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

Efeitos do extrato etanólico e gel das folhas de *Cochlospermum regium* (Schrank) Pilg. em processos inflamatórios, feridas e no controle de *Staphylococcus aureus*

FERNANDA DE OLIVEIRA GALVÃO SANTOS

Dourados - MS
2023

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Orientadora: Profª. Drª. Kelly Mari Pires de Oliveira

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“Pois o Senhor, o seu Deus, estará
com você por onde você andar.”

Josué 1:9

LISTA DE ILUSTRAÇÕES

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE ABREVIATURAS

REVISÃO DE LITERATURA

HIV	Human immunodeficiency virus
MRSA	Methicillin-resistant *Staphylococcus aureus*/*S. aureus* resistente à meticilina
PBP2a	Penicillin binding protein 2a

ARTIGO 1

C	Crust
C. regium	*Cochlospermum regium*
D	Dermis
DC	Cell debris
DEXA	Dexamethasone
EECR	Ethanolic extract of *C. regium* leaves
EP	Epidermis
Epi	Incomplete epidermis
GEECR	Gel of the ethanolic extract of *C. regium*
HE	Hematoxylin and eosin
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
MOR	Morphine
MPO	Myeloperoxidase
N	Neovascularization
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
PRED	Prednisolone
Q	Keratin layer
S.C	Subcutaneously
SEM	Standard error of the mean

UHPLC-ESI-MS/MS	Ultrahigh performance liquid chromatography–electrospray ionization tandem mass spectrometry

ARTIGO 2

ATCC	American Type Culture Collection
CIM	Concentração inibitória mínima
DD	Disco difusão
DP	Difusão em poço
WFO	World Flora Online

ARTIGO 3

AA	Ácido ascórbico
Abs	Absorbância
ABTS	3-etilbenzotiazolina-6 ácido sulfônico
ANOVA	Analysis of Variance/ Análise de variância
ATCC	American Type Culture Collection
C. regium	*Cochlospermum regium*
CC50%	Concentração citotóxica capaz de matar 50% das células
DMEM	Dulbecco's Modified Eagle Medium/ meio de Eagle modificado por Dulbecco
DPPH	1,1-difenil-2-picrilhidrazil
EECR	Extrato etanólico das folhas de *Cochlospermum regium*
GCR	Gel de *C. regium*
IM	Índice de mutagenicidade
MH	Mueller-Hinton
MRSA	Methicillin-resistant *Staphylococcus aureus*/*S. aureus* resistente à meticilina
NaCl	Cloreto de sódio
PBS	Phosphate buffered saline/Tampão fosfato de potássio
UFC	Unidade formadora de colônia

Efeitos do extrato etanólico e gel das folhas de *Cochlospermum regium* (Schrank) Pilg. em processos inflamatórios, feridas e no controle de *Staphylococcus aureus*

RESUMO

Cochlospermum regium (Schrank) pilg., conhecido popularmente como algodãozinho do cerrado, é utilizado na medicina tradicional no tratamento de gastrite, úlceras, artrite, inflamações, infecções, feridas e afecções da pele. Na busca de novas alternativas de produtos terapêuticos, os extratos vegetais se destacam como fontes de recursos naturais para serem incorporados em formulações farmacêuticas. Neste sentido, o presente trabalho teve como objetivo determinar a composição química e avaliar as propriedades biológicas do extrato etanólico e gel das folhas de *C. regium,* além de fornecer informações sobre a fitoquímica, o potencial biológico e a segurança farmacológica de *C. regium*. No primeiro artigo, para caracterização química das folhas de *C. regium* foi realizada cromatografia líquida com espectrometria de massa, e para avaliação da atividade anti-inflamatória do extrato e cicatrizante do gel foram conduzidos testes *in vivo* de pleurisia induzida por carragenina e edema de pata, modelo de dor induzida por formalina e modelo de ferida de excisão. Foram identificados 25 compostos, incluindo quercitrina, galato de metila e 1,2,3,4,6-pentagalloylhexose e comprovada a atividade anti-inflamatória, antinociceptiva e cicatrizante das folhas de *C. regium*. No segundo artigo, para compilar os resultados científicos de pesquisas originais com a espécie *C. regium* foi realizada uma busca em cinco bancos de dados, com pesquisa utilizando o termo "*Cochlospermum regium*". O levamento bibliográfico revelou presença diversificada de compostos biologicamente ativos, bem como, estudos de citotoxicidade, toxicidade e mutagenicidade, que sugerem a segurança da utilização dos extratos de *C. regium*. Sobre a investigação da atividade biológica da planta foi observada uma predominância de estudos antimicrobianos. No terceiro artigo, o extrato etanólico das folhas de *C. regium* foi incorporado em uma formulação de gel e suas propriedades físicas e antibacterianas foram avaliadas, bem como, verificados o potencial antioxidante e biocompatibilidade do extrato. As formulações de géis desenvolvidas apresentaram estabilidade e compatibilidade física quando submetidas a diferentes condições de armazenamento e desempenharam atividade frente a *Staphylococcus aureus*, incluindo um isolado de ferida resistente à meticilina. Somado aos potenciais evidenciados nos

géis, o extrato etanólico das folhas de *C. regium* apresentou atividade antioxidante e biocompatibilidade. Diante dos resultados encontrados nos estudos, foi possível concluir que as folhas de *C. regium* demonstraram ser uma alternativa mais sustentável para a conservação da espécie, visto que, a mesma apresenta alto potencial biológico no tratamento de inflamações, dor, feridas e no controle de *S. aureus*, mostrando resultados mais efetivos contra MRSA comparado com fármaco utilizado comercialmente, sendo promissora para o desenvolvimento de novas formulações terapêuticas de uso tópico.

Palavras-chave: Algodãozinho do cerrado. Plantas medicinais. Atividades biológicas. Formulações tópicas. Resistência bacteriana.

Effects of ethanolic extract and gel of *Cochlospermum regium* (Schrank) Pilg. Leaves on inflammatory processes, wounds, and control of *Staphylococcus aureus*

ABSTRACT

Cochlospermum regium (Schrank) pilg., popularly known as “algodãozinho do cerrado”, is used in traditional medicine to treat gastritis, ulcers, arthritis, inflammations, infections, wounds and skin conditions. In the search for new therapeutic product alternatives, plant extracts stand out as sources of natural resources to be incorporated into pharmaceutical formulations. In this sense, the present work aimed to determine the chemical composition and evaluate the biological properties of the ethanolic extract and gel from *C. regium* leaves, in addition to providing information on the phytochemistry, biological potential and pharmacological safety of *C. regium*. In the first article, for chemical characterization of *C. regium* leaves, liquid chromatography with mass spectrometry was carried out, and to evaluate the anti-inflammatory activity of the extract and healing activity of the gel, transient in vivo tests of pleurisy caused by carrageenan and paw edema were carried out by formalin pain model and excision wound model. 25 compounds were identified, including quercitrin, methyl gallate and 1,2,3,4,6-pentagalloylhexose and proven anti-inflammatory, antinociceptive and healing activity in *C. regium* leaves. In the second article, to compile the scientific results of original research with the species *C. regium*, a search was carried out in five databases, with a search using the term “*Cochlospermum regium*”. The bibliographic survey revealed diverse proposals for biologically active compounds, as well as cytotoxicity, toxicity and mutagenicity studies, which suggest the safety of using *C. regium* extracts. Regarding the investigation of the biological activity of the plant, a predominance of antimicrobial studies was observed. In the third article, the ethanolic extract of *C. regium* leaves was incorporated into a gel formulation and its physical and antibacterial properties were evaluated, as well as the antioxidant potential and biocompatibility of the extract. The gel formulations were designed with stability and physical compatibility when subjected to different storage conditions and influenced activity against *Staphylococcus aureus*, including a methicillin-resistant wound isolate. Added to the potentials evidenced in the gels, the ethanolic extract of *C. regium* leaves showed antioxidant activity and biocompatibility. It was possible to conclude that *C. regium* leaves proved to be a more sustainable alternative for the conservation of the species, as it has high biological potential in the

treatment of inflammation, pain, wounds and control of S. aureus, showing more effective results against MRSA compared to the commercially used drug, being promising for the development of new therapeutic formulations for topical use.

***Keywords*:** Algodãozinho do cerrado. Medicinal plants. Biological activities. Topical formulations. Bacterial resistance.

SUMÁRIO

1. INTRODUÇÃO

A utilização de plantas medicinais contribui de forma adjunta para o tratamento de doenças e promoção da saúde, uma vez que, os compostos produzidos a partir do metabolismo secundário das plantas são responsáveis pelos seus potenciais biológicos (KELLO et al., 2023). Nesta perspectiva, a espécie *Cochlospermum regium* (Schrank) Pilg., conhecida popularmente como algodãozinho do cerrado e pertencente à família Bixaceae Kunth, apresenta relevância medicinal devido a sua ampla utilização popular no tratamento de gastrite, úlceras, artrite, infecções, inflamações, feridas e afecções da pele (CAMILLO et al., 2009; NUNES et al., 2003). No entanto, o uso popular dessa espécie é concentrado no extrativismo de suas raízes, o que potencializa sua destruição e a enquadra na lista de espécies ameaçadas de extinção (IUCN, 2022).

Com intuito de incentivar a preservação dessa espécie e elucidar novos achados científicos em relação a suas bioatividades, o nosso grupo de pesquisa tem estudado os potenciais biológicos das folhas de *C. regium*, evidenciando atividades antimicrobianas e antibiofilme contra *Candida tropicalis*, *Escherichia coli*, *Cryptococcus gattii* e *Staphylococcus aureus* (LEME et al., 2017; ALMEIDA-APOLONIO et al., 2018; GALVÃO et al., 2020). Além disso, nossa triagem fitoquímica anterior revelou que esta planta contém fenóis, flavonóides e taninos, incluindo os compostos ácidos elágico e gálico (GALVÃO et al., 2020).

