracterizada como proteases ativadas em pH ácido, localizadas principalmente em lisossomos, relacionadas a diversas condições patológicas, incluindo processos inflamatórios e infecciosos (MIJANOVIĆ et al., 2019). Ainda, pesquisa realizada por Yang et al. (2015) demonstraram propriedades anti-tumoral de aurantiamida isolada da planta *Clematis terniflora* DC. Enquanto, Mastromarino et al. (2019), apresentaram o efeito antogonista de aurantiamida na de ativação de neutrófilos e quimiotaxia, mostrando um bom potencial anti-inflamatório.

Além disto, o extrato metanólico de *G. celosióides* também apresentou efeito gastoprotetor em ratos Wistar induzidos a úlcera gástrica por indometacina (OLUWABUNMI; ABIOLA, 2015). de Moura et al. (2004) também relataram a atividade antimicrobiana da planta frente a *Staphylococcus aureus* e *Salmonella typhi*. Entretanto, o efeito antitumoral em modelo de colangiocarcinoma utilizando extrato etanólico de *G. celosióides* não foi satisfatório quando comparados a outras plantas e drogas referências; no entratanto, é importante que outras avaliações sejam realizadas (PROMRAKSA et al., 2019). Dosumu et al. (2010) observaram atividade antiparasitária do extrato acetato de etila da planta frente a *Fasciola gigantica, Taenia solium* e *Pheretimapasthuma*, e antimicrobiana frente à *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa, Escherichia coli* e *Salmonella typhi,* mostrando grande potencial antimicrobiano. *G. Celosióides* também apresentou efeito antimalárico em triagem realizada por Gesseler et al. 1994. Já o extrato metanólico apresentou grande potencial antifúngico frente à *Candida albican*s, *Aspergillus niger* e *Trichophyton.* Entretanto, a fração 3-(4-hidroxifenil) metilpropanoato não obteve atividade antimicrobiana nem antiparasitária suficiente (DOSUMU, et al. 2010).

Além das propriedades discutidas acima, Ghonime et al. (2015) avaliaram a ação de imunomodulação e atividade antioxidante *in vitro* de *G. celosióides*, onde foi possível observar a redução da liberação de óxido nítrico (NO), expressão gênica de TNF e inibição seletiva de COX-2 após estímulo de macrófago por lipopolissacarídeo (LPS), mostrando assim o grande potencial anti-inflamatório e analgésico da planta.

Dentre estes possíveis potencias, associados com a identificação de diversos compostos presentes em extratos variados de *G. celosioides* e sua utilização popular, destaca-se a importância

da investigação de seus efeitos analgésicos e anti-inflamatórios, bem como sua avaliação toxicológica.

Figura 1. *Gomphrena celosioides*. Fonte: Marcos José Salvador

2.3 Inflamação

Os mecanismos inflamatórios são processos fisiológicos iniciados em resposta a uma injuria/lesão para inibir um agente agressor e promover reparos teciduais. Porém, apesar de ser um importante mecanismo fisiológico, uma ação excessiva de cascata inflamatória aguda ou crônica pode promover danos teciduais de grande impacto (CRUVINEL et al., 2010).

A inflamação aguda é caracterizada por uma resposta imediata a um agente agressor tecidual, onde ocorre grande migração leucocitária, ativado por liberação de citocinas e quimiciocinas, além do extravasamento proteíco que proporciona a formação de edema. Já a crônica, observa-se uma inflamação ativa persistente, promovendo ao longo do tempo, danos muitas vezes irreversíveis, estimulando a fibrose e/ou necrose tissular, como em casos de doenças autoimunes, exemplo da artrite reumatóide, ou exposição prolongada a um agente agressor (BONET; HERNANDEZ, 2014).

Uma resposta inflamatória tem início imediato após estímulo local, e é mediada pela ação de leucócitos apresentadores de antígeno e/ou liberação de citocinas como, interleucinas pró inflamatórias (IL1, IL2, IL6, IL7), TNF, histaminas, quimiocinas e proteínas do sistema complemento (DE OLIVEIRA et al., 2011).

É importante destacar, que após a liberação dos mediadores inflamatórios, inicia-se a fase vascular da inflamação, onde substâncias como histaminas, são liberadas pelos mastócitos para o líquido extracelular e se ligam a receptores endoteliais H1 e H2 dos vasos sanguíneos estimulando efeitos biológicos, como a vasodilatação e o aumento da permeabilidade vascular, alterando a hemodinâmica local (CRIADO et al., 2010). Após a vasodilatação, é potencializado a marginalização dos leucócitos e como consequência à ativação de moléculas de adesão, iniciando o rolamento e a transmigração leucocitária, melhor descrito no item 2.4 (BONET; HERNANDEZ, 2014). A liberação de citocinas pró inflamatórias como IL-1, IL-2 e TNFα, estimulam o ciclo das prostaglandinas e de leucotrienos liberados pela ativação a partir da cascata de metabolização do ácido aracdônico pela expressão de ciclooxigenases (COX) e lipoxigenases (LOX).

A expressão gênica de COX pode liberar ao menos três isoformas da enzima conhecidas como COX-1, com característica constitutiva, presentes em diversos tecidos e com grande importância fisiológicas como, proteção da mucosa gástrica, da função renal, do endotélio vascular, entre outras. A COX-2, que possuem características induzível atuando diretamente em processos inflamatórios e de dor e COX-3 expressa principalmente em sistema nervoso central (CRUVINEL et al., 2010; OKSUZ et al., 2020).

A isoforma COX-2 é a principal responsável pela produção dos mediadores prostanoides (prostaglandinas e tromboxanos) da inflamação, enquanto a isoforma COX-3, embora seja transcrita através do mesmo gene da COX-1, possuindo praticamente as mesmas características fisiológicas teciduais, expressam polipeptídeos funcionais com grande sensibilidade aos analgésicos e antipiréticos, porém com uma sensibilidade reduzida a ação dos AINES. A expressão gênica de COX-3 já foi identificada e descrita em região de glândula pituitária e no hipotálamo, estando associados a processos de febre e correlacionando o seu potencial com ação agonista de receptores ativados por proliferadores de peroxissoma (PPARs) (ANDRADE et al., 2004; PRZYBYŁA et al., 2020).

Nos leucócitos, parte das moléculas de ácido araquidônico (AA) são metabolizadas pela ação das lipo-oxigenases (5-, 12- e 15-LOX), resultando na formação de leucotrienos (LT). Existem alguns tipos de leucotrienos, incluindo os leucotrienos B4 que são descritos como importantes mediadores de doenças inflamatórias crônicas, estimulando leucócitos polimorfonucleares, ativando a produção de outros leucotrienos, além de atuar como molécula sinalizadora. Essas ações patológicas dos leucotrienos levaram a ser consideradas alvos farmacológicos para ações anti-inflamatórias e analgésicas (BITENCOURT et al., 2013).

Nesse contexto, todos esses mediadores liberados na inflamação são os responsáveis por desenvolver os sinais clássicos como dor, rubor, calor e edema (CRUVINEL et al., 2010). Para

minimizar esses efeios indesejáveis, a utilização de anti-inflamatórios acaba por vezes sendo a escolha, e dentre as drogas mais utilizadas estão os anti-inflamatórios não esteroidais (AINES). Entretanto, apesar de atuar na diminuição dos efeitos inflamatórios, por vezes, os AINES também estimulam efeitos colaterais importantes, pois não são seletivos de COX-2, atuando também na inibição de COX-1, intereferindo diretamente em mecanismos fisiológicos.

Alguns estudos vêm sendo desenvolvidos para produzir novas drogas anti-inflamatórias seletivas que minimizem os danos fisiológicos. Dentre estas drogas, destaca-se a classe dos COXIBES, inibidores específicos de COX-2. Entretanto, efeitos cardiovasculares já foram identificados em medicamentos da classe, e estudos amplos vem sendo desenvolvidos para garantia de segurança de sua utilização (MACÍAS et al., 2010; PATRONO, 2016; BHALA et al., 2013).

2.4 Rolamento, adesão e migração de leucócitos em processos inflamatórios

Os leucócitos são células do sistema imune que tem função fisiológica atuar no combate a agentes agressores (bactérias, fungos, vírus, etc) através do reconhecimento de padrões moleculares associados a lesões celulares (DAMPs) ou a padrões moleculares associados a patógenos (PAMPs) (DA SILVA, 2014). Esses padrões ativados estimulam a liberação de citocinas quimiotáticas e a mudança na tensão hemodinâmica, levando ao direcionamento/marginalização e a migração dos leucócitos para os tecidos, através de mecanismo conhecido como diapedese (KOLACZKOWSKA; KUBES 2013).

Em processos inflamatórios, mecanismos para recrutamento e migração dos leucócitos da corrente sanguínea para os tecidos são ativados através de diversos eventos moleculares especializados que irão garantir uma maior interação entre células leucocitárias e as endoteliais, principalmente das vênulas pós capilares. Entretanto, é relatado que a ativação em excesso da migração leucocitária para o combate a algum agente agressor tecidual, pode desenvolver efeitos adversos e muitas vezes irreversíveis (ALBERTS et al., 2002; BROERING et al., 2019; WAGNER, 2000).

Essa ação pode acontecer em quatro etapas: marginalização, rolamento, adesão e migração transendotelial. É importante destacar que, essas etapas ocorrem após a ativação de moléculas de adesão endotelial (CAMs) (DA SILVA, 2014). As CAMs, após ativadas, atuam na ligação aos leucócitos e dão características específicas através de padrões seletivo e assim caracterizam as respostas inflamatórias. Essas moléculas podem ser divididas em cinco classes, caderinas, mucinas, selectinas, integrinas e a superclasse de imunoglobulinas (SIMON; GREEN, 2005).

A primeira etapa para migração leucocitária, ocorre após a interação entre as CAMs da classe das selectinas e seus ligantes. Essa interação ocorre principalmente pela ligação das selectinas as mucinas e carboidratos expressos nas superfícies dos leucócitos promovendo sua captura e fixação. As selectinas estão divididas em L-Selectina, presente na superfície dos leucócitos, P e E-Selectinas presentes em células endoteliais (RIVERA-NIEVES et al., 2008; KOLACZKOWSKA e KUBES 2013; DA SILVA, 2014).

A P-selectina é produzida constitutivamente, porém não são expressas em superfície de células endoteliais não ativadas, ficando armazenadas em vesículas conhecidas como corpos de Weibel-Palade. Quando a célula endotelial é sensibilizada por citocinas pró-inflamatórias, ocorre a expressão das P-selectinas na superfície de células endoteliais facilitando a ação dos ligantes glicoproteínas P-selectina (PSGL-1) dos leucócitos permitindo o rolamento do mesmo no vaso sanguíneo. Já a E-selectina, expressa em superfície de células endoteliais, são ativadas pela sensibilização de citocinas como IL-1 e TNFα e atuam na transição da fase de rolamento e adesão dos leucócitos, mediando o rolamento principalmente pela ativação parcial de antígeno-1 associado a função linfocitária (LFA-1), PSGL-1 ou ligante de E-selectina (ESL-1). A L-selectina (CD62L) é produzida constitutivamente e estão presentes nas paredes dos granulócitos, monócitos e principalmente dos linfócitos, sendo ativadas pela liberação de IL-8, proteínas do complemento, fatores ativadores plaquetários e leucotrieno B4 (LEY et al., 2007; DA SILVA, 2014).

Uma característica da interação entre os ligantes de selectinas, é a pouca estabilidade entre eles, que fisiologiacamente, diminui a velocidade de deslocamento celular, porém mantém seguindo o fluxo sanguíneo. Nessa fase, a ligação entre glicoproteína dos leucócitos as selectinas e a ação de agentes quimiotáticos, levam a um aumento de número e também de afinidade das integrinas presentes na superfície dos leucócitos às moléculas da família das super imunoglobulinas presentes nas células endoteliais classificadas como moléculas de adesão intercelular (ICAM)-1, ICAM-2, ICAM-3, molécula de adesão de células vasculares (VCAM) e moléculas de adesão de mucosa (Mad-CAM-1) (ALBERTS, 2002; DA SILVA, 2014; SIMON; GREEN, 2005; WAGNER; ROTH, 2000;).

Assim, após a ligação das integrinas com as super imunoglobulinas, que promovem uma espécie de “ancora” fixando os leucócitos no endotélio, ocorre a última interação do leucócito promovendo a transpassagem endotelial, onde a ligação com moléculas de adesão celular endotélio-plaqueta-1 PECAM, presentes nas junções intercelulares do endotélio, estimulam a formação de pseudópodos celulares e facilita a passagem pela parede do endotélio. Após a migração, os leucócitos são atraídos para área de lesão tecidual por quimiotaxia, seguindo o gradiente de concentração das quimiocinas (DA SILVA, 2014; WIMMER, et al., 2019).