De acordo com os potenciais encontrados, com o conhecimento etnofarmacológico e com o aumento da conscientização da saúde pública para a utilização de novas alternativas de origem natural atóxicas e bioativas, essa espécie se destaca como uma opção de incorporação em novos produtos terapêuticos para o tratamento de inflamações, feridas e infecções associadas a *Staphylococcus aureus*, principalmente no que se refere a resistência desse microrganismo aos antimicrobianos tópicos e o surgimento de cepas resistentes à meticilina (Methicillin-resistant *Staphylococcus aureus*: MRSA) (BESSA et al., 2016). Dentre as opções de formulações terapêuticas que podem ser desenvolvidas a base de extratos vegetais, os géis são opções viáveis devido as suas características de espalhabilidade, liberação de compostos, afinidade com água, entre outros fatores que corroboram para o auxílio no tratamento de diferentes doenças (ABDEL GHAFFAR; RADWAN; ALI, 2016; MICALE et al., 2020).

Neste contexto, o presente trabalho teve como objetivo fornecer informações sobre a fitoquímica, o potencial biológico e a segurança farmacológica de *C. regium*, além de determinar a composição química e avaliar a atividade anti-inflamatória e antinociceptiva do extrato etanólico das folhas de *C. regium* (EECR), assim como, desenvolver uma formulação de gel com EECR e avaliar seu efeito cicatrizante e antibacteriano.

2. REVISÃO DA LITERATURA

2.1 Cochlospermum regium

Cochlospermum regium (Schrank) Pilger, membro da família Bixaceae já foi classificado como parte da família Cochlospermaceae Planchon, o que explica a persistência desse termo em alguns trabalhos, no entanto, estudos moleculares indicam sua inclusão na família Bixaceae Kunth. Popularmente conhecida por vários nomes, como algodãozinho-do-campo, algodão-do-campo, algodãozinho-do-cerrado e algodão-bravo, essa espécie é comumente encontrado em campos cerrados e cerrados típicos (FILHO, 2020; POPPENDIECK, 1981).

É um arbusto ou subarbusto que atinge de 0,5 a 2 metros de altura, apresentando raiz engrossada, conhecida como xilopódio, e caule subterrâneo, características que conferem a planta resistência ao fogo e ao pastejo (DIAS & LAUREANO, 2009; POTT & POTT, 1994). Suas folhas, alternadas e simples, longo-pecioladas, palmatificadas, subdigitadas, possuem base obtusa a cordada e 3-5 lobos oval-elipticos. Com flores amarelo-douradas que variam de 6 a 8 cm de diametro e pétalas de coloração amarelo intenso de forma ovaladas-subquadradas, o algodãozinho-do-campo recebe esse nome devido ao seu fruto seco, que produz sementes pilosas com pelos brancos longos semelhantes ao algodão. Seu período reprodutivo, marcado pela perda de folhas e surgimento de flores, ocorre de maio a setembro (POPPENDIECK, 1981; DA SILVA et al., 2020).

Na medicina popular, *C. regium* é utilizado para o tratamento de infecções ginecológicas, infecções da próstata, reumatismo, dores, abcessos, afecções da pele, feridas internas e externas, e também no tratamento de gastrite, ulceras, cravos, espinhas e manchas da pele. Para a realização dos tratamentos medicinais a planta é submetida aos processos de decocção ou infusão, e o produto obtido pode ser utilizado tanto de forma tópica, por banho na região que deseja ser tratada, quanto em forma de ingestão na forma de chá, ou por meio garrafada feita geralmente com vinho branco.

De forma geral, a principal parte indicada e utilizada é a raíz da planta (BIESKI et al., 2012; RODRIGUES; CARLINI, 2006; SÓLON; BRANDÃO; SIQUEIRA, 2009; USTULIN et al., 2009).

O uso frequente de suas raízes é uma preocupação, já que a sua remoção pode resultar na morte da planta, aumentando o risco de extinção da espécie, por essa razão, *C. regium* foi incluído na lista de espécies medicinais prioritárias para conservação, e o interesse em pesquisas direcionadas para explorar outras partes da planta que exibem potencial medicinal sem comprometer a integridade da espécie tem crescido (IUCN, 2018). Portanto, nossa equipe de pesquisa tem se dedicado a investigar o potencial biológico das folhas de *C. regium*, e já relatamos que o extrato etanólico dessas folhas apresenta atividade antimicrobiana e antibiofilme contra *Escherichia coli*, *Candida tropicalis*, *Cryptococcus gattii* e *Staphylococcus aureus* (ALMEIDA-APOLONIO et al., 2018; GALVÃO et al., 2020; LEME et al., 2017). Consequentemente, a utilização e avaliação das folhas de *C. regium* representa uma proposta que contribui para a conservação da espécie.

2.2 *Staphylococcus aureus*

Staphylococcus aureus é uma bactéria esférica, identificada como gram-positiva. Esta espécie bacteriana pode se desenvolver tanto em ambientes aeróbios quanto anaeróbios, tendo a capacidade de se adaptar a ambas as condições. Sua morfologia é caracterizada por crescimento em aglomerados que se assemelham a "cachos de uva", uma disposição típica formada por células bacterianas agrupadas em um padrão irregular (TAYLOR; UNAKAL, 2017). *S. aureus* é a espécie mais notável dentro do gênero *Staphylococcus*, sendo amplamente distribuído na natureza e também fazendo parte da microbiota humana. Sua presença como parte normal da microbiota pode ser encontrada em várias áreas do corpo humano, incluindo a pele, narinas e trato respiratório superior (YAMAZAKI et al., 2017).

No entanto, apesar de ser uma parte comum da microbiota humana, o *S. aureus* pode se tornar patogênico em determinadas situações. Isso ocorre especialmente quando há ruptura das barreiras naturais do corpo ou uma redução na resposta imunológica do indivíduo. Diversos estudos (KARANIKA et al., 2015; MAJCHRZAK et al., 2016; PIO et al., 2016) destacam a associação dessa bactéria com infecções oportunistas, principalmente em pacientes vulneráveis, como,

diabéticos, pacientes com queimaduras na pele, indivíduos HIV-positivos, pacientes em diálise e aqueles internados em ambientes hospitalares por longos períodos.

O amplo espectro de infecções causadas pelo *S. aureus* compreende desde condições cutâneas relativamente simples, como impetigo, foliculite e furúnculos, até formas mais invasivas e sistêmicas, como endocardites, pneumonia, osteomielites, meningite, síndrome do choque tóxico, bacteremias, septicemia e outras complicações (TONG et al., 2015).

Essa diversidade de infecções reflete a notável capacidade adaptativa e patogênica do *S. aureus*, permitindo que ele afete diferentes sistemas do corpo humano, representando, assim, um desafio substancial no cenário clínico. No entanto, é importante destacar que as infecções cutâneas desempenham um papel crucial na disseminação do *S. aureus*, especialmente aquelas causadas por cepas resistentes, como o MRSA (Methicillin Resistant *Staphylococcus aureus*) (AL-MEBAIRIKA et al., 2016). A resistência do MRSA ocorre devido a produção de uma proteína chamada PBP2a, que substitui a proteína alvo original dos antibióticos beta-lactâmicos, resultando na ineficácia desses antibióticos contra a bactéria e dificultando o tratamento eficaz de infecções causadas por esta cepa bacteriana (ZHAN & ZHU 2018).

Enquanto tradicionalmente o MRSA é considerado um problema nos ambientes hospitalares, ocorre um aumento global nos casos de infecções de pele relacionadas à comunidade, afetando até mesmo indivíduos sem histórico de hospitalização (SIDDIQUI; WHITTEN, 2018). Esta disseminação do MRSA na comunidade destaca a importância de estratégias eficazes de prevenção e controle não apenas em ambientes hospitalares, mas também em contextos comunitários para lidar com a ameaça das infecções cutâneas por *S. aureus* resistente a antibióticos.

2.3 Processo inflamatório

A inflamação é a reação do sistema imunológico a danos, esses danos podem estar associados a infecções ou lesões teciduais, o que envolve a ativação de células e substâncias químicas (CHEN et al., 2017). Apresenta inicio com a liberação de mediadores, como histamina e prostaglandinas, causando dilatação dos vasos sanguíneos e aumento da permeabilidade. Isso permite a migração de leucócitos, principalmente neutrófilos, seguidos por macrófagos, para o local da lesão, que podem atuar na remoção de microrganismos e células danificadas. Quando desregulada, a inflamação pode resultar em doenças autoimunes, crônicas ou complicações adversas (ABDULKHALEQ, et al. 2018).

A inflamação exibe cinco sinais distintos, denominados sinais cardinais. O rubor, expresso pela vermelhidão, resulta da vasodilatação, ampliando o fluxo sanguíneo na área afetada. O calor, está associado ao aumento do metabolismo e do fluxo sanguíneo local. O inchaço, conhecido como tumor, surge do acúmulo de fluidos, como plasma e células inflamatórias. A dor, origina-se da irritação das terminações nervosas pela pressão do edema e liberação de substâncias químicas. A perda de função, surge quando a inflamação afeta a funcionalidade normal do tecido ou órgão (MEDZHITOV, 2010).

A necessidade de novos tratamentos para inflamações é essencial, devido às limitações e toxicidades dos tratamentos atuais (PLACHÁ; JAMPÍLEK, 2021). Embora eficazes, muitos medicamentos causam efeitos colaterais, como toxicidade a órgãos e impactos no sistema imunológico. Desenvolver terapias mais específicas é crucial para reduzir esses efeitos, mantendo ou melhorando a eficácia no controle da inflamação. A pesquisa de abordagens inovadoras visa a terapias mais seguras e direcionadas, capazes de controlar a inflamação de maneira mais eficaz e com menor impacto negativo na saúde dos pacientes.

2.4 Formulações a base de extratos vegetais

Dentro do contexto da busca de novas alternativas atóxicas e eficazes no tratamento de infecções e inflamações, pode ser observado uma preocupação mundial crescente em relação às políticas de sustentabilidade, conservação de recursos naturais, redução no impacto ambiental e geração de resíduos, e a busca por produtos mais seguros e biodegradáveis (FONSECA-SANTOS; CORRÊA; CHORILLI, 2015; PAI et al., 2020; SENGAR; VIJAYANANDAN, 2022). Desta forma, a incorporação de extratos vegetais em formulações terapêuticas com a finalidade de se obter produtos naturais menos agressivos à saúde e ao meio ambiente, vem atraindo a atenção do mercado e dos consumidores (ATANASOV et al., 2021; GHAZALI et al., 2017).

Além disso, essas formulações se apresentam promissoras, visto que o Brasil possui uma vasta biodiversidade, proporcionando o desenvolvimento de tecnologias de produção econômica, ecológica e segura. As aplicações desses produtos desenvolvidos são amplas e podem ser atribuídas a diferentes contextos, como por exemplo no tratamento de inflamações, feridas e infecções de pele por *S. aureus* (ALI et al., 2019; ARDEKANI et al., 2019; KARUNANIDHI et al., 2017; MULLER et al., 2018; ZEPON et al., 2019). *Cochlospermum regium*, se destaca neste contexto de novas aplicações terapêuticas, levando em consideração suas utilzações na medicina tradicional e os

novos artigos que trazem descobertas quanto ao potencial biológico desta espécie (ALMEIDA-APOLÔNIO et al., 2018; GALVÃO et al., 2020; LEME et al., 2017).

Assim, as formulações desenvolvidas a base de extratos vegetais, como géis, apresentam destaque devido as suas características de espalhabilidade e liberação dos compostos bioativos (ABDEL GHAFFAR; RADWAN; ALI, 2016; MICALE et al., 2020), podendo ser produzidas por meio de técnicas simples e de baixo custo, que valorizam o meio ambiente (CHIRAYATH et al., 2019). A comprovação científica da atividade biológica e de sua segurança são necessárias para o desenvolvimento correto e seguro de novos produtos.

3. OBJETIVOS

GERAL

Identificar compostos químicos, avaliar atividade anti-inflamatória e antinociceptiva do extrato etanólico das folhas de *C. regium*, desenvolver uma formulação de gel com extrato etanólico das folhas de *C. regium* e verificar seu efeito cicatrizante e antibacteriano. Destacar os resultados científicos de pesquisas originais com *C. regium* associados a estudos fitoquímicos, atividades biológicas e segurança farmacológica.

ESPECÍFICOS

- Identificar os componentes químicos presentes no EECR;
- Avaliar a atividade anti-inflamatória e antinociceptiva do EECR, por meio dos modelos de edema de patas, pleurisia e formalina em camundongos;
- Incorporar o EECR em formulações de géis;
- Avaliar a atividade cicatrizante do gel por meio de modelo de ferida excisional em ratos Wistar;
- Verificar a establidade e compatiblidade física das formulações desenvolvidas;
- Analisar atividade antibacteriana *in vitro* e *ex vivo* do gel contra *Staphylococcus aureus* e isolado de *S. aureus* resistente à meticilina;
- Avaliar a atividade antioxidante do EECR;
- Analisar a biocompatiblidade do EECR
- Realizar uma revisão da literatura destacando os resultados científicos de pesquisas originais com *C. regium* sobre estudos fitoquímicos, atividades biológicas e segurança farmacológica;

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5. APÊNDICES

5.1 Artigo 1: **Chemical composition and effects of ethanolic extract and gel of *Cochlospermum regium* (Schrank) Pilg. Leaves on inflammation, pain, and wounds**

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Chemical composition and effects of ethanolic extract and gel of *Cochlospermum regium* (Schrank) Pilg. Leaves on inflammation, pain, and wounds

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ABSTRACT

Ethnopharmacological relevance: *Cochlospermum regium* is well-known as "Algodãozinho do cerrado" in folk Brazilian medicine, and is used to fight infections, inflammation and skin disorders.
Aim of the study: To identify the phytochemical constituents and the effects of the ethanolic extract of *C. regium* leaves (EECR) on inflammation and pain, and the effects of *C. regium* gel (GEECR) on wound healing.
Materials and methods: Animals were treated with EECR (30–300 mg/kg) or GEECR (1.25 and 2.5%) and studies were conducted using carrageenan-induced pleurisy and paw edema tests, formalin-induced pain model, and excision wound model.
Results: In total, 25 compounds, including quercitrin, methyl gallate, and 1,2,3,4,6-pentagalloylhexose, with highest detectability were identified. The treatments reduced leukocyte migration, nitric oxide production, protein extravasation, edema, mechanical hyperalgesia, pain in both phases (neurogenic and inflammatory), cold hypersensitivity, and improved wound closure and tissue regeneration.
Conclusions: The present findings established the anti-inflammatory, anti-nociceptive, and wound healing potential of the leaves of *C. regium*, confirming the potential therapeutic effect of this plant.

1. Introduction

Medicinal plants have great relevance in the treatment of several diseases, thereby generating a high impact on health promotion. They are one of the main sources of bioactive substances, which are used as precursors for pharmacological drug development, and continue to be an important medicinal source for the majority of the population (Khan et al., 2020; Vieira et al., 2020). Data from the World Health Organization (WHO, 2019) revealed that approximately 80% of the countries relies on traditional and complementary medicine, such as medicinal plants, for the primary treatment of diseases.

Plant-based medicines have recently been gaining popularity in the pharmaceutical industry worldwide as they are cheaper, easier to prepare, and have fewer side effects than conventional synthetic medicines, leading to greater accessibility and safety (Fridlender et al., 2015). Therefore, discovering new medicinal plants is important because of the growing demand for phytomedicines worldwide and for developing commercially important industrial products that can improve the quality of life of patients.

Cochlospermum regium (Schrank) Pilg., an essential ethno-medicinal, ornamental, and forage plant, belongs to the Bixaceae family, according to World Flora Online (WFO, 2022). It is commonly called as "Algodãozinho do Cerrado", and is predominantly found in the Brazilian

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Abbreviations

C	Crust
C. regium	*Cochlospermum regium*
D	Dermis
DEXA	dexamethasone
DC	cell debris
EECR	Ethanolic extract of *C. regium* leaves
EP	Epidermis
Epi	Incomplete epidermis
GEECR	Gel of the ethanolic extract of *C. regium*
HE	Hematoxylin and eosin
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
MOR	Morphine
MPO	myeloperoxidase
N	Neovascularization
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
PRED	prednisolone
Q	keratin layer
S.C	Subcutaneously
SEM	Standard error of the mean
UHPLC-ESI-MS/MS	Ultrahigh performance liquid chromatography–electrospray ionization tandem mass spectrometry

mid-west region. It has long been used in folk medicine as an antibacterial, analgesic, and anti-inflammatory agent to treat skin conditions, such as wounds and skin infections (Nunes and Carvalho, 2003; Nunes et al., 2003). Previous studies by our research group revealed that the leaves of this plant exhibit antimicrobial and anti-biofilm activities against *Candida tropicalis*, *Escherichia coli*, *Cryptococcus gattii*, and *Staphylococcus aureus* (Leme et al., 2017; Almeida-Apolonio et al., 2018; Galvão et al., 2020). Furthermore, our previous phytochemical screening revealed that this plant contains phenols, flavonoids, and tannins, including the compounds ellagic and gallic acids (Galvão et al., 2020).

As scientific evidence has not been collected on the traditional wound healing and anti-inflammatory applications of *C. regium* leaves, we evaluated the anti-inflammatory and anti-nociceptive activities of *C. regium* extract and the topical healing activities of *C. regium* gel in mouse and rat models. We also determined the chemical composition of *C. regium* extract. To the best of our knowledge, this is the first study that investigates the *in vivo* biological activity of *C. regium* leaves.

2. Material and methods

2.1. Reagents

Solvents employed for chemical characterizations were all of HPLC grade (Tedia® and J. T. Baker®); ultrapure water (resistivity of 18.2 MΩ × cm; Mili-Q®; Millipore, USA). Quitosan was purchased from Polymar® (Fortaleza, Brazil). Acetone, acetic acid, and ethyl alcohol were purchased from Vetec® (Rio de Janeiro, Brazil). Carrageenan, dexamethasone, prednisolone, and formalin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Morphine sulfate was purchased from Dimorf® (Cristalia, Brazil). Solosite Wound Gel was purchased from Smith & nephew® (Watford, England).

2.2. Plant material and ethanolic extract preparation

C. regium leaves were collected in Dourados, Mato Grosso do Sul, in October 2020, identified by Dra. Zefa Valdivina Pereira and registered in the Herbarium of the Universidade Federal da Grande Dourados (UFGD; register number DDMS 5001). The leaves were dried in an air circulating oven at 40 °C for 96 h, and crushed using a knife mill. The dried and powdered material obtained (64.7 g) was extracted by sonication (Quimis® Q335D; frequency 40Hz) for 45 min in two extractive steps with ethanol (2 × 640 mL) to obtain the ethanolic extract of *C. regium* (EECR) (5.3 g; 8.2%) after complete solvent elimination in a fume hood. License for research on Brazilian biodiversity was obtained (Access register SisGen/MMA number AA49C66).

2.3. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analyses

A portion (5.0 mg) of EECR was fractionated by washing with ethyl acetate to yield two fractions, F1 (soluble; 1.2 mg) and F2 (insoluble; 3.8 mg), which were analyzed in both negative and positive ionization modes via ultra-high performance liquid chromatography–electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS) using an Agilent 6545 Q-TOF LC/MS system. An isopropanol solution of the ethyl acetate-soluble fraction (F1) was prepared at 1,000 ppm, and diluted ten-fold using CH_3CN–H_2O 1:1 (v/v). The F2 fraction was directly dissolved in ACN-H_2O 1:1 (v/v). Samples (at 100 ppm) were analyzed using an Agilent Zorbax Eclipse Plus C18 column (Rapid Resolution HD 2.1 × 50 mm, 1.8 μm). Flow rate, column temperature, and injection volume were 0.3 mL/min, 33 °C, and 3 μL, respectively. The mobile phase comprised of water containing 0.1% (v/v) of formic acid (A) and acetonitrile (B). The elution program involved an initial linear gradient (0–14 min for 5–100% B) followed by isocratic elution (14–16 min for 100% B). The operating parameters were as follows: capillary voltage of 3,000 V; skimmer voltage of 65 V; dry gas temperature of 320 °C; drying gas of 12 L/min; nebulizer gas pressure of 35 psi; sheath gas flow of 10 L/min, and sheath gas temperature of 300 °C. MS1 data were acquired in the range of *m/z* 100 to 1,500 with a scan rate of 3 spectra per second, whereas MS2 data were recorded from *m/z* 70 to 1,500 using a scan rate of 5 spectra per second. LC-MS/MS data were processed using MassHunter B.08.00 (Agilent) and MZmine 2.53 software.