2.5 Dor

A Associação Internacional para o Estudo de Dor (IASP) define dor como uma sensação subjetiva após injuria ou lesão tecidual, sendo conceituada a partir do processamento nociceptivo. Esse conceito, entretanto, vem sendo discutido e evidências mostram que, além dessa associação, existam também a relação de dor sem lesão tecidual e sim por alterações neurofuncionais em processos de sinapses pela ação de neuromediadores e neurotransmissores, o que dificulta a compreensão de sua etiologia (ROCHA et al., 2007). Sendo assim, podemos dividir os estímulos de dor em periféricos e central (neuropática) (ASHMAWI; FREIRE, 2016).

O estímulo periférico da dor inicia quando receptores sensoriais primários (nociceptores) localizados nas fibras nervosas periféricas são sensibilizados, sinalizando as células neurais a promover alteração no potencial de ação, levando a exocitose de neurotransmissores na raiz dorsal da medula espinhal. Esse processo estimula o sistema nervoso central a sensibilizar o córtex cerebral e interpretar a sensação dolorosa (LEVINE; TAIWO, 1994; MITSI; ZACHARIOU, 2016).

É importante destacar, que o estímulo aos nociceptores para sensibilização das fibras podem acontecer por ações mecânica, ou de temperatura e também por substâncias químicas como ácidos, acetilcolina, bradicinina, histamina, tromboxanos, interleucinas, fator de necrose tumoral (TNFα), serotonina, prostaglandinas, leucotrienos, fator de ativação plaquetário, radicais lívres, potássio, fator de crescimento nervoso (NGF) e monofosfato cíclico de adenosina (AMPc), além de migração leucocitária para reparo tecidual levando a liberação de citocinas inflamatórias no local (ROCHA et al., 2007; SNEDDON, 2018).

As fibras nocipetivas podem ser divididas em fibras Aδ e C, sendo as fibras Aδ, com presença de mielina em sua constituição, possuindo característica de velocidade rápida e saltatória de transmissão e está relacionada principalmente ao estímulo agudo da dor, e as fibras C que possuem característica de células desmielinizadas com transmissão do impulso mais lenta (ROCHA et al., 2007; LEVINE; TAIWO, 1994).

Além disso, a dor central, também caracterizada como dor neuropática, provém de uma cascata de reações moleculares e liberação de neuromoduladores e neurotransmissores que sensibilizam regiões do sistema nervoso central e dificulta a compreensão de sua etiologia. Os agentes etiológicos da dor neuropática já foram descritos como doenças autoimunes, acidente vascular encefálico, infecções e traumas encefálicos ou medular, entre outras (GARCIA et al, 2016). Ainda destaca-se, que centro de dopamina do mesencéfalo compreende ação direta na dor

neuropática, sendo modulado a partir de estímulos agudos ou crônicos. Estudos indicam, também, que a dor modulada a partir do sistema dopaminérgico tem ação direta no comportamento estando relacionado com desenvolvimento de ansiedade e depressão (PINHEIRO et al., 2014; MITSI; ZACHARIOU, 2016). É importante destacar, que esses mecanismos ainda podem ser ativados por ação imunológica e inflamatória a partir da liberação de citocinas como TNF-α e IL-1β após macrófagos serem ativados em região onde ocorre lesão neural (MOALEM; TRACEY, 2006; BALIKI et al., 2015)

O entendimento dos mecanismos de dor pode influenciar na descoberta de tratamentos para inibição dos estímulos dos nociceptores e bloqueio da transmissão da sinalização para a dor.

2.6 Modelos experimentais de Inflamação e Dor

Modelos experimentais para inflamação e dor estão descritos na literatura para auxiliar na busca por novas moléculas anti-inflamatórias e analgésicas onde podemos citar a os modelos de indução estimulados por Carragenina e Zymosan para fase aguda e "Complete Freund's adjuvante" (CFA) para fase crônica (DALMARCO et al., 2008; KAWAI; AKIRA, 2007).

A carragenina é uma substância caracterizada como polissacarídeos sulfatados provenientes de algas vermelhas sendo a espécie predominante a *Chondrus crispus*, é um agente inflamatório muito utilizado em modelos *in vivo* na indução de inflamação aguda devido sua ação de estimular o exsudato principalmente em cavidades como pleura e peritoneal, além de formação de edema em região intraplantar e estímulo a hiperalgesia (WINTER et al., 1962).

Destaca-se ainda, que a ação da carragenina em modelo de indução inflamatória ocorre em duas fases, sendo a primeira com aumento de histaminas, serotonina e bradicina enquanto a segunda fase promovendo o aumento de liberação de prostaglandinas e óxido nítrico aumentando a sensibilidade local. Além disso, outros relatos da literatura, demonstraram um aumento na liberação de interleucinas pró inflamatórias, TNF-α e aumento da expressão de moléculas de adesão ao endotélio como P-selectina e ICAM-1. (WINTER et al., 1962; LEVY, 1969; CUZZOCREA et al., 2006; DALMARCO et al., 2008).

Outro modelo de indução de processo inflamatório agudo é o Zymosan, extraída de um fungo *Saccaromyces cerevisae* que age no diretamente no estímulo a COX-2, além do estímulo a formação de edema e exsudato através da migração leucocitária. O zymosan sensibiliza receptores Tool-like ativando NF-KB devido a grande liberação de interleucinas pró inflamatórias, muito utilizado em modelos de indução de artrite e peritonite em fase aguda. (OZINSKY et al., 2000; SATO et al., 2003; KAWAI; AKIRA, 2007)

Entre os modelos experimentais de estímulo a inflamação e dor crônica destaca-se o "Complete Freund's adjuvante" (CFA) caracterizado por antígenos de *Mycobacterium* inativada que após a injeção intraplantar estimula a expressão de diversas isoformas de NO em região medular espinhal, além disso, o estímulo por CFA induz poliartrite migratória em ratos devido uma resposta imune modulada pelo antígeno bacteriano e não por ação infecciosa, essa resposta se assemelha com a artrite reumatoide humana (KURAOKA-OLIVEIRA et al., 2020).

2.7 Toxicologia de Plantas Medicinais

As plantas são grandes produtoras de moléculas químicas com diversas atividades biológicas já apresentadas, entretanto, mesmo sendo um produto natural, elas também podem produzir substâncias tóxicas ao organismo, levando a necessidade de identificação correta e conhecimentos desses produtos antes de sua utilização como agentes terapêuticos (COLOMBO et al., 2010).

Diversos efeitos toxicológicos já foram identificados em produtos naturais como por exemplo, em plantas popularmente utilizadas como medicinais que demonstraram ação hepatotóxicas, abortivas, nefrotoxica, dermatites, distúrbio de sistema nervoso, teratogênica, entre outras (CAMPOS et al., 2016).

A toxicidade apresentada por uma espécie vegetal pode sofrer várias influências, desde o período da coleta (efeito sazonal na produção de compostos químicos) até modo de armazenamento após coleta, além de possíveis interferentes externos como contaminantes fúngicos, pesticidas e metais pesados (RATES, 2001).

Para minimizar os riscos potenciais à saúde humana de agentes toxicológicos presentes em plantas medicinais, estudos sobre a toxicidade são realizados com o objetivo de identificar potenciais nocivos, o grau de letalidade, identificar a toxico-cinética e a relação dose-resposta desses produtos substâncias (VALADARES, 2006).

A Organização para Cooperação Econômica e Desenvolvimento (OCED), preconizou e desenvolveu diretrizes para avaliações toxicológicas, afim de padronizar modelos experimentais para o auxilio a descobertas de potenciais produtos nocivos. Vale destacar, que produtos a base de plantas medicinais passam obrigatoriamente por avaliações toxicológicas com o objetivo a predição de possíveis eventos adversos e determinação da dose inicial para estudos clínicos (CAZARIN, *et al*. 2004).

Entre as técnicas de avaliação toxicológica pré-clínica, destaca-se modelos *in vitro* para determinação de potencial citotóxico e *in vivo* como modelos de toxicidade aguda e subaguda

seguindo guideline 423 e 427, respectivamente. (ORGANIZATION FOR ECONOMIC COOPERATION AND DEVELOPMENT; OECD).

3 OBJETIVOS

GERAL

Avaliar o potencial anti-inflamatório, analgésico e a toxicidade do extrato etanólico das folhas de *Gomphrena celosioides.*

ESPECÍFICOS

Verificar a atividade anti-inflamatória *in vivo* do extrato etanólico de *Gomphrena celosioides;*

avaliar a atividade de redução da migração leucocitária *de* extrato etanólico de *Gomphrena celosioides;*

avaliar a atividade analgésica extrato etanólico de *Gomphrena celosioides;*

verificar a toxicidade aguda e subaguda do extrato etanólico de *Gomphrena celosioides in vivo.*

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5 APÊNDICES

Artigo 1. Antiarthritic and anti-hyperalgesic properties of ethanolic extract from Gomphrena celosioides Mart. (Amaranthaceae) aerial parts

Artigo 2. Toxicological analysis of the ethanolic extract of Gomphrena celosioides

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Research Article

Antiarthritic and Antihyperalgesic Properties of Ethanolic Extract from *Gomphrena celosioides* Mart. (Amaranthaceae) Aerial Parts

Luis Fernando Benitez Macorini,[1,2] Joyce Alencar Santos Radai,[1] Rafael Souza Maris,[1,2] Saulo Euclides Silva-Filho,[3] Maicon Matos Leitao,[1,2] Sérgio Faloni de Andrade,[4] Dayanna Isabel Araque Gelves,[5] Marcos Jose Salvador,[5] Arielle Cristina Arena,[1,6] and Cândida Aparecida Leite Kassuya[1]

[1]*School of Health Sciences, Federal University of Grande Dourados (UFGD), Dourados, MS, Brazil*
[2]*School of Health Sciences, University Center of Grande Dourados (UNIGRAN), Dourados, MS, Brazil*
[3]*College of Pharmaceutical Sciences, Food and Nutrition, Federal University of Mato Grosso do Sul (UFMS), Campo Grande, MS, Brazil*
[4]*Research Center for Biosciences and Health Technologies (CBIOS), Lusofona University, Lisbon, Portugal*
[5]*Institute of Biology, Department of Plant Biology, PPG BTPB, PPG BCE, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil Institute of Biology, Brazil*
[6]*Department of Structural and Functional Biology, Institute of Biosciences of Botucatu, UNESP—University Estadual Paulista—Botucatu, São Paulo State, Botucatu, Brazil*

Correspondence should be addressed to Cândida Aparecida Leite Kassuya; candida2005@gmail.com

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Gomphrena celosioides Mart. (Amaranthaceae) is used in folk medicine as a natural analgesic, and in Brazil, the species of genus *Gomphrena* is used for rheumatism. However, scientific evidence which supports its popular use as an analgesic is scarce. This study assessed the antiarthritic and antihyperalgesic activities of the ethanolic extract obtained from *G. celosioides* aerial parts on Swiss or *C57BL/6* mice. The antiarthritic and antihyperalgesic potential of *Gomphrena celosioides* was evaluated using paw edema, mechanical hyperalgesia, cold allodynia, carrageenan-induced pleurisy, articular inflammation zymosan-induced, Freund's complete adjuvant-induced inflammation zymosan-induced peritonitis, and carrageenan-induced adhesion and rolling experiment models. All doses of *G. celosioides* (300, 700, and 1000 mg/kg) significantly reduced edema formation in all the intervals evaluated, whereas the mechanical hyperalgesia was reduced 3 hours after the carrageenan injection. The cold hyperalgesia was significantly decreased 3 (700 mg/kg) and 4 hours (700 and 1000 mg/kg) after the carrageenan injection. Ethanolic extract of *G. celosioides* at 1000 mg/kg reduced the total leukocyte number, without interfering in the protein extravasation in carrageenan-induced pleurisy model. Ethanolic extract of *G. celosioides* (300 mg/kg) was also able to reduce significantly the leukocyte migration in zymosan-induced articular edema, while a reduction of the adhesion and migration and leukocyte rolling was induced by the ethanolic extract of *G. celosioides* (300 mg/kg) in zymosan-induced peritonitis. In Freund's complete adjuvant-induced inflammation model, an edema formation and mechanical hyperalgesia reduction were induced by the ethanolic extract of *G. celosioides* on day 22, whereas the cold allodynia was reduced on day 6 of treatment with the extract. These results show that ethanolic extract of *G. celosioides* has antihyperalgesic and antiarthritic potential in different acute and persistent models, explaining, at least in part, the ethnopharmacological relevance of this plant as a natural analgesic agent.

Antiarthritic and anti-hyperalgesic properties of ethanolic extract from *Gomphrena celosioides* Mart. (Amaranthaceae) aerial parts

Luis Fernando Benitez Macorini[1,2]; Joyce Alencar Santos Radai[1]; Rafael Souza Maris[1,2]; Saulo Euclides Silva-Filho[3]; Maicon Matos Leitao[1,2]; Sérgio Faloni de Andrade[4]; Dayanna Isabel Araque Gelves[5], Marcos Jose Salvador[5]; Arielle Cristina Arena[1,6]; Cândida Aparecida Leite Kassuya[1*]

[1]School of Health Sciences, Federal University of Grande Dourados (UFGD), Dourados, MS, Brazil.
[2]School of Health Sciences, University Center of Grande Dourados (UNIGRAN), Dourados, MS, Brazil.
[3]College of Pharmaceutical Sciences, Food and Nutrition, Federal University of Mato Grosso do Sul (UFMS), Campo Grande, MS, Brazil.
[4]Research Center for Biosciences and Health Technologies (CBIOS), Lusofona University, Lisbon, Portugal.
[5]Institute of Biology, Department of Plant Biology, PPG BTPB, and PPG BCE, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil Institute of Biology.
[6]Department of Structural and Functional Biology, Institute of Biosciences of Botucatu, UNESP – Univ. Estadual Paulista - Botucatu, São Paulo State, Brazil.