2.4. Preparation of 1.25 and 2.5% C. regium leaf ethanolic extract gel

Gel of the ethanolic extract of *C. regium* (GEECR) was formulated by preparing a 3% chitosan (Polymar) solution in 1% acetic acid, which was stirred for 24 h to completely dissolve the solid. Subsequently, EECR was incorporated into the solution at concentrations of 1.25% and 2.5%.

2.5. In vivo biological activity tests

2.5.1. Animals

The study was performed on male and female Swiss mice (20–30 g) and male Wistar rats (250–350 g) obtained from the Central Animal Laboratory of UFGD. The animals were kept in clean polypropylene boxes at approximately 22 ± 1 °C, exposed to 12/12h light/dark cycle, with free access to food and water. The experiments were carried out with 5 groups of 6 animals and were approved by the Ethics Committee Use of animals of the UFGD (process 041/2019) in accordance with the norms required by the Conselho Nacional de Controle de Experimentos Animais (CONCEA).

2.5.2. Carrageenan-induced pleurisy

Female Swiss mice were divided into 5 groups (n = 6) and treated

orally with EECR (30, 100, or 300 mg/kg); subcutaneously (s.c.) with dexamethasone as the positive control (1.0 mg/kg), and the negative control group received 0.9% saline (vehicle) orally. To induce pleurisy, 100 μL of 1% carrageenan suspension was intrapleurally injected into the animals, as described by Velo et al. (1973). After 4 h, the animals were euthanized, their thoracic cavities were washed with phosphate-buffered saline (PBS, 1 mL), and the pleural exudates were collected to estimate the total leukocyte count, protein levels, and nitric oxide production.

2.5.3. Carrageenan-induced paw edema, mechanical hyperalgesia, cold allodynia, and myeloperoxidase (MPO) activity

Male Swiss mice were divided into 5 groups (n = 6) and treated orally with EECR (30, 100, and 300 mg/kg), with prednisolone as the positive control (3.0 mg/kg), and the negative control group received 0.9% saline (vehicle) orally. After 60 min, the animals received a subcutaneous injection of 100 μL of carrageenan (1%) into the right hind paw (Velo et al., 1973). Three and 4 h after the carrageenan injection, paw volume, mechanical hyperalgesia, and sensitivity to cold were measured using digital plethysmometer, electronic von Frey test, and acetone drop method, respectively. Furthermore, at the end of the test, the paw skin of previously treated mice was removed for indirect assessment of neutrophil migration through myeloperoxidase (MPO) quantification (De Young et al., 1989).

2.5.4. Formalin-induced spontaneous pain model

Acute inflammatory pain was induced in male Swiss mice by injecting formalin solution, as described by Hunskaar and Hole (1987). The animals were divided into 5 groups (n = 6) and 1 h before formalin injection, were treated orally with EECR (30, 100, and 300 mg/kg); with morphine as the positive control (5 mg/kg s. c.), and the negative control group received 0.9% saline (vehicle) orally. Immediately after induction, the pain reaction time (paw licking) was evaluated in two phases, the first, from 0 to 5 min, which is the response to neurogenic pain, and the second, from 15 to 30 min, which is the response to inflammatory pain. Cold allodynia was determined via the acetone drop method.

2.5.5. Wound healing activity assessment

2.5.5.1. Excision wound model. Male Wistar rats were divided into 5 groups (n = 6) and anesthetized before and during wound creation. Trichotomy of the dorsal region of all the animals was performed using an electric clipper. After shaving, a 6 mm circular incision was made on the back of the animals using a punch for skin biopsy and scissors, and tweezers for tissue removal. Subsequently, the wounds were topically treated with 1.25% GEECR, 2.25% GEECR, Gel Base, and Commercial Solosite Wound Gel as the positive control, once a day for 10 days. A non-treated control group was also maintained. The wound area of each animal was measured using a digital caliper to analyze wound contraction, and photographs were taken on days 1, 5, and 10. The percentage of wound contraction was calculated using the initial and final wound size (Ramsey et al., 1995).

2.5.5.2. Histopathological analysis. Skin samples were excised from all animals with a 1 cm safety margin around the lesion, fixed in 10% buffered formalin, dehydrated in ethyl alcohol, paraffinized, diaphanized, and stored. For visualization under light microscopy, skin fragments were cut into 4 μm thickness, and stained with hematoxylin and eosin (HE) (Cunha et al., 2009).

2.6. Statistical analyses

The results are expressed as mean ± standard error of the mean (SEM) or percentage. One-way analysis of variance followed by the Newman-Keuls or Bartlett's post-test was used to compare the results. Differences were considered statistically significant at $p < 0.05$. The analyses were generated using GraphPad Prism software (version 5.0).

3. Results and discussion

3.1. Chemical characterization by LC-ESI-QTOF-MS/MS

In total, 25 compounds, one trisaccharide (**1**), one cyclytol (**2**), 12 hydrolyzable tannins (**3**, **5**–**7**, **10**, **11**, **14**, **17**, **19**, **20**, **22**, and **24**), gallic acid (**4**) and its methyl ester (**9**), two depsides (**8** and **18**), one anthraquinone derivative (**13**), five glycosylated flavonoids (**12**, **15**, **16**, **21**, and **23**), and one bilin-type breakdown product of chlorophyll (**25**), were found in the EECR extract through LC-MS/MS analyses. The chemical structures of **1**–**25** were proposed based on the interpretation of their MS data and a comparison with those available in the literature (Cavaliere et al., 2005; Clifford et al., 2007; Kachlicki et al., 2008; Valgimigli et al., 2012; Müller et al., 2014; Wyrepkowski et al., 2014; Geng et al., 2016; Singh et al., 2016; Chang et al., 2019; Sinosaki et al., 2020; Świątek et al., 2021). MS^2 fragmentation data obtained for compounds **1**–**25** are listed in Table 1.

Compound **1**, which was annotated as a trisaccharide, exhibited an $[M+Na]^+$ peak at *m/z* 527.1588 and showed consecutive loss of two hexosyl moieties to give the $[C_6H_{12}O_6Na]^+$ ion [*m/z* 203.05] (Valgimigli et al., 2012). Based on the fragmentation patterns, compounds **2**, **4**, and **9** were annotated as quinic acid, gallic acid, and methyl gallate, respectively (Clifford et al., 2007; Sinosaki et al., 2020). Methyl gallate showed a typical loss of $CH_3^{\bullet}$ radical in both + and − ionization modes, and its identity as a methyl ester was endorsed by the CH_3OH loss from the protonated molecule. The acidic natures of **2** and **4** were evidenced by the CO_2 elimination. The molecular formula of **4** was $C_7H_5O_5$ as it was detected as $[M+H]^+$ and $[M-H]^-$ ions, with *m/z* 171.0289 and 169.0142, respectively. In addition to a peak relative to the $[M–CO_2–H]^-$ ion, peaks of fragment ions at *m/z* 124.03, 97.03, 81.03, and 79.02, were also observed in the ESI(−)-MS^2 spectrum of **4**, which is consistent with the previous reports (Clifford et al., 2007; Sinosaki et al., 2020), indicating that this compound is gallic acid. The elimination of two H_2O molecules from the deprotonated quinic acid gave an ion with *m/z* 155.04, and it generated three anions (*m/z* 137.02, 127.04, and 111.05) through the fragmentation pathways of H_2O, CO, and CO_2 elimination. The $[C_7H_{12}O_6–2H_2O–CO_2–H]^-$ anion produced from **2**, in turn, generated the phenoxide ion through a dehydration reaction.

Compounds **3**, **6**, **10**, **14**, **17**, **19**, **20**, **22**, and **24** showed fragmentation patterns typical of galloyl-hexose derivatives (Chang et al., 2019). Invariably, their ESI(−)-MS^2 spectra exhibited a peak at *m/z* 169.01, which was interpreted to be related to the detection of deprotonated gallic acid molecules. Loss of a hexosyl moiety (162 Da) was also observed in all cases. With the exception of **3**, which was annotated as monogalloylhexose, all the other aforementioned tannins displayed one or more steps of gallic acid (−170 Da) and/or galloyl moiety (−152 Da) loss: digalloylyhexose (**6**) $[M-152Da-H]^-$ (*m/z* 331.07) and $[M-170Da-H]^-$ (*m/z* 313.05); trigalloylhexose (**10**) $[M-152Da-H]^-$ (*m/z* 483.08), $[M-170Da-H]^-$ (*m/z* 465.06), $[M-152Da-152Da-H]^-$ (*m/z* 331.07), and $[M-152Da-170Da-H]^-$ (*m/z* 313.05); tetragalloylhexose (**14**) $[M-152Da-H]^-$ (*m/z* 635.09), $[M-170Da-H]^-$ (*m/z* 617.07), $[M-152Da-152Da-H]^-$ (*m/z* 483.08), $[M-152Da-170Da-H]^-$ (*m/z* 465.06), $[M-152Da-152Da-152Da-H]^-$ (*m/z* 331.06), and $[M-152Da-152Da-170Da-H]^-$ (*m/z* 331.06); 1,2,3,4,6-pentagalloylhexose (**17**) $[M-152Da-H]^-$ (*m/z* 787.10), $[M-170Da-H]^-$ (*m/z* 769.09), $[M-152Da-152Da-H]^-$ (*m/z* 635.09), $[M-152Da-170Da-H]^-$ (*m/z* 617.08), $[M-152Da-152Da-170Da-H]^-$ (*m/z* 465.07), $[M-152Da-170Da-170Da-H]^-$ (*m/z* 447.06), and $[M-152Da-152Da-170Da-170Da-H]^-$ (*m/z* 295.05); 4-*O*-{3,4-dihydroxy-5-[(3,4,5-trihydroxybenzoyl)oxy]benzoyl}-1,2,3,6-tetrakis-*O*-(3,4,5-trihydroxybenzoyl)hexopyranose (**19**) $[M-152Da-H]^-$ (*m/z* 939.11), $[M-152Da-152Da-H]^-$ (*m/z* 787.10), $[M-152Da-170Da-H]^-$ (*m/z* 769.06), $[M-152Da-152Da-152Da-H]^-$ (*m/z* 635.09), $[M-152Da-152Da-170Da-H]^-$ (*m/z* 617.08),

Table 1
Chemical constituents tentatively identified in the EECR by UHPLC-ESI-MS/MS.