*Corresponding author:

Cândida Aparecida Leite Kassuya, E-mail: candida2005@gmail.com

ABSTRACT

Gomphrena celosioides Mart. (Amaranthaceae) is used in folk medicine as a natural analgesic, and in Brazil, the species of genus *Gomphrena* are indicated for rheumatism. However, scientific evidence which support its popular use as an analgesic is scarce. This study assessed the antiarthritic and anti-hyperalgesic activities of the ethanolic extract obtained from *G. celosioides* aerial parts on Swiss or *C57BL/6* mice. The antiarthritic and anti-hyperalgesic potential of *Gomphrena celosioides* was evaluated using paw edema, mechanical hyperalgesia, cold allodynia, carrageenan-induced pleurisy, articular inflammation zymosan-induced, Freund's Complete Adjuvant induced inflammation zymosan-induced peritonitis, and carrageenan-induced adhesion and rolling experiment models. All doses of *G. celosioides* (300, 700 and 1000 mg/kg) significantly reduced edema formation in all the intervals evaluated, whereas the mechanical hyperalgesia was reduced 3 hours after the carrageenan injection. The cold hyperalgesia was significantly decreased 3 (700 mg/kg) and 4 hours (700 and 1000 mg/kg) after the carrageenan injection. Ethanolic extract of *G. celosioides* at 1000 mg/kg reduced the total leukocyte number, without interfering in the protein extravasation in carrageenan-induced pleurisy model. Ethanolic extract of *G. celosioides* (300 mg/kg) was also able to reduce significantly the leukocyte migration in zymosan-induced articular edema, while a reduction of the adhesion and migration, and leukocyte rolling was induced by the ethanolic extract of *G. celosioides* (300 mg/kg) in zymosan-induced peritonitis. In Freund's Complete Adjuvant induced inflammation model, an edema formation and mechanical hyperalgesia reduction were induced by the ethanolic extract of *G. celosioides* on day 22, whereas the cold allodynia was reduced on day 6 of treatment with the extract. These results show that ethanolic extract of *G. celosioides* has anti-hyperalgesic and antiarthritic potential in different acute and persistent models, explaining, at least in part, the ethnopharmacological relevance of this plant as a natural analgesic agent.

Keywords: *Gomphrena celosioides*; Arthritis; Analgesic; Inflammation; Carrageenan.

List of Abbreviations

ANOVA - Analysis of variance
C57BL/6 – C57 Black 6
CFA - Freund's complete adjuvant
COX - Cyclooxygenase
DEXA – Dexamethasone
EDTA - Ethylenediaminetetraacetic acid
EDTA - Wthylenediaminetetraacetic acid
EECG – Ethanolic Extract of *Gomphrena celosioides*
ESI-MS - Electrospray ionisation mass spectrometry
ICAM - Intercellular adhesion molecule
IL-1β – Interleucina 1 β
Indo - Indometacin
NFkB - Factor nuclear kappa B)
NO - Nitric Oxide
NSAIDs - Non-steroidal anti-inflammatory
PBS - Phosphate-buffered saline
TLR2 - Toll-like 2
TNF – Tumor Necrosis Factor
VCAM - Vascular cell adhesion molecule

1. INTRODUCTION

Scientific evidence has demonstrated that products from natural sources, including medicinal plants, are promising for the development of safe alternatives for the treatment of pain management and inflammatory diseases [1]. Thus, the ethnopharmacologically guided research has contributed with the identification of new therapeutic agents obtained from plants [2], which often have fewer adverse effects, and are important for patients who use medications for long periods.

Gomphrena celosioides Mart. (synonyms *G. serrata and G. decumbens*), an annual herb known popularly as "Perpétua Brava", belongs to the Amaranthaceae family [3] and can be found in America, Australia and Indo-Malaysia. In Brazil, this species occurs in savanna vegetation, napeadic grassland, high altitude grassland, and caatinga [4]. This plant is used for several folk medicinal purposes, such as for the treatment of several liver-related and dermatological diseases, dysmenorrhea, bronchial infections, renal disorders and also as an analgesic [4, 5, 6, 7, 8].

Several chemical compounds with high therapeutical potential, such as hydrocarbons, alcohol, steroids, terpenes, ecdysteroids, flavonoids, saponins, butacyanine, and ketoses have already been isolated from *G. celosioides* [9]. Moura et al. [10] identified and isolated chemical compounds from *G. celosioides* aerial parts, including vanillic acid, 4-hydroxy-benzoic acid, and 4-hydroxy-3-methoxybenzoic acid, in addition to stigmasterol, sitosterol and campesterol. Dosumu et al. [11] identified and isolated 3-(4-Hydroxyphenyl) methylpropenoate from the methanol extract of *G. celosioides*. These same authors also found aurantiamide and aurantiamide acetate from the n-hexane extract of *G. celosioides* [12].

Despite its importance in folk medicine, there are few scientific studies which validate its therapeutic effects, especially the analgesic activity. Some studies using the

aerial parts of *G. celosioides* extract have already reported its antihypertensive [8], antitumor, antimicrobial [10], cytotoxic, anti-inflammatory and analgesic properties [13]. In a study carried out by Vasconcelos et al. [8], the ethanolic extract of *G. celosioides* showed diuretic effect and reduced the blood pressure in rats, demonstrating potential as an antihypertensive drug. Oluwabunmi et al. [6] showed a gastroprotective effect of the methanolic extract obtained from leaves, while Moura et al. [10] found an antimicrobial effect of the crude extract of the plant against *Staphylococcus aureus* and *Salmonella typhi*. In another study, the ethyl acetate and methanol extracts were active against *Fasciola gigantica*, *Taenia solium* and *Pheretima pasthuma*, corroborating the popular use of *G. celosioides* in the treatment of infectious diseases [11].

Although *G. celosioides* is a species widely used in folk medicine with important bioactive compounds, few scientific studies are found in the literature to confirm its popular indication, especially regarding its antiarthritic and anti-hyperalgesic potential. Thus, this study aimed to evaluate the analgesic and antiarthritic activities of the ethanolic extract of the *G. celosioides* aerial parts in different acute and persistent inflammation models.

2. MATERIALS AND METHODS

2.1. Plant material and preparation of ethanolic extract

G. celosioides aerial parts were collected (lat: -19.666667 long: -51.183333 WGS84) and identified by Dr. Josafá Carlos de Siqueira. A voucher specimen (SCAB 4051) is deposited in the herbarium of Pontifical Catholic University, Rio de Janeiro. The preparation of ethanolic extract of *G. celosioides* (EEGC) was performed according Vasconcelos et al. [8].

2.2. Animals

Male and female *Swiss* or *C57BL6* mice (weighing 20-30 g; 60-65 days of age) were provided by the Central Animal House of the Federal University of Grande Dourados/Mato Grosso do Sul, Brazil. The animals were housed at 22 ± 2 ^{o}C under a 12/12 h light/dark cycle and free access to food and water. Prior to the experiments, the animals were fasted overnight, with water provided *ad libitum*. The experimental protocols were in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethical Committee in Animal Experimentation of the Federal University of Grande Dourados (Protocol number: 09/2018). The experimental design is shown in Figure 1.

2.3. Reagents

Carrageenan, Dexamethasone, Zymosan, Indomethacin, Acetone, and Bradford reagent were purchased from Sigma-Aldrich Co.LLC (St. Louis, MO, USA).

2.4. Paw edema, mechanical hyperalgesia and cold allodynia induced by carrageenan

Swiss male mice were allocated into five groups: negative control group (treated with saline 0.9 %, p.o.), positive control group (DEXA; Dexamethasone 1 mg/kg, s.c.), and three groups treated with different doses of ethanolic extract of *G. celosioides* (EEGC) (300, 700 or 1000 mg/kg, p.o.). One hour after the treatment, all animals received 50 µL of carrageenan (300 µg/paw, s.c.) in the right hind paw, and saline solution in the left hind paw (used as a control). The paw volume was measured in time intervals (1, 2, and 4 h) using a plethysmometer device. Mechanical hyperalgesia was evaluated by the electronic Von Frey pressure-increasing test at time intervals 3 and 4 h [14]. Sensitivity

to cold was performed by the acetone drop test described by Decosterd et al. [15], at time intervals 3 and 4 h. Acetone (30 µL) was released over the right paw of the animals. Right after, the number of times in which occurred the paw rising reaction was evaluated. Minimum and maximum cutoff points were assigned at 5 and 20 s, respectively.

2.5. Model of carrageenan-induced pleurisy in mice

Swiss female mice (50 days of age) were treated and allocated into five groups: negative control group (Saline solution 0.9% p.o.), positive control group (DEXA, 1 mg/kg, s.c.), and three groups treated with different doses of EEGC (300, 700 or 1000 mg/kg p.o.). One hour after the treatment, 1 mL of carrageenan (300 µg/cavity, diluted in sterile saline) was injected into the animals by intrapleural pathway as described by Vinegar et al. [16]. After 4 h of the carrageenan injection, the animals were anesthetized and euthanized (ketamine/xylazine solution 1:1). The exudate was collected by aspiration and put into tubes. The leukocyte count was performed in the Neubauer chamber, and the total protein was determined by the Bradford method using a commercial kit Bioagency®.

2.6. Leukocyte recruitment and mechanical hyperalgesia evaluation in experimental model of zymosan-induced arthritis

The experimental model of zymosan-induced arthritis was carried out as previously reported [17]. The right knee joints of the animals received 200 µg/cavity of zymosan (in 10 µL sterile saline; intra-articularly injected), while the contralateral knee joint received an equal volume of saline. Thirty minutes before zymosan injection, the mice were treated orally with vehicle (saline) or EEGC (300 mg/kg). The additional mice group received only saline in the articular cavity and was treated with vehicle (naive group). At times of 3 and 4 h after zymosan injection, the mechanical hyperalgesia was

evaluated using a digital analgesimeter (Von Frey, Insight®), a pressure transducer which records the applied force (in grams) in paw until the moment of paw withdrawal. At time of 6 h after zymosan injection, the animals were anesthetized and euthanatized, the knee joint was exposed by surgical incision and washed twice with 5 µL of phosphate-buffered saline (PBS) that containing ethylenediaminetetraacetic acid (EDTA). The supernatant was diluted to a final volume of 50 µL with PBS/EDTA to determine the total cells counts.

2.7. Zymosan-induced peritonitis

Swiss male mice were allocated into four groups: naive group (saline 0.9 %, p.o.), negative control group (saline 0.9 %, p.o.), positive control group (DEXA, 1 mg/kg s.c.) and EEGC (300 mg/kg, p.o.). Peritonitis was induced by 1 mg/kg of Zymosan administrated intraperitoneally 30 min after the treatment in each animal [18]. The naive group received saline solution for control. The zymosan-induced peritonitis was assessed 6 h after the administration. After this period, the animals were euthanized, and their peritoneal cavity was washed with 1 mL of PBS/EDTA. Then, the solution containing the wash was centrifuged, and the supernatant was used for the Nitric Oxide (NO) dosage and the precipitate was re-suspended in 1 mL of PBS/EDTA for the total leukocytes analysis using the KX-21n Roche® equipment. The nitric oxide determination nitrite was measured by methods of Griess. A Griess solution was prepared, where 50 µL of the solution and 50 µL of the sample were added in a 96-well microplate, after a 15 min wait, the reading was performed in a spectrophotometer at 580 nm. A nitrite curve using Sodium Nitrite at 5, 10, 30, and 60 µM concentrations was also performed [19].

2.8. *In situ intravital microscopy analysis for rolling and adhesion events of leukocytes in the mesenteric microcirculation*

The leukocyte rolling and adhesion was performed after the induction of leukocyte migration by an injection of carrageenan (500 µg/cavity, i.p.) in sterile saline. The mice were treated orally with EEGC (300 mg/kg), vehicle (saline) or indomethacin (5 mg/kg) as a reference drug, 30 min before the carrageenan injection. The additional mice group was injected only with saline in the peritoneal cavity. After 2 h the carrageenan or saline injection, the animals were anesthetized (ketamine/xylazine solution 1:1). A lateral surgical incision was performed in the abdominal wall to the exposure the mesentery and observation of *in situ* microcirculation. The mice were kept on a heated plate, with temperature maintained at 37 ºC, adapted to the chariot of an optical microscope with a video camera and monitor to project and record the images. The preparation was kept moist and warm with Ringer Locke's solution that contained 1% gelatin. The vessels were considered the post-capillary venules with 10-18 µm diameter. The number of rolling and adherent leukocytes was counted as 10 min intervals. Leukocyte adherence was determined when cells remained static in the endothelium for 30 s or more [20].