N°.	t_R (min)	FM	Ionized molecules and ion fragments (m/z)					Annotation	Sample (s)
			MS	Error	MS	Error	MS^2 [+ (#) and - (*) modes]		
1	0.767	$C_{18}H_{32}O_{16}$	527.1588 $[M+Na]^+$	0.0			#365.10 → 305.08; 203.05; 185.04	Trisaccharide (3 × hexose)	F2
2	0.819	$C_7H_{12}O_6$	193.0711 $[M+H]^+$	−0.5	191.0559 $[M-H]^-$	+1.6	*191.06 → 173.05; 155.04; 137.02; 127.04; 111.05; 93.03; 85.03	Quinic acid	F1[$]; F2
3	1.032	$C_{13}H_{16}O_{10}$	355.0635 $[M+Na]^+$	−1.7	331.0667 $[M-H]^-$	+0.6	*331.07 → 313.05; 271.04; 241.03; 211.05; 193.02; 169.01; 151.00; 125.02; 123.01	Monogalloylhexose	F1[$]; F2
4	1.703	$C_7H_5O_5$	171.0289 $[M+H]^+$	−2.6	169.0142 $[M-H]^-$	+3.0	#171.03 → 153.02; 135.01; 125.02; 109.03; 107.01; 97.03; 81.04; 79.02; *169.01 → 125.02; 124.02; 97.03; 81.03; 79.02	Gallic acid	F1[$];F2
5	1.703	$C_{14}H_{15}O_{10}Na$	367.0639 $[M+H]^+$ 345.0817 $[M+Na]^+$	−0.6 -1.4	365.0486 $[M-H]^-$ 343.0670 $[M-Na]^-$	+0.4 + 1.4	#367.06 → 349.05; 331.04; 197.04; 345.08 → 171.03; 153.02; 125.02; 109.03; 85.05; 79.02; *365.05 → 191.06; 169.01; 125.02; 343.07 → 191.06; 173.05	Sodium salt of Galloylquinic acid	F2
6	3.366	$C_{20}H_{20}O_{14}$			483.0769 $[M-H]^-$	−1.2	*483.08 → 331.07; 313.05; 271.04; 169.01	Digalloylhexose	F2
7	3.833	$C_{21}H_{20}O_{14}$	497.0926 $[M+H]^+$		495.0777$[M-H]^-$		#497.09 → 153.02; *495.08 → 343.07; 191.06	Digalloylquinic acid	F2
8	3.884	$C_{14}H_{10}O_9$	323.0397 $[M+H]^+$	−1.9	321.0254 $[M-H]^-$	+2.2	#323.04 → 153.02; 125.02; *321.03 → 169.01; 125.02	Digallic acid	F1[$]; F2
9	4.140	$C_8H_8O_5$	185.0446 $[M+H]^+$	−2.2	183.0298 $[M-H]^-$	+2.7	#185.05 → 170.02; 153.02; 135.01; 126.03; 125.02; 107.01; 79.02; *183.03 → 168.01; 124.02; 78.01	Methyl gallate	F2
10	4.452	$C_{27}H_{24}O_{18}$	659.0851 $[M+Na]^+$	−1.4	635.0879 $[M-H]^-$	−0.9	#659.09 → 489.06; 337.06; 301.02; 153.02; *635.09 → 483.08; 465.06; 423.04; 331.07; 313.05; 271.05; 169.01	Trigalloylhexose	F2
11	4.452	$C_{41}H_{28}O_{27}$			951.0732 $[M-H]^-$	−0.8	*951.07 → 933.06; 915.04; 463.05; 301.00; 273.00; 169.01	Geraniin	F2
12	4.763	$C_{21}H_{20}O_{11}$	449.1076 $[M+H]^+$	−1.8	447.0932 $[M-H]^-$	+1.0	#449.11 → 413.09; 395.08; 383.07; 377.06; 365.07; 353.06; 339.09; 329.06; 325.07; 311.05; 299.06; 287.05; *447.09 → 429.08; 411.07; 387.07; 369.06; 357.06; 339.05; 327.05; 297.04; 285.04	Luteolin-*C*-hexoside	F1[$]; F2
13	5.124	$C_{27}H_{22}O_{17}$	619.0921 $[M+H]^+$	−2.3			#619.09 → 467.07; 449.07; 305.03; 279.05; 261.04; 237.04; 153.02; 125.02	Rufigallol-galloyl-hexoside	F1; F2
14	5.126	$C_{34}H_{28}O_{22}$	811.0955 $[M+Na]^+$	−1.8	787.0991 $[M-H]^-$	−0.4	#811.09 → 641.07; 623.06; 489.06; 471.05; 319.04; 175.00; 153.02; *787.10 → 635.09; 617.07; 483.08; 465.06; 331.06; 313.05; 169.01	Tetragalloylhexose	F1[$]; F2
15	5.178	$C_{21}H_{20}O_{10}$	433.1131 $[M+H]^+$	−0.9	431.0985 $[M-H]^-$	+1.6	#433.11 → 415.10; 397.09; 379.08; 367.08; 361.07; 351.09; 343.08; 313.07; 297.09; 283.06; *431.10 → 341.07; 311.06; 283.06; 269.04	Apigenin-*C*-hexose	F1; F2
16	5.333	$C_{21}H_{20}O_{12}$	465.1032 $[M+H]^+$	−0.2	463.0885 $[M-H]^-$	+1.8	#465.10 → 319.05; 303.05; *463.09 → 317.03; 316.02; 301.04; 300.03; 287.02; 271.02; 179.00; 151.00	Myricetin-rhamnose	F1; F2
17	5.385	$C_{41}H_{32}O_{26}$	963.1076 $[M+Na]^+$	−0.4	939.1114 $[M-H]^-$	+1.1	#963.11 → 793.08; 641.07; 623.06; 471.05; 455.06; 301.03; 283.02; 175.00; 153.02; *939.11 → 787.10; 769.09; 725.10; 635,09; 617,08; 599.07; 465.07; 447.06; 431.06; 403.07; 295.05; 277.04; 169.01	1,2,3,4,6-Pentagalloylhexose	F1[$]; F2
18	5.385	$C_{15}H_{12}O_9$	337.0555 $[M+H]^+$	−1.4	335.0406 $[M-H]^-$	+0.9	#337.06 → 153.02; 135.01; 125.02; 107.01; 97.03; 79.02; *335.04 → 183.02; 168.01; 124.02	2,3-Dihydroxy-5-(methoxycarbonyl)phenyl 3,4,5-trihydroxybenzoate	F2
19	5.695	$C_{48}H_{36}O_{30}$	1115.1160 $[M+Na]^+$	−2.6	1091.1211 $[M-H]^-$	−0.2	*1091.12 → 939.11; 787.10; 769.06; 725.10; 635.09; 617.08; 599.07; 483.07; 465.07; 447.06; 321.03; 169.01	4-*O*-{3,4-Dihydroxy-5-[(3,4,5-trihydroxybenzoyl)oxy]benzoyl}-1,2,3,6-tetrakis-*O*-(3,4,5-trihydroxybenzoyl)hexopyranose	F2
20	5.695	$C_{48}H_{35}NaO_{30}$			1113.1023 $[M-H]^-$	−0.9	*1113.10 → 961.09; 809.08; 791.07; 769.09; 747.07; 639.06; 487.05; 469.04; 451.03; 343.00; 169.01	Sodium salt of **19**	F2

(*continued on next page*)

Table 1 (*continued*)

N°.	t_R (min)	FM	Ionized molecules and ion fragments (*m/z*)					Annotation	Sample (s)
21	5.695	$C_{21}H_{20}O_{11}$	449.1090 $[M+H]^+$	+1.4	447.0931 $[M-H]^-$	+0.8	#449.11 → 303.05; 285.04; 257.04; 247.06; 229.05; 201.05; 183.04; 165.02; 153.02; 137.02; 121.03; 111.04; *447.09 → 301.03; 300.03; 284.03; 271.02; 255.03; 243.03; 227.03; 179.00; 151.00	Quercitrin	F1; F2
22	5.954	$C_{55}H_{40}O_{34}$	1267.1279 $[M+Na]^+$	−1.6	1243.1323 $[M-H]^-$	0.0	#1267.13 → 1097.11; 945.10; 927.06; 793.08; 775.09; 757.06; 623.07; 471.06; 453.04; 409.06; 301.04; 327.01; 175.00; 153.02; *1243.13 → 1091.12; 939.11; 787.10; 769.09; 725.10; 635.08; 617.08; 599.07; 465.06; 447.06; 321.03; 169.01	2,3-*Bis*-O-{3,4-dihydroxy-5-[(3,4,5-trihydroxybenzoyl)oxy] benzoyl}-1,4,6-*tris*-*O*-(3,4,5-trihydroxybenzoyl)-hexopyranose	F2
23	6.161	$C_{28}H_{24}O_{15}$	601.1200 $[M+H]^+$	+1.1	599.1037 $[M-H]^-$	0.0	#601.12 → 455.06; 303.05; 153.02; 85.03; 71.05; *599.10 → 447.09; 301.03; 300.03; 271.02; 255.03; 179.00; 151.00	Galloylquercitrin	F1; F2
24	6.163	$C_{62}H_{44}O_{38}$	1419.1318 $[M+Na]^+$	+0.7	1395.1438 $[M-H]^-$	+0.4	#1419.13 → 1249.13; 1097.11; 945.09; 927.08; 793.09; 775.07; 731.06; 623.05; 605.05; 479.03; 471.05; 453.04; 327.00; 153.02; *1395.14 → 1243.13; 1091.12; 939.11; 787.10; 769.09; 617.08; 447.05; 169.01	Octagalloyl hexose	F2
25	15.76	$C_{35}H_{36}N_4O_5$	593.2764 $[M+H]^+$	0.0	591.2610 $[M-H]^-$	+0.4	#593.28 → 575.26; 561.25; 547.27; 533.25; 519.24; 505.22; *591.26 → 559.23; 515.24; 500.22; 497.24; 487.25; 471.22	Phyllobilin, possibly pheophorbide A	F1; F2

$Apparently, trace amount.