2.9. Paw edema and mechanical hyperalgesia induced by Freund's Complete Adjuvant (CFA)

The persistent model of edema and mechanical hyperalgesia induced by Freund's Complete Adjuvant (CFA) in male *C57BL6* mice was performed to study the analgesic and anti-inflammatory properties with prolonged treatment with EEGC. The animals were allocated into three groups: control group (saline 0.9%, p.o.), EEGC group (100 mg/kg, p.o.) and positive control group (DEXA, 1 mg/kg s.c.).

At time zero, 20 µL of a suspension containing dead *Mycobacterium tuberculosis* and added in paraffin oil (85%) and moneleate (15%) were injected into the right hind

paw. The nociceptive threshold was estimated at 3 and 4 h after CFA and was then analyzed on days 6, 11, 16 and 22 using the Von Frey electronic test [21]. In addition, CFA-induced edema was resolved at 2 and 4 h intervals on days 6, 11, 16, and 22 after CFA injection with a plethysmometer.

Cold sensitivity was measured by the acetone drop test as described by Eliav et al. [22]. A blind needle attached to a syringe was used to release 30 µL of acetone in the paw of CFA animals from the CFA model experiment on days 6, 11, 16, and 22, and the duration (in s) of paw withdrawal was evaluated. The minimum and maximum cutoff points were assigned to 0.5 and 20 s, respectively. Paw removals due to locomotion or weight change were not counted.

2.10. Statistical analysis

The data are presented as the mean ± SEM (standard error of the mean). Differences among means were evaluated by one-way analysis of variance (ANOVA), followed by the Newman-Keuls post-hoc test, using GraphPad Prism software. Statistical differences were considered significant when $P < 0.05$.

3. RESULTS

3.1 Effects of EEGC on the paw edema, mechanical hyperalgesia and cold allodynia induced by carrageenan

All doses of EEGC (300, 700 and 1000 mg/kg) reduced the edema formation in the first, second and fourth hour after the carrageenan administration with maximum inhibition of 61±5%, 53±6% and 68±5% for at 300, 700 and 1000 mg/kg, respectively. The values were similar to those of the animals treated with dexamethasone, which had

its maximum anti-edematogenic activity in the fourth hour reducing 68±4% paw edema (Figure 2A, B, and C).

Furthermore, the treatment with all doses of extract (300, 700, and 1000 mg/kg) after 3 h of the carrageenan injection reduced the mechanical hyperalgesia (Figure 3). EEGC exhibited maximal activity on the mechanical hyperalgesia at 300 mg/kg with 91±22%, a reduction similar to those observed with dexamethasone treatment (87±8%). However, it was not possible to observe the same reduction after the fourth hour (Figure 3A and B). In relation to cold allodynia, EEGC treatment promoted a reduction at 700 mg/kg of EEGC in the third hour, and in the fourth hour occurred the allodynia reduction in the doses of 700 and 1000 mg/kg with maximum inhibition of 58±14% (Figure 3C and D).

Effects of EEGC on carrageenan-induced pleurisy

The EEGC treatment at the dose of 300 mg/kg showed a significant reduction (58±14%) of the leukocyte migration compared to the control group, indicating a possible reduction of the inflammatory process (Figure 4A). However, the treatment with the extract did not show reduction in the protein extravasation to the pleural cavity (Figure 4B).

3.3 Effects of EEGC on zymosan-induced articular inflammation and peritonitis

In another model of articular inflammation that EEGC treatment at dose of 300 mg/kg promoted a reduction of hyperalgesia and leukocyte migration compared to the control group with maximum inhibition of 52±3% and 81±4%, respectively (Figures 5 and 6). There was a significant reduction of 46±10% induced by EEGC in the total leukocytes migration in peritonitis at 300 mg/kg dose (Figure 7), therefore EEGC did not alter significantly the nitric oxide (NO) levels (Figure not shown). The dexamethasone group inhibited significantly the hyperalgesia and leukocyte migration in articular injection (Figures 5 and 6) and also the leukocytes migration in peritonitis (Figure 7).

A significant reduction in cell adhesion to the endothelium and in cells rolling provoked by EEGC administration (300 mg/kg) were observed with 40±7% and 48±6% of inhibition, respectively. As expected, the reference drug (indomethacin) at a dose of 5 mg/kg decreased the adhesion (45±5% of inhibition) and consequently the rolling of leukocytes (65±4% of inhibition) (Figure 8A and B).

3.4 Effects of EEGC on CFA inflammatory model

The dose of 100 mg/kg of EEGC was tested in CFA model of chronic inflammation for 22 days and the oral EECG treatment was able to reduce significantly the edema volume (maximal inhibition of 25±18%) after this period. The dexamethasone reference drug showed a reduction in the paw edema on the sixteenth day, when compared to the control group (Figure 9A).

EEGC (100 mg/kg) and dexamethasone groups blocked the development of the mechanical hyperalgesia by induced CFA on day 22 of the treatment (Figure 8B), while the cold allodynia had an inhibition of sensitivity until the 16th day, possessing its maximum effect both by EEGC (44±21%) and dexamethasone (67 ± 11%) on the sixth day of treatment (Figure 9C).

DISCUSSION

Despite the therapeutic benefit, non-steroidal anti-inflammatory (NSAIDs) and disease-modifying anti-rheumatoid drugs have important adverse effects [23], which reinforce the need to search for other safe and efficient therapeutic alternatives. The results of this study contribute with this search showing that EEGC has great anti-arthritic and anti-hyperalgesic potential, corroborating the popular use already reported.

In the carrageenan-induced acute paw edema model, all doses of EEGC exhibited a similar result to the dexamethasone (a reference drug), demonstrating an anti-edematogenic potential of this extract. This model is associated with an acute inflammatory process and has several mediators for inflammatory response induction. In the first and second hours, the inflammatory effect is mediated by histamine, serotonin and kinins, while in the next phase (3 to 6 hours) it is mediated by an increase of the prostaglandin production and COX-2 activation [24, 25]. The EEGC showed great potential in reducing the peripheral inflammatory process, the mechanical hyperalgesia and the cold allodynia in the paw edema model, actions which may be related to the direct action of the cytokine expression and release of NO in the tissues.

Studies show that the release of proinflammatory cytokines activates the expression of cyclooxygenases, such as COX-1 and COX-2 [26]. These enzymes play an important role in the production of prostaglandins and leukotrienes from arachidonic acid [27]. Several physiological functions such as gastric mucosa protection, regulation of gastric juice release, vascular tone control, and metabolism are related to the action of these molecules [28]. However, physiological effects of COX action such as hyperalgesia, increased body temperature (fever), and inflammatory processes are also found. Studies also show that COX-1 is traditionally known as the constitutive or inducible isoform while COX-2 is known as inducible isoform in the inflammatory process. The selective COX-2 NSAIDs drugs did not frequently show gastric ulcer induction, which is a common adverse effect observed by traditional NSAIDs. The carrageenan-induced edema was inhibited by EEGC in this study and may be related with an inhibition of the prostaglandin production [29, 30].

The main anti-inflammatory and analgesic drugs used by the population are within the class of NSAIDs. However, the majority of these drugs are not characterized by the

selectivity to cycloxygenases, except for Coxibes, which acts in the selective inhibition of COX- 2 [31]. Some studies show that Coxibes drugs, after prolonged use, have adverse effects such as direct action on the cardiovascular system [32]. The anti-inflammatory effect was also evaluated in an acute model of pleurisy induced by carrageenan. The carrageenan administration induces the formation of exudate, changes in coloidosmotic pressure and infiltration of polymorphonuclear leukocytes in the pleural cavity, in addition to the release of pro-inflammatory mediators [33]. Doses of 700 and 1000 mg/kg of EEGC reduced the total leukocyte migration in the pleural cavity however did not reduce the protein extravasation.

The antiarthritic activity of EEGC was evaluated by zymosan-induced arthritis in mice. Zymosan is an isolate from the cell wall of the yeast *Saccharomyces cerevisiae* characterized as a polysaccharide that acts in macrophage Toll-like 2 (TLR2) receptors and subsequently in the activation of proinflammatory mechanisms [21, 34]. The EEGC decreased the total leukocytes of the articular lavage, indicating a reduction in diapedesis [35].

Our research group identified caffeic acid, ferulic acid, vanillic acid, catechin in EEGC [8]. Among the biologically active compounds contained in this extract we point out important anti-inflammatory agents such as caffeic acid, ferulic acid, vanillic acid, catechin [36] that can be related to the therapeutic effects exhibited by EEGC. Calixto et al. [37] showed that anti-inflammatory effect of vanillic acid is related to the inhibition of the neutrophil recruitment and also to the NFkB activation. Vanillic acid can also inhibit the COX-2 and NO expression induced by LPS *in vitro* [38].

Zymosan-induced peritonitis was also evaluated in this study. The adhesion, rolling and leukocyte migration to the peritoneal cavity was decreased by EEGC, with a similar reduction produced by the reference drug indomethacin. Indomethacin decreases

the expression of adhesion molecules such as L-selectin, E-selectin, I-CAM and VCAM [20, 39]. These molecules play important role in the leukocyte adhesion and in the rolling to the focus of the inflammatory process, although others factors are is important to the leukocyte migration phenomenon. NO is an important mediator in the leukocyte migration, promoting vasodilation, and reducing the recruitment, adhesion, rolling and leukocyte migration during inflammatory response favoring diapedesis [40, 41]. Since EEGC did not increase the NO levels induced by zymosan, it led us to conclude that the EEGC mechanism of action was not involved in the NO pathway. EEGC maybe act by the same pathway of indomethacin.

Based on the results obtained in acute models, the oral dose of 100 mg/kg EEGC was tested in the CFA model to evaluate EEGC anti-arthritic and anti-hyperalgesic properties. EEGC was effective against mechanical and cold hyperalgesia induced by CFA confirming the popular use of *G. celosioides* as an analgesic. In addition, it is possible to report that the mechanical hyperalgesia and cold hyperalgesia processes are characterized as pain indicators since are since they result from the sensitization and the pain pathway and type C nerve fiber caused by the CFA inflammatory persistent process [42, 43].

In conclusion, the ethanolic extract of *G. celosioides* aerial parts showed antiarthritic and anti-hyperalgesic activities in different evaluated models, decreasing leukocyte recruitment, rolling, adhesion, and migration to the inflammatory focus. Although, these results corroborate the popular statement, other studies should be conducted to evaluate the mechanisms of action and to identify the compound responsible for these effects.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflict interest

The authors declare that they have no conflicts of interest in this work.

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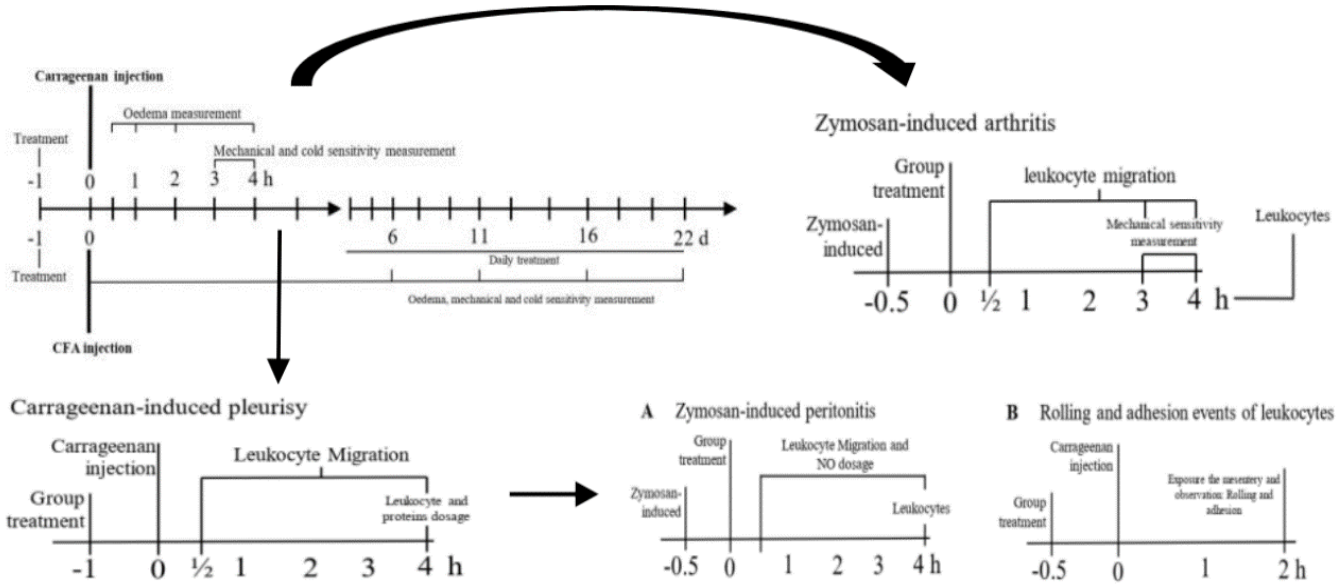

Figure 1. The experimental design.

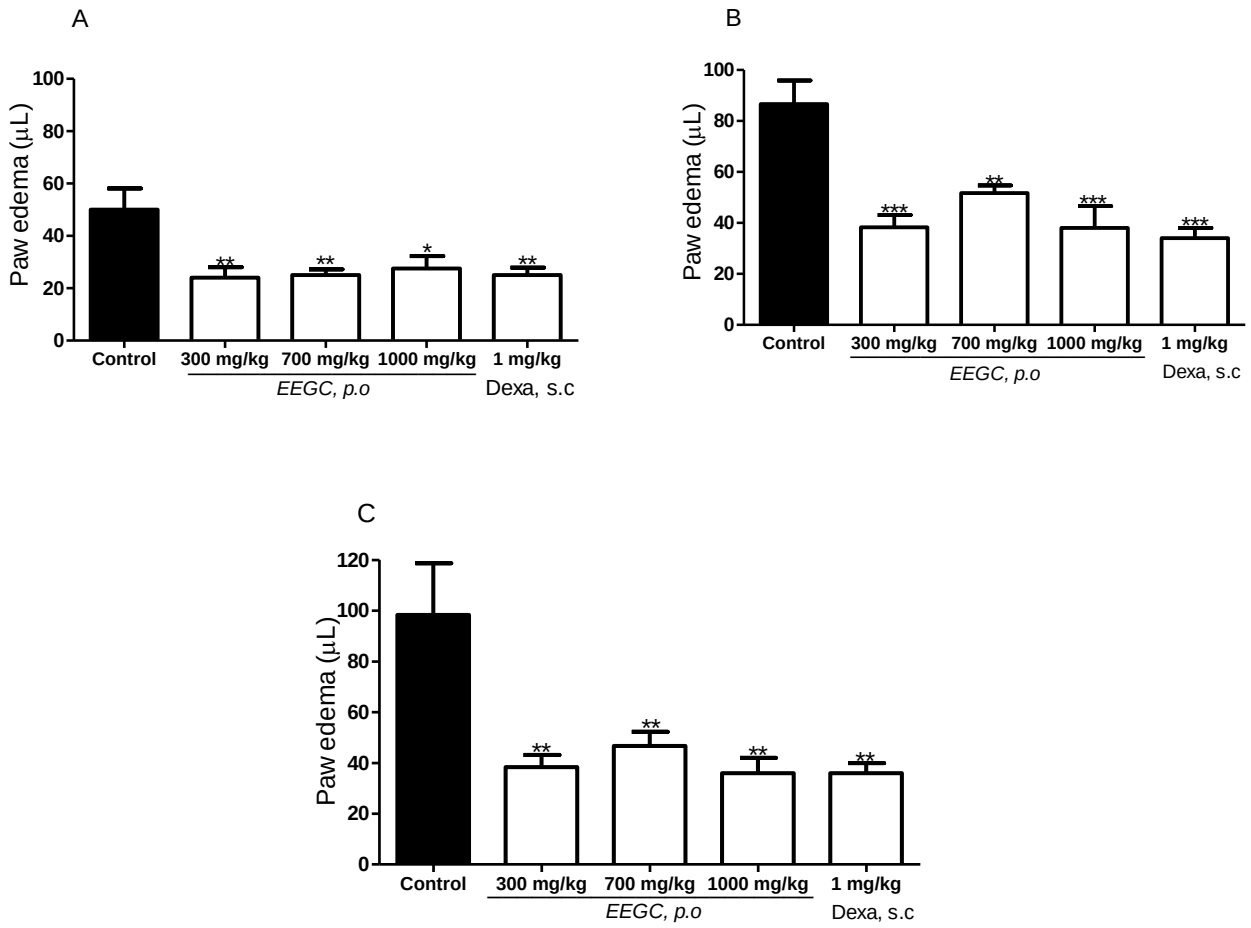

Figure 2. Effect of oral administration of EEGC at 1 (A), 2 (B) and 4 (C) hour after carrageenan-induced edema. The control (saline 0.9%, p.o.), EEGC (300, 700, or 1000 mg/kg, p.o.), and DEXA (dexamethasone 1 mg/kg, i.p.) groups were treated after 1 hour with carrageenan. The bars express the mean ± SEM compared to the control group. * P <0.05; ** P <0.01 and *** P <0.001. One-way analysis of variance followed by the Newman-Keuls test.

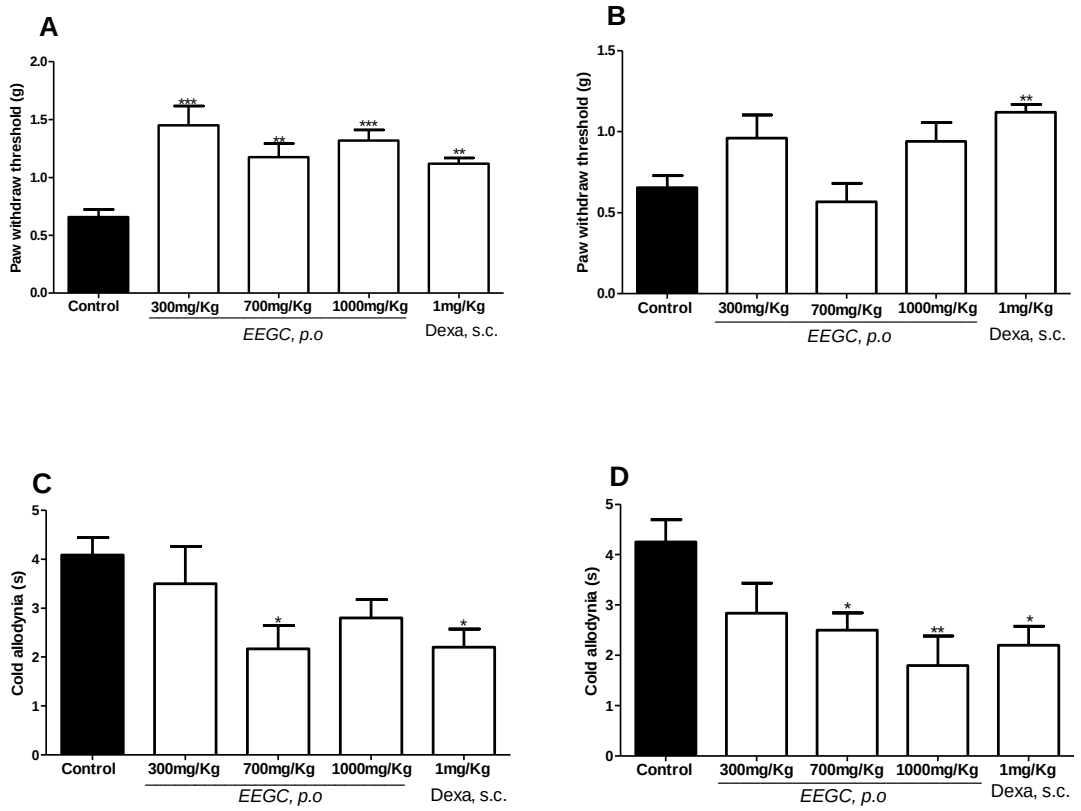

Figure 3. Effect of oral administration of EEGC at 3 h and 4 h after carrageenan-induced mechanical sensitivity (A and B) and cold hypersensitivity (C and D). The control (saline 0.9%, p.o.), EEGC (300, 700, or 1000 mg/kg, p.o.), and Dexa (dexamethasone 1 mg/kg, s.c.) groups were treated after 1 hour with carrageenan. The bars express the mean ± SEM compared to the control group. ** P <0.01. One-way analysis of variance followed by the Newman-Keuls test.

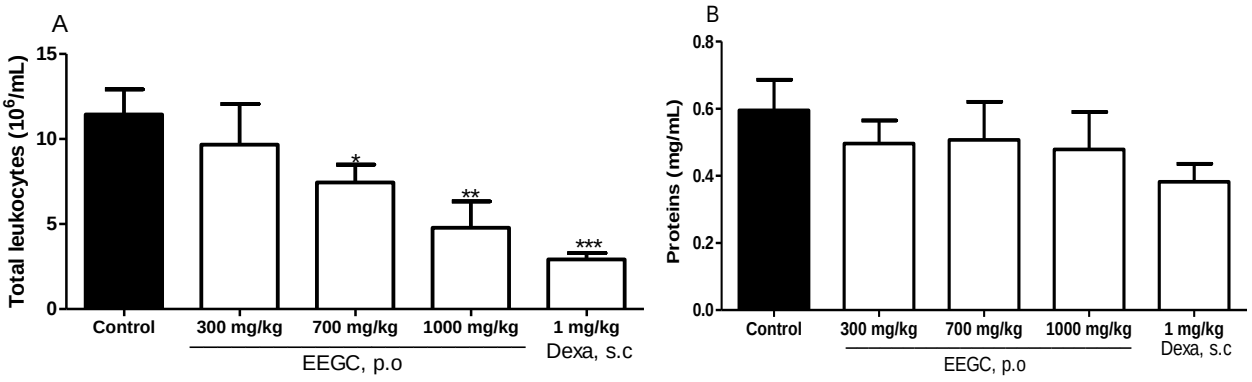

Figure 4. Effect of the oral administration of EEGC on acute inflammation induced by intrapleural injection of carrageenan in mice. In (A) leukocyte migration x 10^6 cells/cavity, (B) proteins (mg/ml). The control group received saline solution (0.9 %), and the EEGC groups received 300, 700, or 1000 mg/kg. The bars express the mean ± SEM compared to the control versus treated group. * P <0.05; ** P <0.01. One-way analysis of variance followed by the Newman-Keuls test.

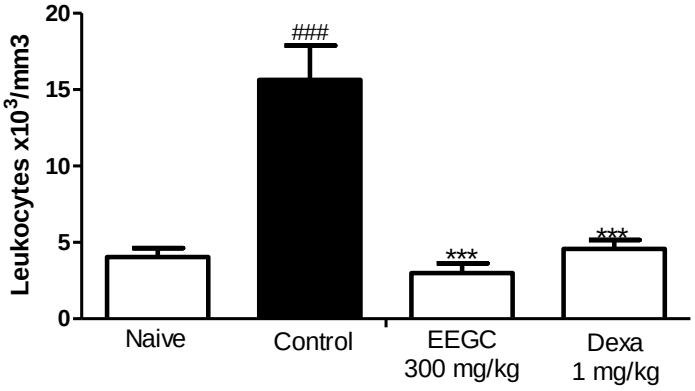

Figure 5. Effect of the oral administration of EEGC on the leukocyte recruitment in articular inflammation induced by zymosan model in mice. The figures show the values after the induction of arthritis in the naive, control (saline, 0.9 %, p.o.), EEGC (300 mg/kg, p.o.), and Dexa (dexamethasone 1 mg/kg, s.c.) groups. The bars express the mean ± SEM, ### or *** P <0.001. ###Control versus Naïve. ***EEGC versus Control. One-way analysis of variance followed by the Newman-Keuls test.

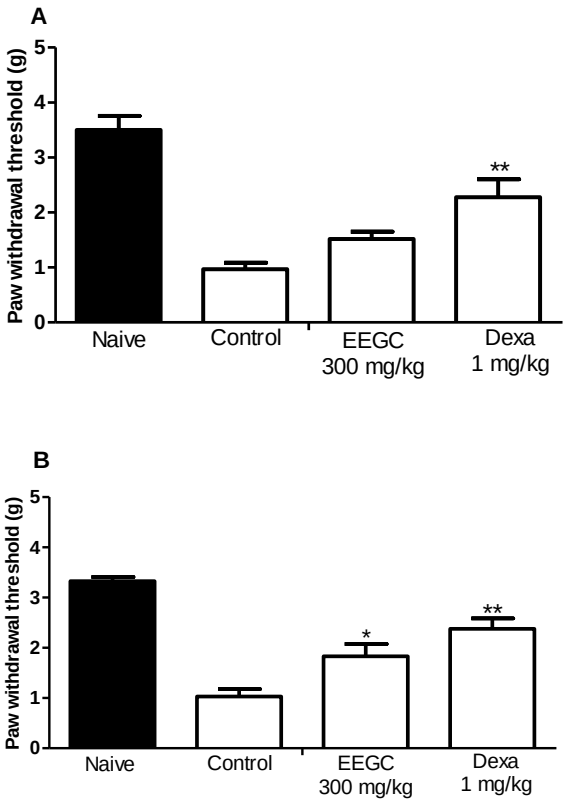

Figure 6. Effect of the oral administration of EEGC on the increase of mechanical sensitivity (paw withdrawal threshold) in articular inflammation induced by zymosan model in mice. The figures show the values at 3 (A) and 4 (B) h after the procedure of induction of articular inflammation in the naive, control up (saline 0.9 %, p.o.), EEGC (100 mg/kg, p.o.), and Dexa (1 mg/kg, s.c.) groups. The bars express the mean ± SEM compared with the control vs. treated group. * P <0.05; ** P <0.01 # or *** P <0.001. One-way analysis of variance followed by the Newman-Keuls test.