$[M-152Da-152Da-152Da-152Da-H]^-$ (*m/z* 483.07), $[M-152Da-152Da-152Da-170Da-H]^-$ (*m/z* 465.07), and $[M-152Da-152Da-170Da-170Da-H]^-$ (*m/z* 465.07); 2,3-*bis*-*O*-{3,4-dihydroxy-5-[(3,4,5-trihydroxybenzoyl)oxy]benzoyl}-1,4,6-tris-*O*-(3,4,5-trihydroxybenzoyl)-hexopyranose (**22**) $[M-152Da-H]^-$ (*m/z* 1091.12), $[M-152Da-152Da-H]^-$ (*m/z* 939.11), $[M-152Da-152Da-152Da-H]^-$ (*m/z* 787.10), $[M-152Da-152Da-170Da-H]^-$ (*m/z* 769.09), $[M-152Da-152Da-152Da-152Da-H]^-$ (*m/z* 635.08), $[M-152Da-152Da-152Da-170Da-H]^-$ (*m/z* 617.08), $[M-152Da-152Da-152Da-152Da-170Da-H]^-$ (*m/z* 465.06), and $[M-152Da-152Da-152Da-170Da-170Da-H]^-$ (*m/z* 447.06); and octagalloyl hexose (**24**) $[M-152Da-H]^-$ (*m/z* 1243.13), $[M-152Da-152Da-H]^-$ (*m/z* 1091.12), $[M-152Da-152Da-152Da-H]^-$ (*m/z* 939.11), $[M-152Da-152Da-152Da-152Da-H]^-$ (*m/z* 787.10), $[M-152Da-152Da-170Da-H]^-$ (*m/z* 769.09), $[M-152Da-152Da-152Da-152Da-170Da-H]^-$ (*m/z* 617.08), and $[M-152Da-152Da-152Da-152Da-170Da-170Da-H]^-$ (*m/z* 447.05). Compound **11** was also found to be a tannin and its MS data were consistent with those described for geraniin (Świątek et al., 2021).

Compounds **7**, **8**, **13**, **18**, and **25** were annotated as digalloylquinic acid, digallic acid, rufigallol-galloyl-hexoside, 2,3-dihydroxy-5-(methoxycarbonyl)phenyl 3,4,5-trihydroxybenzoate, and pheophorbide A, respectively. The identity of **7** was tentatively established based on successive losses of the two galloyl moieties observed in its ESI(−)-MS^2 spectrum. This fragmentation pathway led to an anion with *m/z* 191.06, for which chemical formula $C_7H_{11}O_6^-$ was proposed, which is consistent with the deprotonated molecules of quinic acid (Clifford et al., 2007). By endorsing the nature of the substituents attached to the cyclytol core, the ESI(+)-MS^2 spectrum of **7** showed a peak at *m/z* 153.02, which is compatible with the detection of the trihydroxylated phenylacylium ion $C_7H_5O_4^+$. For compound **8**, the galloyl moiety elimination was followed by CO_2 loss to give the pyrogallol anion (Wyrepkowski et al., 2014). The chemical structure proposed for this depside was corroborated by the data obtained in the positive ionization mode, as peaks at *m/z* 153.02 and 125.02 (attributed to the successive losses of gallic acid and CO from the $[M+H]^+$ ion) were observed in the ESI(+)-MS^2 spectrum. The fragmentation pattern of **18** was similar to that of **8**, suggesting that it was also depside. However, the MS spectra of **18** exhibited peaks corresponding to ionized molecules at *m/z* 337.0555 $[M+H]^+$ and 335.0406 $[M-H]^-$, which were 14 u higher than those observed for **8**. In addition, compound **18** showed a characteristic loss for a carbomethoxy group, namely $CH_3^•$ radical elimination in the negative ionization mode, and thus was annotated as a dimer of gallic acid and methyl gallate. This was further supported by the elimination of a ~184 Da fragment from the $[M+H]^+$ ion. Compound **13** was detected as an $[M+H]^+$ ion, and it showed successive losses of a galloyl moiety (−152 Da) and a hexose moiety (−162 Da), to give a [M-152 Da–162 Da + H]$^+$ cation with *m/z* 305.03, which corresponded to protonated rufigallol. Compound **25** was easily detected in both ionization modes and exhibited a fragmentation pattern similar to that reported for phyllobilins, which are polyfunctional products with chlorophyll degradation (Müller et al., 2014). Among others, peaks at *m/z* 575.26 ($[M-H_2O + H]^+$), 547.27 ($[M–H_2O–CO + H]^+$), 561.25 ($[M-CH_3OH + H]^+$), and 519.24 ($[M-C_3H_6O_2+H]^+$; loss of propanoic acid) were observed in its ESI(+)-MS^2 spectrum. In addition, the peak at *m/z* 515.24, corresponding to the $[M–CH_3OH–CO_2–H]^-$anion, was prominent in its ESI(−)-MS^2 spectrum. These data suggested that **25** contained one ketone carbonyl, one carbomethoxy, and one carboxyl functional groups, the last one being integrated with a carboxyethyl side chain. Therefore, this compound was annotated as pheophorbide A.

Compounds **12**, **15**, **16**, **21**, and **23** were annotated as luteolin-*C*-hexoside, apigenin-*C*-hexose, myricetin-rhamnose, quercitrin, and galloylquercitrin (Cavaliere et al., 2005; Kachlicki et al., 2008; Geng et al., 2016; Świątek et al., 2021). The elimination of a hexosyl moiety was verified in ESI(−)-MS^2 spectra of both **12** and **15**. In the ESI(−)-MS^2 spectra of the compounds **16** and **21**, the loss of a rhamnose moiety (−146 Da) was observed. Fragmentation patterns of **16** and **21** contrasted with those of **12** and **15**, as 12 and 15 were fragmented in a more complex manner, and the fragmentations preferentially occurred on the glycosidic moiety. Hence, compounds **12** and **15** were tentatively identified as *C*-glycosides, whereas **16** and **21** were identified as

O-glycosides. The nature of the aglycones was suggested based on of the following data: **12** ([M-162 Da-H]$^-$: *m/z* 285.04), **15** ([M-162 Da-H]$^-$: *m/z* 269.04), **16** ([M-146 Da-H]$^-$: *m/z* 317.03), and **21** ([M-146 Da-H]$^-$: *m/z* 301.03). The annotation of **21** was proposed by the detection of the fragment ions at *m/z* 285.04, 257.04, 247.06, 229.05, 201.05, 183.04, 165.02, 153.02, 137.02, and 121.03 in the ESI(+)-MS^2 spectrum of this flavonoid (Scigelova et al., 2011). The MS^2 data of **23** were closely related to that of **21**, primarily in the negative ionization mode. However, the [M-H]$^-$ ion of **23** was 152 u higher than that of **21**, leading to the proposal that **23** is a derivative of **21** containing a galloyl moiety. This suggestion was corroborated by the elimination of a 152 Da fragment from the [M-H]$^-$ ion to give an anion with *m/z* 447.09, which fragmented to generate ions with the same *m/z* values as those observed in the ESI(−)-MS^2 spectrum of **21**.

Among the compounds found in the EECR extract, compounds **2**, **9**–**12**, **16**–**18**, **21**, **23,** and **25**, particularly methyl gallate (**9**), 1,2,3,4,6-pentagalloylhexose (**17**), and quercitrin (**21**), were of pharmacological interest, and showed higher detectability in the negative and positive ionization modes based on the peaks shown in the chromatogram (Supplementary material 1 and 2). Therefore, these compounds are suspected to be the most abundant compounds in EECR. Compounds **9** and **17** possess anti-inflammatory activity as they inhibit edema formation, leukocyte migration, and inflammatory mediator production (Correa et al., 2016; Rosas et al., 2019). Additionally, **21** reported have anti-inflammatory activity, which is related to the inhibition of pro-inflammatory mediators, including the production of nitric oxide, and a decrease in the infiltration of macrophages and neutrophils during the inflammatory process (Camuesco et al., 2004; Tang et al., 2019).

3.2. Effects of EECR on carrageenan induced pleurisy

The anti-inflammatory properties of EECR were first evaluated in a carrageenan-induced pleurisy model, commonly used to assess inflammation, by examining the production of pleural exudate, which was characterized by leukocyte migration and the release of pro-inflammatory mediators (Dhalendra et al., 2013). Oral administration of EECR at 100 and 300 mg/kg significantly decreased ($p < 0.01$) leukocyte migration and nitric oxide production, indicating its anti-inflammatory potential. It also inhibited protein extravasation, which showed that EECR modulated vasodilation (Fig. 1). The maximum parameter inhibition observed with EECR (300 mg/kg) was 78% for leukocyte migration, 65% for protein extravasation, and 53% for nitric oxide production, and the effect was demonstrated in a dose-dependent manner. A similar pattern parameter inhibition was observed in the dexamethasone group compared to control groups, with 88% for leukocyte migration, 73% for protein extravasation, and 76% for production of nitric oxide. Inflammatory events are causative agents of human morbidity and mortality. Therefore, the use of products that can control of the inflammatory response contributes to clinical anti-inflammatory therapy (Baue et al., 1998).