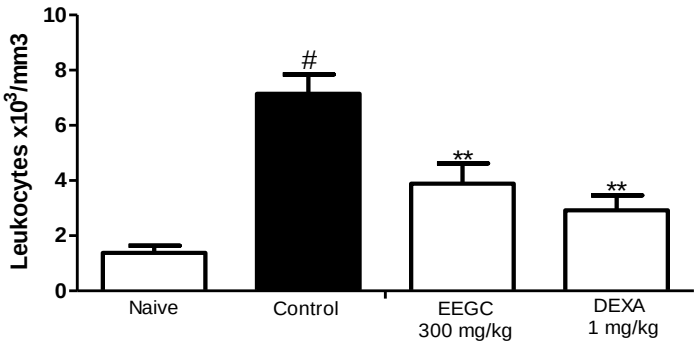

Figure 7. Effect of the oral administration of EEGC on leukocyte migration after peritoneal injection of zymosan in mice. Mice were pretreated with EEGC (300 mg/kg, p.o.), dexamethasone (Dexa, 1 mg/kg, i.p) or vehicle (saline solution, 0.9 %, p.o.). After 60 min, naive mice were injected with saline (i.p.), while all other groups received zymosan. The bars express the mean ± SEM compared with the control vs. treated group, ### or *** P <0.001. #Control versus Naive. ***EEGC versus Control. One-way analysis of variance followed by the Newman-Keuls test.

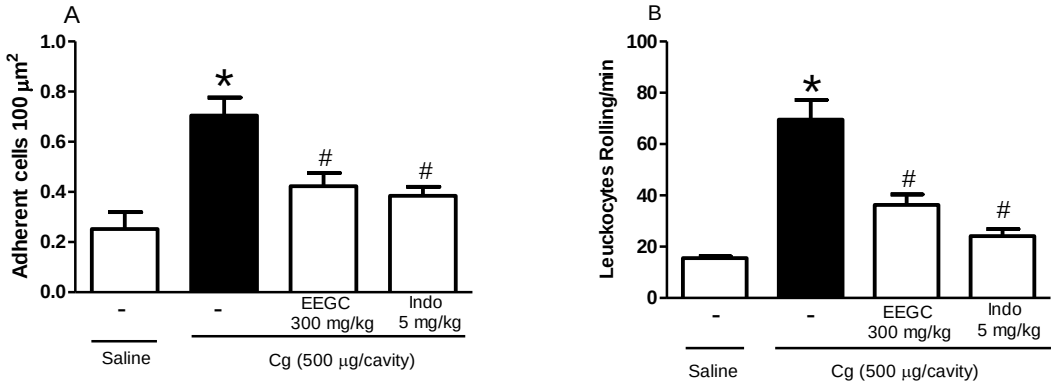

Figure 8. Effect of EEGC on leukocyte rolling (A) and adhesion (B) induced by carrageenan. Mice were orally pretreated with EEGC (300 mg/kg), indomethacin (Indo - 5 mg/kg) or vehicle. After 60 min, saline or carrageenan was injected i.p. Leukocyte rolling and adhesion were evaluated by intravital microscopy in the mesentery 2 h later. The bars express the mean ± SEM compared with the control vs. treated group, ### or *** P <0.001. ###Control versus Naive; ***EEGC versus Control. One-way analysis of variance followed by the Newman-Keuls test.

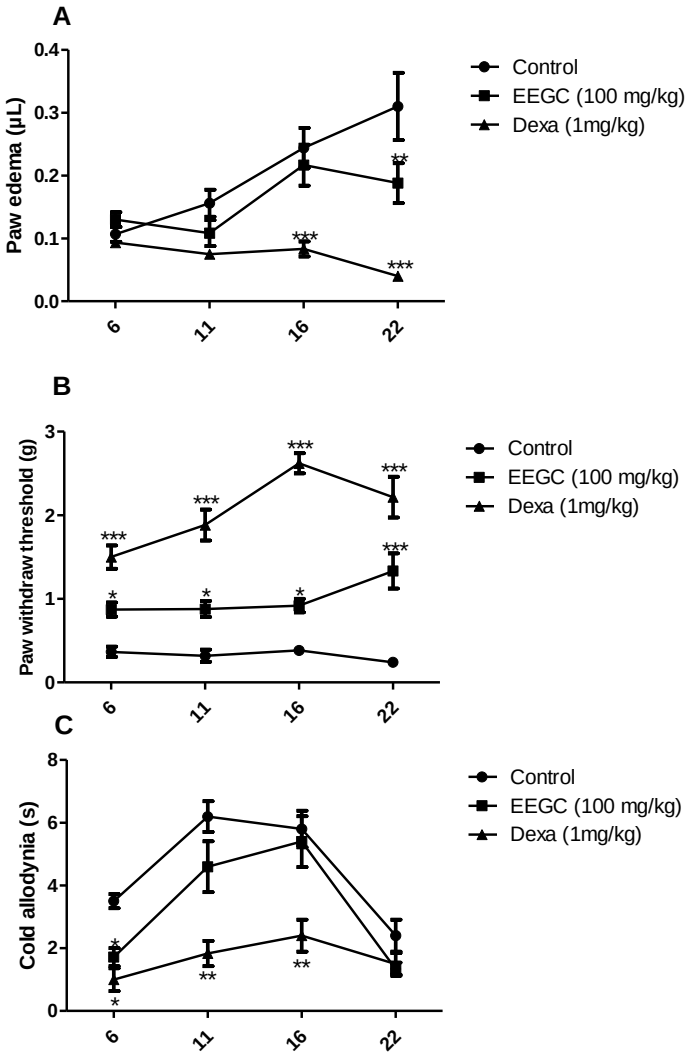

Figure 9. Effect of the oral administration of EEGC on increases in paw edema (A), mechanical sensitivity (paw withdrawal threshold) (B), cold hypersensitivity (C) in persistent inflammation induced by CFA model. Mice were treated once/day for 21 days with saline (0.9 %, p.o., control), EEGC (100 mg/kg, p.o.) and dexamethasone (1 mg/kg, i.p.) after CFA. The points are expressed as the mean ± SEM compared with the control vs. treated group. *P < 0.05; **P < 0.01 or ***P<0.001. One-way analysis of variance followed by the Newman-Keuls test.

Artigo 2. Submetido para Drug Chemical and Toxicology
Qualis capes: B2
Fator de impacto: 2.405

Acute and subacute oral toxicity evaluation of the ethanolic extract from the aerial parts of *Gomphrena celosioides* Mart. in rodents

Luis Fernando Benitez Macorini[a,b], Rafael Souza Maris[a,b], Thiago Chaves Teixeira[b], Caren Naomi Aguero Ito[a], Angela Midori Kuraoka-Oliveira[b], Flávia Barbieri Bacha[b], Angela Julieth Buitrago Mejia[c], Marcos José Salvador[c], Cândida Aparecida Leite Kassuya[b], Arielle Cristina Arena[d]

[a]*School of Health Sciences, Federal University of Grande Dourados (UFGD), Dourados, MS, Brazil.*

[b]*School of Health Sciences, University Center of Grande Dourados (UNIGRAN), Dourados, MS, Brazil*

[c]*Institute of Biology, Department of Plant Biology, PPGBCE, and PPGCF, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil*

[d]*Institute of Biosciences of Botucatu, Department of Structural and Functional Biology, University. Estadual Paulista – Botucatu (UNESP), São Paulo, Brazil.*

Running Title: **Toxicological profile of *Gomphrena celosioides***

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*Author for correspondence:

Arielle Cristina Arena

Address: Department of Structural and Functional Biology, Institute of Biosciences of Botucatu, São Paulo State University (UNESP), Distrito de Rubião Junior, s/n, Caixa Postal – 510; CEP: 18618970; Botucatu – SP.

Tel: + 55 14 38800495

E-mail address: arielle.arena@unesp.br (A. C. Arena)

ABSTRACT

Gomphrena celosioides Mart. is a medicinal species used to treat lung problems, fever, cough, infectious diseases, heart and digestive problems in America, Australia, and Indomalaysia. Although some studies have demonstrated the pharmacological properties of this species, few studies have focused on its adverse effects. This study aimed to evaluate the toxicological potential of ethanolic extract from *G. celosioides* (EEGC) in acute and subacute toxicity models in rodents. EEGC (2000 mg/kg) was administered orally to male and female Wistar rats for the acute toxicity test. For the subacute toxicity model, Swiss mice received orally 75, 150 or 300 mg/kg of EEGC for 28 consecutive days. In the acute toxicity test, the animals treated with 2000 mg/kg EEGC showed no clinical signs of toxicity, indicating that the LD_{50} is higher than this dose. The repeated treatment with EEGC, did not cause significant changes in the behavior, body weight, or hematobiochemical parameters. In addition, there were no significant differences in the histology of the target organs of toxicity. In conclusion, EEGC, at the tested doses, showed low toxicological potential after a single dose or repeated administration to rodents and can be considered safe. However, other aspects of toxicity should be performed.

Keywords: Amaranthaceae; *Gomphrena celosioides;* acute toxicity model, subacute toxicity model; mice, rats.

Introduction

Gomphrena celosioides Mart. (synonyms *G. serrata* and *G. decumbens*), a herbknown belonging to the Amaranthaceae family, is a native medicinal plant widely found in America, Australia, and Indomalaysia (Prachi *et al.* 2009; Vieira *et al.* 1994). This species is used in the folk medicine to treat pulmonary disorders, fever, infectious diseases, cough, renal, heart, and digestive problems (Vieira *et al.* 1994). In Brazil, the roots are used for tea preparations for children with severe diarrhea due to its anti-diarrheic activity, while in India, juice preparations administered twice a day, for 10 days, are used to cure urolithiasis (Prachi *et al.* 2009).

Due to the ethnopharmacological relevance of this herb, our research group showed that the ethanolic extract from the aerial parts of *G. celosioides* (EEGC) is a natural diuretic agent that also reduces arterial pressure (Vasconcelos *et al.* 2018; 2017). We also identified antiarthritic and antihyperalgesic properties of this extract (Macorini *et al.* 2020). In addition, the ethyl acetate and hexanic extracts and 3-(4-hydroxyphenyl) methylpropenoate obtained from the whole plant *G. celosioides* has shown antimicrobial and anthelmintic properties (Dosumu *et al.* 2010), while the aqueous extract of the leaves of *G. celosioides* exhibited hepatoprotective (Sangare *et al.* 2012), anti-lithiasis (Goswami and Srivastava 2015), anti-inflammatory and analgesic (Oladele *et al.* 2009) activities. Other relevant property of this species includes gastroprotective action (Oluwabunmi and Abiola 2015).

EEGC phytochemical analyses showed the presence of malic acid, caffeic acid, ferulic acid, catechin, vanillic acid, irisone B, dimethoxyflavone, and caffeoyl glucose (Vasconcelos *et al.* 2017). In other study, Dosumu *et al.* (2014) identified the presence of

aurantiamide and aurantiamide acetate from a *G. celosioides* whole plant hexanic extract. Other chemical compounds with high therapeutic potential such as stigmasterol, campesterol and sitosterol were isolated in the ethanolic, methanolic and hexanic extracts of the aerial parts of *G. celosioides* (De Moura *et al.*,2004).

Although the chemical compounds found in the *G. celosioides* possess important biological activities, many of them can be toxic (Galati and O'Brien 2004; *Yang et al.* 2018), which makes it urgent to investigate the toxicity of this species. A study demonstrated that the aqueous extract of the *G. celosioides* whole plant did not induce toxicity (100 to 4000 mg/kg by intraperitoneal injection) in acute toxicity model in rats (Souleymane *et al.* 2014). However, the oral use of ethanolic extract of *G. celosiodes* has not been evaluated in the literature regarding its toxic potential, especially after prolonged use. Thus, this study investigated the acute toxicity (determination of LD_{50}), as well as the subacute toxicity after daily oral treatment with an ethanolic extract from *G. celosioides* during 28 days in rodents.

Materials and methods

Plant material, extraction and preparation

The *G. celosioides* aerial parts were collected in Paranaíba, Mato Grosso do Sul, Brazil [lat:−19.666667long:−51.183333 WGS84] and identified by Dr. Josafá Carlos de Siqueira from the Pontifical Catholic University of Rio de Janeiro (PUC-RJ), Brazil. A voucher specimen (SPFR-2962) was deposited in the herbarium of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo (FFCLRP/USP). The preparation of EEGC and their phytochemical analysis by ESI-MS/MS were performed according to Vasconcelos et al. (2017).