3.3. Effects of EECR on carrageenan-induced paw edema and measurement of MPO activity

The carrageenan-induced paw edema model is another well-established *in vivo* method used to evaluate anti-inflammatory activity. It is characterized by a progressive increase in injected paw volume, and evaluation is conducted by measuring paw edema, mechanical hyperalgesia, and cold sensitivity (Nantel et al., 1999). Peak of inflammation is observed at the third hour of the test and is related to the increased production of prostaglandins, particularly prostaglandin E2 (PGE2) (Möller et al., 2008), and at this time treatment with EECR at 100 and 300 mg/kg reduced paw volume by 46% and 57% (Fig. 2). In the fourth hour, the extract continued to act, showing a reduction of 52% and 67%, respectively, for 100 and 300 mg/kg EECR treatment. The positive control (prednisolone) demonstrated significant reduction in paw volume at all time points. In the inflammatory process, pain induction is mediated by PGE2 production, which causes nociceptor sensitization and a decrease in the activation threshold of type C nerve fibers, leading to hyperalgesia (Posadas et al., 2004; Zhang and An, 2007). EECR at a dose of 300 mg/kg also reduced hyperalgesia in animals at the third and fourth hour after carrageenan injection, with a reduction of 87% and 89%, respectively, showing similar efficacy to the drug prednisolone (Fig. 3). Treatment with EECR also reduced sensitivity to cold after carrageenan injection (Fig. 4). The most effective dose was 100 mg/kg, with approximately 78% decrease in 3 h after injection.

Neutrophil migration to the hind paw was measured using carrageenan-induced MPO activity (Fig. 5). Neutrophils are important cells in host defense and are rich in MPO. Therefore, the presence of MPO activity indirectly indicates an inflammatory process induced by carrageenan (De Young et al., 1989). Oral administration of 100 and 300 mg/kg of EECR inhibited MPO activity by approximately 42% and 55%, respectively. MPO activity was also decreased by prednisolone treatment.

3.4. Effects of EECR on nociception test

We also conducted the formalin-induced nociception test wich is a biphasic model used to assess neurogenic pain (first phase), primarily involving the direct stimulation of receptive neurons and C fibers leading to inflammatory pain (second phase), which involves the release of inflammatory mediators, such as bradykinin, serotonin, and prostaglandins (Hunskaar and Hole, 1987). Oral EECR administration reduced both phases of formalin-induced spontaneous pain (Fig. 6), suggesting that the extract is effective on pain mechanisms both centrally and peripherally. Doses of 100 and 300 mg/kg acted similarly on paw licking, with a reduction of 68% and 78% in the first phase and 70% and 71% in the second phase, respectively. Morphine, known to inhibit both phases of pain, reduced paw licking by 83% in the first phase and 72% in the second phase. In addition, EECR treatment (300 mg/kg) resulted in significant reduction in cold allodynia by approximately 86%, which is similar to the findings with morphine treatment. These results indicate interactions between the EECR and the cyclooxygenase system, and corroborate our findings on the anti-inflammatory and analgesic activities of the EECR.

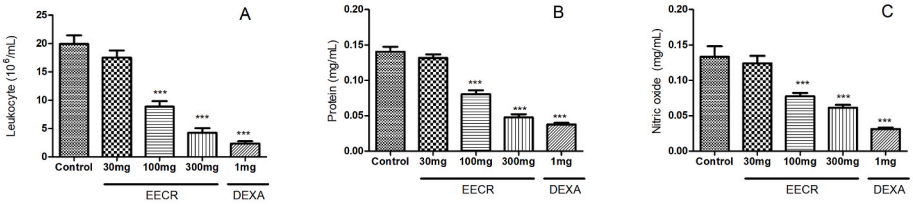

Fig. 1. Effect of treatment with EECR and dexamethasone on pleurisy model. Control: untreated group; A: leukocyte migration; B: protein extravasation; C: nitric oxide production. The bars represent the mean ± SEM. ***$p < 0.001$ compared to the control group according to One-way ANOVA and Newman-Keuls post-test.

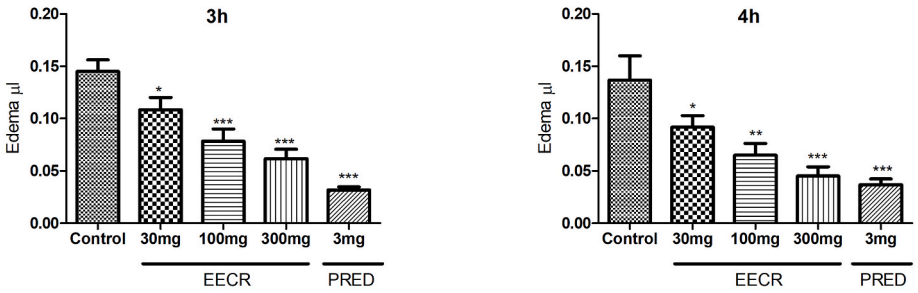

Fig. 2. Effect of EECR and prednisolone on paw edema 3 and 4 h after carrageenan injection. Control: untreated group. The bars represent the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control group according to One-way ANOVA and Newman-Keuls post-test.

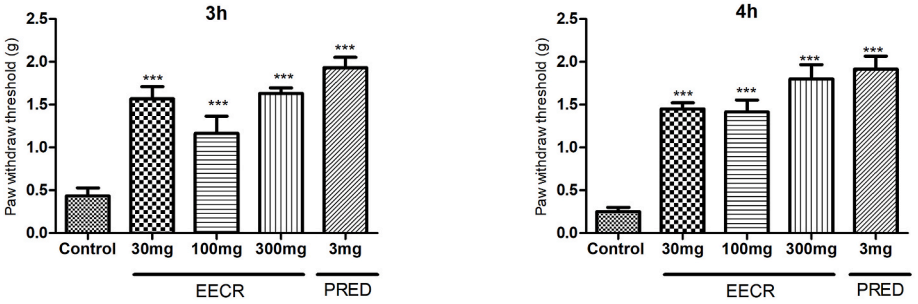

Fig. 3. Effect of EECR and prednisolone on mechanical hyperalgesia 3 h and 4 h after carrageenan injection. Control: untreated group. The bars represent the mean ± SEM. ***p < 0.001 compared to the control group according to One-way ANOVA and Newman-Keuls post-test.

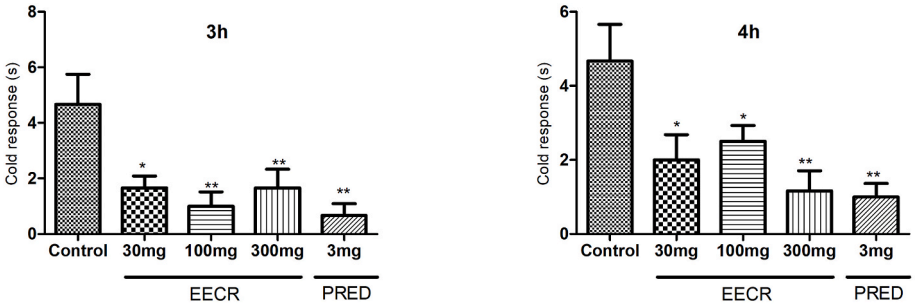

Fig. 4. Effect of EECR and prednisolone on the cold nociceptive 3 h and 4 h after carrageenan injection. Control: untreated group. The bars represent the mean ± SEM. *p < 0.05 and **p < 0.01 compared to the control group according to One-way ANOVA and Newman-Keuls post-test.

3.5. Effect of GECCR on wound healing

The therapeutic efficacy of the gels of the ethanolic extract of *C. regium* on healing was further evaluated using an excision wound model. According to the Wound Healing Society, wounds are defined as any damage or rupture of the integrity of the skin, which can be cellular and anatomical, resulting from physical, chemical, thermal, immunological, or microbial injury to the tissue (Tessema et al., 2018). Topical application of medications to wounds is one of the crucial methods in wound therapy. Wound photographs from the groups treated topically with GEECR 1.25%, GEECR 2.25%, Gel Base, Solosite Wound Gel®, and control group, taken on days 1, 5 and, 10 are shown in Fig. 7. The wound contraction rates are shown in Fig. 8. Wound contraction was measured to assess the healing process, which is essential for the restoration of the disturbed functional state of the skin (Fitriana et al., 2016). At the end of the treatment period, the groups treated with 1.25 and 2.25% GEECR demonstrated effective wound closure, with contraction rates of 92 and 91%, respectively, indicating that the gel with the lowest extract concentration was more effective. Furthermore, analysis between the groups revealed significant differences in wound contraction in both groups treated with GEECR. Macroscopic observations were confirmed using microscopic results. Histopathological analysis of the wounds 10 days post-treatment is shown in Fig. 9. The group treated with GEECR 1.25% showed complete and organized re-epithelialization and dermis with a predominance of neovascularization, characterizing the formation of granulation tissue, whereas the control group presented fine and incomplete re-epithelialization in the presence of inflammatory cells. This confirms the success of the gel as a therapeutic agent in wound closure.

4. Conclusion

The present study revealed for the first time that the ethanolic extract of the leaves of *C. regium* possesses anti-inflammatory and antinociceptive activities. When formulated in a gel base, it has significant wound healing activity when applied topically to treat cutaneous wounds in rats. LC-MS/MS analyses revealed an abundance of 25 compounds, including methyl gallate, 1,2,3,4,6-pentagalloylhexose, and quercitrin, which are associated with these biological properties. Therefore, the popular use of *C. regium* leaves for the treatment of inflammatory conditions, pain, and wound healing is convincing, and our results encourage further research on the benefits of formulations

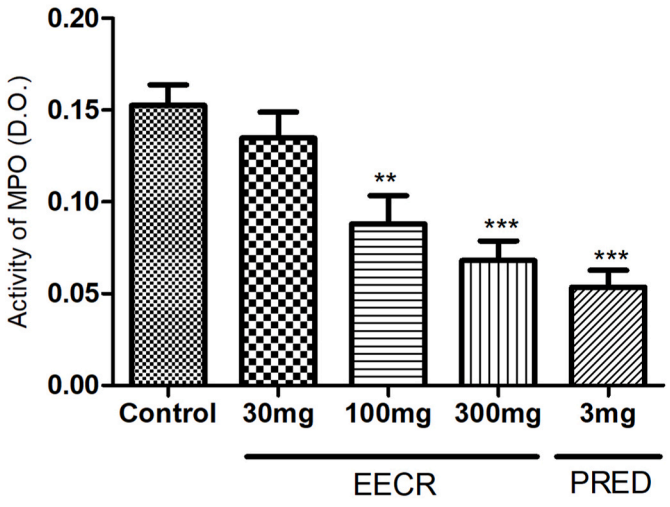

Fig. 5. Effect of EECR and prednisolone in myeloperoxidase (MPO) activity. Control: untreated group. The bars represent the mean ± SEM. **p < 0.01 and ***p < 0.001 compared to control group according to One-way ANOVA and Newman-Keuls post-test.

containing plant extracts.

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CRediT authorship contribution statement

Fernanda Galvão: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Elisangela dos Santos:** Methodology, Formal analysis, Investigation. **Fabiana Gomes da Silva Dantas:** Conceptualization, Methodology, Validation. **José Irlan da Silva Santos:** Methodology, Investigation. **Talita da Paz Costa Sauda:** Methodology, Investigation. **Ariany Carvalho dos Santos:** Investigation, Resources, Formal analysis. **Roosevelt Isaias Carvalho Souza:** Investigation, Resources, Formal analysis. **Luciano da Silva Pinto:** Investigation, Resources, Formal analysis. **Carlos André Ferreira Moraes:**

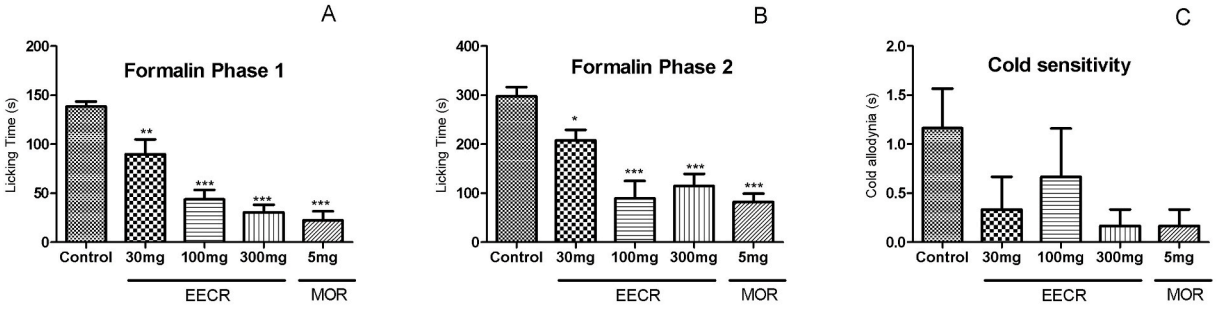

Fig. 6. Effect of EECR and morphine on formalin test. Control: untreated group; A,B: Nociceptive behavior in phase 1 and 2, respectively; C: Cold sensitivity. The bars represent the mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to the control group according to one-way ANOVA and Newman-Keuls post-test.

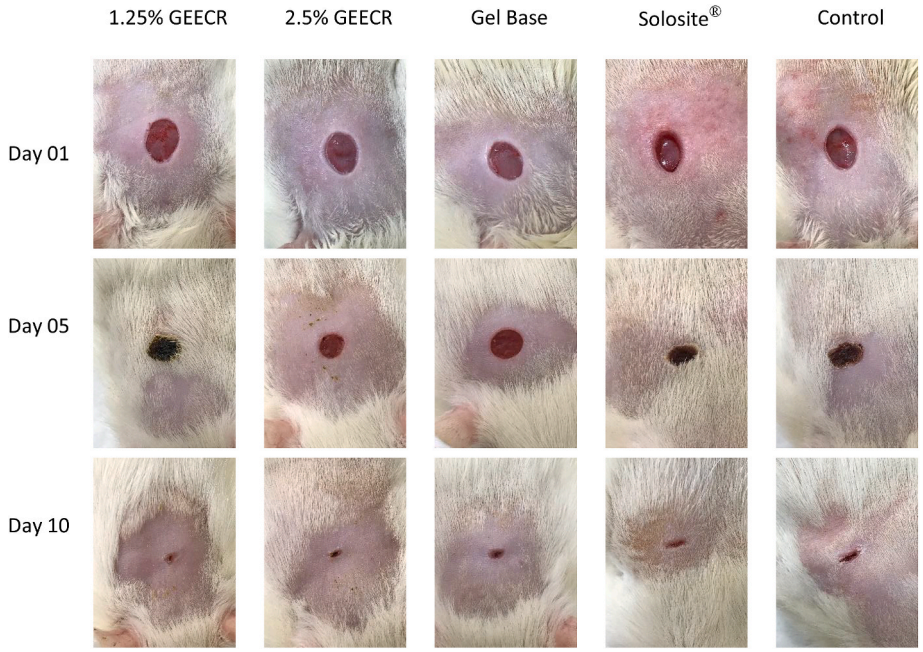

Fig. 7. Wound assessment on days 1, 5, and 10. Control: untreated group.

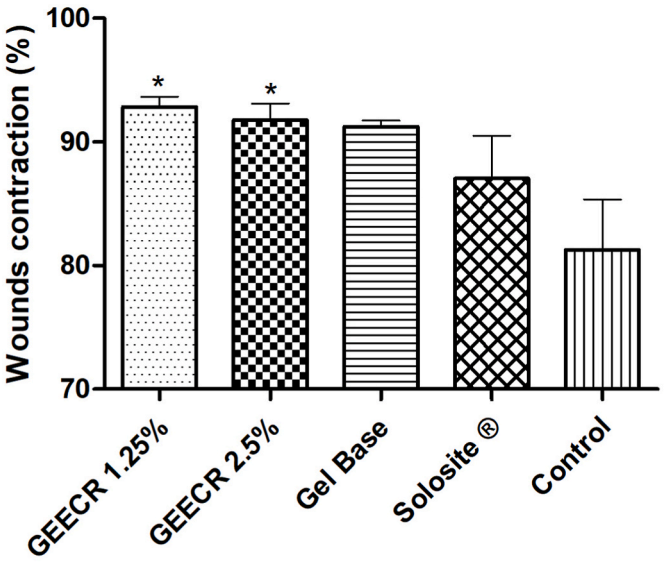

Fig. 8. Effect of topical treatment with 1.25% GEECR; 2.25% GEECR; Gel Base; Comercial Solosite ® and a control group without any treatment, on the contraction of the skin wounds in rats. The bars represent the mean ± SEM. *p < 0.05 compared to the control group according to one-way ANOVA followed by Bartlett's test.

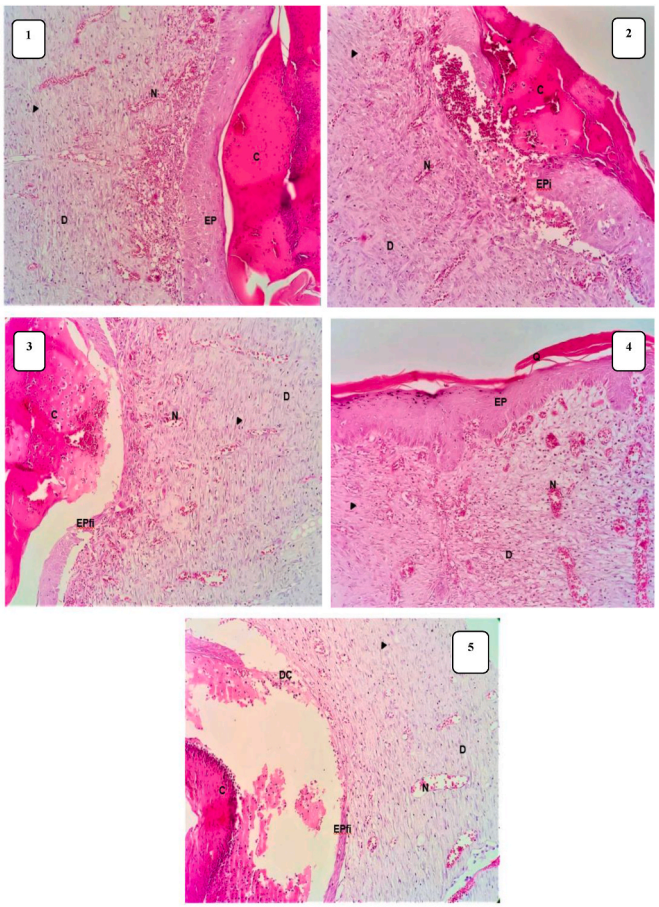

Fig. 9. Histological photographs of wound tissues collected after treatment with (1) Comercial Solosite ®, (2) GEECR 1.25%, (3) GEECR 2.5%, (4) Gel Base, (5) Untreated rat. EP: epidermis; EPi: incomplete epidermis; D: dermis; N: neovascularization; C: crust; Arrowhead: fibroblast; Q: keratin layer; DC: cell debris. HE, obj. 20X.

Investigation, Formal analysis. **Andréia Sangalli:** Conceptualization, Resources, Validation. **Candida Aparecida Leite Kassuya:** Methodology, Resources, Validation. **Cláudio Rodrigo Nogueira:** Conceptualization, Methodology, Investigation, Resources, Formal analysis, Writing - Review & Editing. **Kelly Mari Pires de Oliveira:** Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision.

Declaration of competing interests

The authors declare that they have no competing interests.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115881.

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