Animals

Adult male (n=10; 90 days old, 350-400 g) and female (n=10; 90 days old, 200-250 g) *Wistar* rats from the Central Animal Facility of São Paulo State University (UNESP) were used for the acute toxicity study. For evaluation of the subacute toxicity test, male and female *Swiss* mice (n=5; 60 days old, 25 g) from the Central Biotherium of Federal University of Grande Dourados (UFGD) were used. The animals were kept under standardized conditions (temperature of 23°C, photoperiod of 12 h light/12 h dark) with water and food *ad libitum*. The experimental protocols were approved by the Ethical Committee in Animal Experimentation of the Federal University of Grande Dourados (protocol number 06/2018).

Toxicity studies

Acute and subacute toxicity studies were based on the Organisation for Economic Co-operation and Development (OECD) Guidelines 425 and 407 (OECD 2008a,b). Since both rats and mice are recommended for the use in acute and subacute toxicity studies (OECD) and we have a limited amount of EEGC, we decided to use rats for the acute toxicity study and mice for the subacute toxicity model.

Acute oral toxicity

EEGC was administered at a single dose of 2000 mg/kg orally (by gavage) to one male and one female *Wistar* rat under an 8 h fast. After 48 h, the same dose was administered to other rats (four males and four females), totaling five animals per sex at this same dose. In parallel, five males and five females received vehicle (0.9% saline, 1.0 mL/kg) to establish a control group (OECD 2008a). The animals were observed periodically during the first 24 h after EEGC administration and then once a day for 14 days. The five Hippocratic screening parameters were analyzed (Malone and Robichaud 1962): conscious state (general activity); activity and coordination of the motor system and muscle tone (response to the touch of the tail and strength to grab); reflexes (cornea

and ear); activity in the central nervous system (tremors, convulsions, sedation, anesthesia and ataxia); and activity in the autonomic nervous system (tearing, cyanosis, ptosis, salivation and piloerection). The consumption of water and food and body weights were also recorded daily (OECD 2008a). At the end of the observation period, the animals were anaesthetized (ketamine and xylazine, 25 and 10 mg/kg, respectively), and the organs (heart, lung, spleen, liver, kidney, uterus and ovary) were removed, weighed and examined macroscopically.

Subacute oral toxicity

Swiss mice were randomly divided into four groups (n = 10 animals/group, 5 males and 5 females). Three different doses of EEGC (75, 150 or 300 mg/kg) were administered per group orally (by gavage) daily for 28 consecutive days (OECD 2008b). The control group received only vehicle (0.9% saline, 1.0 mL/kg). The doses were chosen based on a previous study performed by our research group that showed that the highest dose (300 mg/kg) of this extract was the lowest dose that exhibited antihyperalgesic and antiarthritic effects (Macorini *et al.* 2020). During treatment, the body weights, food and water consumption, and possible signs of toxicity were observed and recorded daily, following the Hippocratic screening and behavioral analysis. Clinical examination was performed once daily. Study models of tail suspension and elevated plus-maze were used for behavioral assessment. At the end of the observation period, all animals were anesthetized (ketamine and xylazine, 25 and 10 mg/kg, respectively). Blood samples were collected by cardiac puncture for subsequent hematological and biochemical analyses. The following biochemical parameters were analyzed with Cobas C111 using commercial kits (Roche): urea, creatinine, total protein, albumin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Hematological analysis measured the total leukocyte count, erythrocytes and platelets, in addition to the levels of hemoglobin,

hematocrit and red cell distribution width with a Kx21N unit (Sysmex). After collecting blood, the vital organs (heart, lung, kidney, liver and spleen) were removed and weighed. Samples of all organs were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin and examined by light microscopy. Histological analysis aimed to assess the tissue integrity of the organs. The parameters analyzed were degeneration, necrosis, apoptosis, leukocyte infiltration, congestion, extravasation of blood, and fibrosis (Traesel *et al.* 2014).

Statistical analysis

The data are presented as the mean ± SEM (standard error of the mean). Differences among means were evaluated by one-way analysis of variance (ANOVA), followed by the Newman-Keuls post-hoc test, using GraphPad Prism software. Statistical differences were considered significant when $P < 0.05$.

Results

In the acute toxicity test, a dose of 2000 mg/kg EEGC did not cause the death of any animal. The exposed male and female rats showed no behavioral or body weight gain changes during the treatment period (Table 1). Similarly, food and water consumption, as well as the weights of the vital and reproductive organs (Table 2) were not altered by the treatment in both sexes. At necropsy, no macroscopic alterations were found in the organs.

After repeated exposure, no clinical signs of toxicity that could be associated with local or systemic toxicity was observed in both sexes. The animals were responsive and active to stimuli, and there were no deaths in the treated groups. The behavioral signs remained normal, with no difference among groups. In relation to body mass, reduced

weight gain was observed in the males treated with 75 mg/kg EEGC, which was associated with lower food intake when compared to the control group, and a higher body mass gain was observed in the males given 300 mg/kg EEGC without presenting a difference in food consumption. The females treated with 150 and 300 mg/kg EEGC also presented a decrease in weight gain without changes in food or water intake (Table 3).

Animals of both sexes exposed to treatment did not show alterations in the weights of their hearts, spleens, livers, lungs or kidneys (Table 4). Additionally, the biochemical and hematological parameters were similar among groups (Tables 5 and 6). The histological analyses also did not present alterations after treatment with EEGC (data not shown).

Discussion

EEGC has demonstrated pharmacological potential for the development of a product with several therapeutic properties (Macorini *et al.* 2020). However, the lack of sufficient scientific studies on their safety, especially after a repeated administration, has also attracted attention. The present study was the first to show that repeated oral exposure to ethanolic extract in a dosage with scientifically proven pharmacological effect is safe to rodents, and provides valuable data on the toxicological profile of this species.

Phytochemical studies have already demonstrated the presence of several compounds in EEGC (Vasconcelos *et al.* 2017; Dosumu *et al.* 2014), which, although they have important therapeutic properties, can cause adverse effects. Exposure to medicinal species preparations containing flavonoids, terpenes and saponins can induce nephrotoxicity (Xu *et al.* 2020). In this way, toxicity assays are required by regulatory agencies before using any medicinal plant as an herbal medicine (Brasil 2004).

In the present study, a single oral dose of 2000 mg/kg EEGC was safe for rats, since there were no clinical signs of toxicity. We can estimate that the LD_{50} for oral administration is over 2000 mg/kg body weight. According to OECD Guideline 425, this extract is considered to have low toxicity and can be included in category 5 (OECD 2008a). The aqueous extract of the whole plant *G. celosioides* was previously tested in rats at the dose of 4000 mg/kg and also did not induce acute toxicity (Souleymane *et al.* 2014), corroborating with our findings. Thus, based on the method of acute toxicity classification, EEGC was not toxic after a single oral administration.

Although the data obtained after a single exposure to the extract are important, regulatory agencies also request analyses after repeated and prolonged exposure to characterize the toxicological profile of a new pharmacological product (Sayyad *et al.* 2017). Repeated administration of medicinal plants is common, and these data help establish a dose-response relationship and determine doses for subchronic tests (OECD 2008b). Since *G. celosioides* whole plant is used as a juice preparation to cure urolithiasis over 10 days of administration (Prachi *et al.* 2009), we evaluated the exposure to three different doses of EEGC in a subacute toxicity assay. The results demonstrated that the animals did not exhibit clinical or behavioral alterations during the treatment that could be associated with local or systemic toxic effects from EEGC.

However, lower weight gain associated with a lower food intake was observed in the males treated with the lowest dose of EEGC, while the males exposed to a higher dose presented an increase in weight gain without a change in food consumption. In females, the intermediate and high doses significantly inhibited the weight gain of mice, and this alteration was not related to food intake. Changes in weight gain, as observed in this study, are indicators for evaluating the toxicity of natural products (Upadhyay *et al.* 2019) and may be due to either decreases in food and water consumption or organ injury caused

by the test substance (Berenguer Rivas *et al.* 2013). At the end of treatment, toxicological target organs were removed and weighed, and no alterations in weight or macroscopic morphology were observed in either sex.

As the liver is considered the primary organ of detoxification, biochemical evaluations of some hepatic enzymes (such as ALT and AST) or total protein and albumin contents may indicate a toxic action of the extract (Singh *et al.* 2016). In this study, the biomarkers of hepatic injury investigated were similar among all groups. Furthermore, the hepatic histology was normal and had a preserved morphology.

Several compounds present in this extract can be toxic to the kidneys (Xu *et al.* 2020). Thus, some important parameters of renal injury, including creatinine and urea contents were evaluated in this study. The amounts of these biomarkers is fundamental in the analysis of physiological mechanisms of glomerular filtration (Njinga *et al.* 2020). However, the doses of EEGC used in the subacute test did not change the renal biomarkers. Since the histology of the kidney was also not altered, these results indicate the absence of nephrotoxicity in the extract doses tested in this study.

Hematological parameters are among the most susceptible to potential toxic agents and may indicate alterations in hematopoiesis leading to diseases such as anemia, coagulation (platelets) or those immunological in nature (Abid and Mahmood 2019). Repeated treatment with EEGC did not interfere with the hematological parameters evaluated in this study. These parameters are considered to be the most sensitive to the toxic effects of substances (Li *et al.* 2010), which indicates the absence of hematotoxicity of the doses of extract used in this study.

According to the main parameters used in the subacute toxicity tests (hematological, biochemical and histopathological parameters), the doses of EEGC used in this study possess low toxicity. In addition to not showing toxicity, the highest dose

(300 mg/kg) of this extract was the lowest dose that presented antihyperalgesic and antiarthritic effects (Macorini *et al.* 2020). However, it is important to note that the toxicity of any natural product is associated with the amount or dose used. Compounds with low toxicity can be toxic if used at high dosages (Hill 1997). Additionally, components present in the extracts can interact with allopathic medicines, resulting in an increase or decrease in toxicological effects (Woolston 2017).

In conclusion, our findings show that the EEGC was not toxic after a single exposure, with an LD_{50} greater than 2000 mg/kg. The tested doses during its prolonged use have low toxicological potential and could be used in a safe way. However, for the development of an EEGC-based herbal medicine, other toxicological tests, especially chronic toxicity assays, should be performed.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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ATTACHMENT

Table 1. Body weight gain and food and water consumption of male and female rats treated with a single dose of EEGC.

Parameters	**Control**	**2000 mg/kg**
Initial weight (g)	269.70 ± 14.32	265.06 ± 10.49
Final weight (g)	281.34 ± 16.46	272.70 ± 12.29
Body weight gain (%)	4.63 ± 2.33	3.37 ± 1.09
Food intake (g/day)	17.87 ± 0.71	17.34 ± 0.35
Water intake (mL/day)	26.44 ± 0.83	27.46 ± 0.81

Values are expressed as the mean±SEM. $p>0.05$ (Student's test), n= 5 animals/group per sex.

Table 2. Relative organ weights (g/100 g of body weight) of male and female rats treated with a single dose of EEGC.

Parameters	Control	2000 mg/kg
Initial weight (g)	281.34 ± 16.46	272.70 ± 12.29
Left kidney	0.35 ± 0.01	0.38 ± 0.01
Liver	3.83 ± 0.19	3.91 ± 0.19
Spleen	0.21 ± 0.02	0.26 ± 0.02
Heart	0.36 ± 0.01	0.38 ± 0.01
Lung	0.33 ± 0.02	0.39 ± 0.03
Left ovary	0.02 ± 0.00	0.02 ±0.00
Uterus	0.19 ± 0.03	0.17 ± 0.01

Values are expressed as the mean±SEM. p>0.05 (Student's test), n= 5 animals/group per sex.

Table 3. Body weight gain and food and water consumption of male and female mice treated with repeated doses of EEGC.

Parameters	Control	75 mg/kg	150 mg/kg	300 mg/kg
Males				
PND 1 (g)	26.52 ± 0.29	29.54 ± 0.33	27.28 ± 0.29	20.80 ± 0.48
PND 6 (g)	27.85 ± 0.29	28.85 ± 0.48	28.32 ± 0.33	26.28 ± 0.36
PND 13 (g)	29.13 ± 0.27	29.67 ± 0.40	28.61 ± 0.36	28.25 ± 0.22
PND 20 (g)	30.31 ± 0.28	31.03 ± 0.51	28.95 ± 0.31	29.67 ± 0.28
PND 27 (g)	32.21 ± 0.32	31.97 ± 0.48	31.04 ± 0.30	29.77 ± 0.36
Body weight gain (%)	21.45 ± 2.19	8.22 ± 1.26**	13.78 ± 1.37	43.75 ± 3.71*
Food intake (g/day)	29.50 ± 1.05	20.04 ± 0.48*	27.71 ± 1.19	27.14 ± 1.00
Water intake (mL/day)	41.43 ± 2.5	27.77 ± 0.78	47.41 ± 2.61	45.63 ± 3.37
Females				
PND 1 (g)	20.96 ± 0.29	19.62 ± 0.39	21.37 ± 0.29	21.20 ± 0.26
PND 6 (g)	21.15 ± 0.32	19.57 ± 0.76	20.75 ± 0.25	21.27 ± 0.31
PND 13 (g)	22.29 ± 0.35	20.57 ± 0.48	22.49 ± 0.28	21.82 ± 0.28
PND 20 (g)	24.19 ± 0.41	21.72 ± 0.60	21.74 ± 0.36	22.38 ± 0.31
PND 27 (g)	24.41 ± 0.46	22.26 ± 0.50	22.90 ± 0.43	22.75 ± 0.41
Body weight gain (%)	16.45 ± 1.63	13.45 ± 1.21	7.15 ± 0.86*	7.31 ± 0.71*
Food intake (g/day)	20.49 ± 0.90	19.42 ± 0.78	19.28 ± 0.90	20.22 ± 0.91
Water intake (mL/day)	27.14 ± 1.09	24.11 ± 1.20	25.18 ± 1.23	23.39 ± 1.21

PND: Postnatal day
Values are expressed as the mean±SEM. * $p < 0.05$ (ANOVA/Tukey test) compared to control group, n = 5 animals/group per sex.

Table 4. Relative organ weights (g/100 g of body weight) of male and female mice treated with repeated doses of EEGC.

Parameters	Control	75 mg/kg	150 mg/kg	300 mg/kg
Males				
Liver	4.06 ± 0.07	4.33 ± 0.10	3.88 ± 0.10	4.15 ± 0.07
Kidney	0.79 ± 0.01	0.79 ± 0.02	0.78 ± 0.02	0.80 ± 0.02
Spleen	0.38 ± 0.01	0.43 ± 0.03	0.35 ± 0.01	0.42 ± 0.02
Lung	0.66 ± 0.02	0.52 ± 0.01	0.56 ± 0.01	0.66 ± 0.02
Heart	0.53 ± 0.01	0.49 ± 0.01	0.47 ± 0.01	0.50 ± 0.03
Females				
Liver	4.93 ± 0.06	4.88 ± 0.12	4.44 ± 0.2	4.49 ± 0.14
Kidney	0.75 ± 0.01	0.70 ± 0.02	0.66 ± 0.02	0.74 ± 0.02
Spleen	0.65 ± 0.07	0.59 ± 0.05	0.51 ± 0.06	0.47 ± 0.02
Lung	0.70 ± 0.04	0.68 ± 0.02	0.56 ± 0.02	0.63 ± 0.04
Heart	0.54 ± 0.01	0.49 ± 0.01	0.54 ± 0.02	0.58 ± 0.02

Values are expressed as the mean±SEM. $p>0.05$ (ANOVA/Tukey test) compared to control group, n = 5 animals/group per sex.

Table 5. Biochemical parameters of the mice treated with repeated doses of EEGC.

Parameters	Control	75 mg/kg	150 mg/kg	300 mg/kg
Males				
Total proteins (g/dL)	43.28 ± 0.36	43.30 ± 0.72	43.34 ± 0.97	37.33 ± 3.29
Albumin (g/dL)	20.18 ± 0.88	15.58 ± 1.52	20.62 ± 1.30	17.25 ± 0.84
ALT (U/L)	36.73 ± 0.42	42.08 ± 2.20	36.68 ± 1.0	47.63 ± 6.99
Creatinine (mg/dL)	0.22 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.17 ± 0.02
Urea (mg/dL)	50.92 ± 0.66	50.64 ± 1.10	48.42 ± 0.93	46.12 ± 0.83
Females				
Total proteins (g/dL)	35.80 ± 2.57	44.33 ± 1.65	42.90 ± 0.56	41.20 ± 0.87
Albumin (g/dL)	18.50 ± 1.75	22.79 ± 2.00	22.31 ± 1.50	19.22 ± 1.30
ALT (U/L)	41.65 ± 3.45	40.35 ± 1.32	35.55 ± 1.46	39.10 ± 1.70
Creatinine (mg/dL)	0.18 ± 0.01	0.15 ± 0.01	0.18 ± 0.01	0.15 ± 0.01

Urea (mg/dL)	46.30 ± 3.45	52.43 ± 1.95	48.78 ± 1.91	41.20 ± 0.43

Values are expressed as the mean±SEM. p>0.05 (ANOVA/Tukey test) compared to control group, n = 5 animals/group per sex.

Table 6. Hematological parameters of male and female mice treated with repeated doses of EEGC.

Parameters	**Control**	**75 mg/kg**	**150 mg/kg**	**300 mg/kg**
Males				
Erythrocytes (10^6/µL)	8.82 ± 0.29	8.97 ± 0.13	8.38 ± 0.34	8.86 ± 0.22
Hemoglobin (g/dL)	13.70 ± 0.35	14.03 ± 0.15	13.10 ± 0.30	13.80 ± 0.53
Hematocrit (%)	49.17 ± 1.55	49.23 ± 0.35	45.10 ± 1.65	48.10 ± 1.15
MCV (fL)	55.73 ± 1.42	54.90 ± 0.44	53.75 ± 1.01	54.30 ± 0.17
MCH (pg)	15.53 ± 0.47	15.67 ± 0.32	15.60 ± 0.41	15.57 ± 0.25
MCHC (g/dL)	27.87 ± 0.29	28.50 ± 0.46	29.07 ± 0.68	28.70 ± 0.44
Leukocytes (10^3/ µL)	3.67 ± 0.85	4.77 ± 0.81	4.34 ± 1.25	4.03 ± 0.91
Females				
Erythrocytes (10^6/µL)	8.33 ± 0.14	8.543 ± 0.11	8.67 ± 0.06	8.70 ± 0.29
Hemoglobin (g/dL)	13.67 ± 0.20	13.56 ± 0.19	14.00 ± 0.06	14.00 ± 0.35
Hematocrit (%)	49.03 ± 0.63	47.36 ± 0.85	48.67 ± 0.33	47.43 ± 1.57
MCV (fL)	57.47 ± 0.73	55.43 ± 0.52	54.07 ± 0.35	54.50 ± 0.32
MCH (pg)	16.03 ± 0.32	15.90 ± 0.15	15.53 ± 0.13	16.13 ± 0.27
MCHC (g/dL)	28.37 ± 0.37	28.63 ± 0.15	28.77 ± 0.08	29.57 ± 0.40
Leukocytes (10^3/ µL)	4.50 ± 0.20	4.60 ± 0.36	4.95 ± 0.30	4.88 ± 0.22

Values are expressed as the mean±SEM. p>0.05 (ANOVA/Tukey test) compared to control group, n = 5 animals/group per sex. MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration).

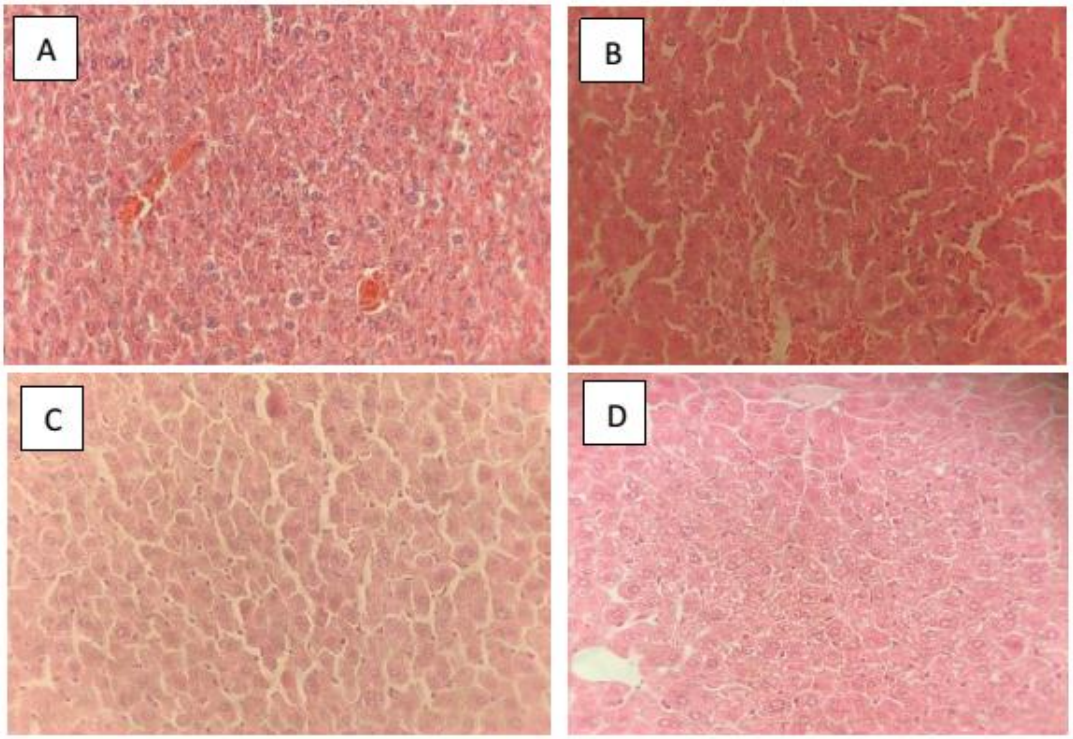

Figura 1. Histopathological analysis of subacute hepatic toxicity of Swiss female mice treated with ethanol extract from Gomphrena celosioides leaves at doses of 75, 150 and 300 mg / kh, p.o., and saline control, p.o. for 28 days. A- Saline Control; B- EXGC 75 mg / kg; C- 150 mg / kg; D- 300 mg / kg.

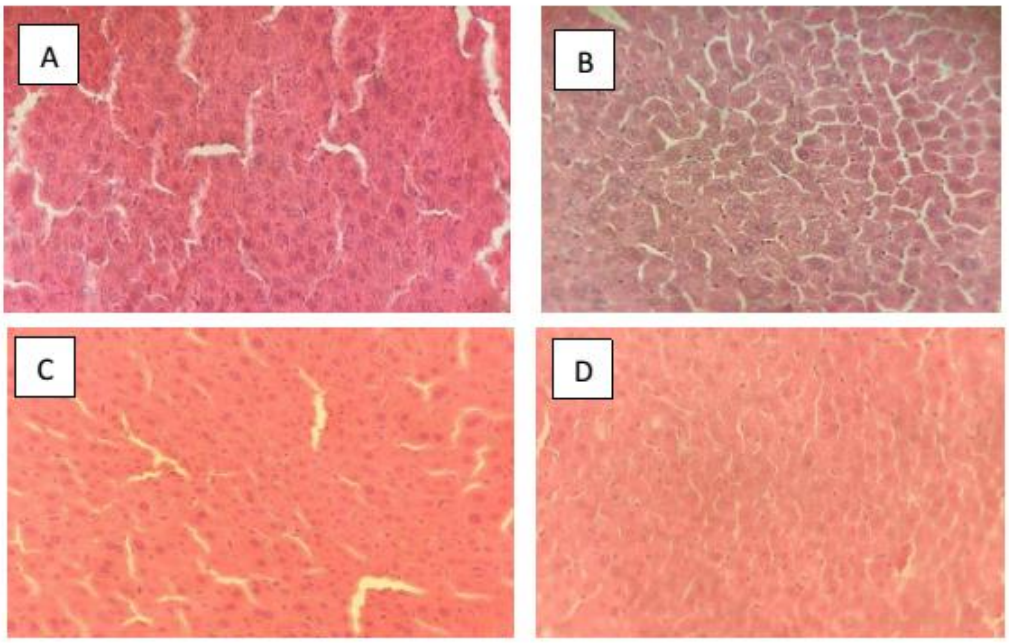

Figure 2. Histopathological analysis of subacute hepatic toxicity in male Swiss mice treated with ethanolic extract of Gomphrena celosioides leaves at doses of 75, 150 and 300 mg / kh, p.o., and saline control, p.o. for 28 days. A- Saline Control; B- EEGC 75 mg/kg; C- EEGC 150 mg/kg; D- EEGC 300 mg/kg.

6. CONCLUSÃO

Em conjunto, essa tese apresenta a atividade anti-inflamatória e analgésica frente à modelos *in vivo* do extrato etanólico das folhas de *Gomphrena celosioides* corroborando com o uso popular, além de demonstrar em modelos de toxicidade aguda e subaguda *in vivo* que não houve alterações comportamentais, histológicas, bioquímicas, hematólogicas e peso dos órgãos o que reforça o grande potencial da planta em desenvolvimento de produto fitoterápico.

7. ANEXOS

7.1 Carta de aprovação do comitê de ética em uso de animais

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

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 26 de junho de 2018.

CERTIFICADO

Certificamos que a proposta intitulada ***"Avaliação farmacológica e toxicológica do extrato etanólico de Gomphrena celosioides"***, registrada sob o protocolo de nº 06/2018, sob a responsabilidade de *Cândida Aparecida Leite Kassuya e Luis Fernando Benitez Macorini* – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata, subfilo Vertebrata* (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados, em reunião de 16/03/2018.

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização	*28/06/2018 a 22/01/2020*
Espécie/linhagem/raça	*Rattus norvegicus / Mus musculus*
Nº de animais	92 *Wistar / 170 Swiss*
Peso/idade	200 g / 20 g
Sexo	86 machos e 8 fêmeas / 170 machos
Origem	Biotério Central UFGD

Melissa Negrão Sepulvida
Coordenadora CEUA

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