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PROGRAMA DE PÓS–GRADUAÇÃO EM BIOTECNOLOGIA
REDE NORDESTE DE BIOTECNOLOGIA – RENORBIO

CLEYDLENNE COSTA VASCONCELOS LOPES

EFEITOS DA *Arrabidaea chica* (Humb. & Bonpl.) **Verlot** em **OSTEOARTRITE.**

São Luís – MA
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Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Rede Nordeste de Biotecnologia – RENORBIO, ponto focal – Universidade Federal do Maranhão – UFMA, como requisito parcial para obtenção do título de Doutora em Biotecnologia.

Área de concentração: Biotecnologia em recursos naturais.

Orientadora: Profa. Dra. Maria do Socorro de Sousa Cartágenes.

Co–orientador: Prof. Dr. João Batista Santos Garcia.

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BANCA EXAMINADORA

_____
Profa. Dra. Maria do Socorro de Sousa Cartágenes
(Universidade Federal do Maranhão – Presidente)

_____
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1º avaliador

_____
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2º avaliador

_____
Profa. Dra. Flávia Maria Mendonça do Amaral – UFMA
3º avaliador

_____
Prof. Dr. Marcelo Souza de Andrade – UFMA
4º avaliador

DEDICATÓRIA

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“Consagre ao Senhor tudo o que você faz, e os seus planos serão bem-sucedidos.”

Provérbios 16:3

“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis."

José de Alencar

RESUMO

A osteoartrite (OA) é uma doença de grande impacto socioeconômico, multifatorial e complexa, caracterizada por remodelação óssea, inflamação sinovial e perda de cartilagem, e cuja dor está entre os principais sintomas. Pode levar a incapacidade e comprometimento funcional do indivíduo, e possui tratamento considerado desafiador. Neste contexto, a busca de terapias alternativas para a OA é essencial, dentre as possíveis fontes de tratamento destacamos as plantas medicinais. *Arrabidaea chica* (Humb. & Bonpl.) Verlot é uma planta conhecida mais popularmente como pariri ou crajiru, e está presente na Relação Nacional de Plantas Medicinais de Interesse ao SUS (RENISUS) em função de seu uso na medicina tradicional e popular, sobretudo para tratar afecções de pele, mas também por ações anti-inflamatórias, cicatrizantes, anti-hipertensiva, hepatoprotetor, antiparasitária, antitumoral, dentre outras. Destacando assim, seu grande potencial farmacológico e interesse para novas investigações científicas. Deste modo, o objetivo desta pesquisa foi avaliar a ação anti-inflamatória, anti-nociceptiva e regenerativa de *A. chica* no controle da OA, além de sua composição química. Para isso, o material vegetal (folhas de *A. chica*) foi coletado, seco a 40 ºC, pulverizado, e em seguida embebido em álcool etílico a 70% sob maceração, sendo posteriormente filtrado e concentrado em rotaevaporador a 40 °C, obtendo-se e o extrato hidroetanólico, parte deste extrato foi também submetida a partição líquido-líquido, para obtenção das frações (hexânica, butanólica e acetato de etila) de *A. chica.* Posteriormente, o extrato e as frações obtidos foram submetidos a análises *in vitro*, em ensaio de inibição da ciclo-oxigenase 1 (COX–2) e 2 (COX–2). O extrato e frações também tiveram seu efeito observado em estudos *in vivo*, em que ratos, da espécie *Rattus norvegicus* passaram por indução experimental de OA no joelho, com monoiodoacetato de sódio (MIA) - (2 mg/kg), e foram tratados com extrato hidroetanólico de *A. chica* nas doses de 50, 150 e 450 mg/kg, e com as frações hexânica, acetato de etila e butanólica de *A. chica,* na dose de 5 mg/kg, administrados por via oral/diariamente, durante 26 dias. Os animais foram avaliados clinicamente a cada 7 dias, através dos testes de atividade: motora (Rotarod), Incapacitância/distribuição de peso nas patas (Weight Bearing), hiperalgesia mecânica (Randall-Selitto) e alodínia mecânica (Von Frey). No 29º dia os animais foram eutanasiados e procedeu–se a coleta dos órgãos (fígado, baço e rim) e joelhos para análises histopatológicas posteriores. Foram realizadas também análises químicas por HPLC-MS e CG-MS, para identificação dos compostos presentes no extrato e na fração hexânica de *A. chica*, estes foram submetidos ao ensaio *in silico* de docagem molecular, tendo como alvo a COX-2, a fim de nortear a escolha dos compostos mais promissores para o tratamento de OA. Os resultados obtidos mostraram que o extrato e frações de *A. chica* promove inibição da COX–1 e 2, sendo que a fração hexânica apresentou os melhores resultados, sendo capaz de inibir a COX-2 em mais de 90%, já na dose de 10µg/mL e com maior afinidade pela COX-2, do que pela COX-1. O extrato e frações de *A. chica,* também induziram melhoras significativas nos parâmetros de incapacidade, atividade motora, hiperalgesia e alodínia decorrentes da OA. O extrato e frações também produziram melhoras na condição radiológica dos joelhos, contudo nas avaliações histopatológicas estas melhoras só foram significativas nos tratamentos com as frações hexânica e acetato de etila, que obtiveram respectivamente, scores médios de 2,9 (±1,8) e 3,7 (±1,6), sendo significativamente menores que grupo salina (CTL-), com média 6,0 (± 0,5). Cabendo ainda ressaltar que as frações tiveram excelentes resultados em doses muito baixas, de 5mg/kg. As análises histopatológicas do fígado, baço e rim também mostram que as doses administradas do extrato e frações não produzindo toxicidade aos animais. Quanto à composição química do extrato, foram identificados 22 compostos, dentre os quais 12 são relatados pela primeira vez nesta espécie, em sua maioria, os compostos identificados são importantes flavonoides, que tem grande potencial anti-inflamatório destacado na literatura.

As análises *in silico* sugeriram a existência de interações muito favoráveis entre alguns desses flavonoides e a enzima COX–2, principalmente para a amentoflavona (energia livre de ligação de –9,21 kcal/mol), quercetina-*O*-galato (energia livre de ligação de –8,86 kcal/mol) e crisoeriol-*O*-glucosídeo (energia livre de ligação de –8,45 kcal/mol). Na fração hexânica foram identificados 20 compostos, sendo estes ácidos graxos, terpenos e fitoesteróis, dentre os quais destacamos o fitol com componente majoritário, o qual tem significativos registros de atividade analgésica e anti-inflamatória. A partir da docagem molecular destes 20 compostos com a COX-2, também foram sugeridas interações muito favoráveis, entre o alfa-tocoferol (Vitamina E), escaleno e beta-sitosterol, com energias livres de ligação de -10,4, -10,4 e -9,8 kcal/mol, respectivamente. A partir destes dados, conclui–se que o extrato e frações de *A. chica* possuem propriedades analgésicas e anti-inflamatórias e podem ser úteis para o tratamento da OA, e que as atividades aqui evidenciadas podem estar ligadas aos flavonoides, terpenos e fitoesteróis, que tem o potencial de intervir na via do ácido araquidônico, reduzindo o processo inflamatório.

Palavras–chave: dor, inflamação, plantas medicinais, *Bignoniaceae*, antinocicepção, pariri.

ABSTRACT

Osteoarthritis (OA) is a disease of great socioeconomic impact, multifactorial and complex, characterized by bone remodeling, synovial inflammation and loss of cartilage, and whose pain is among the main symptoms. It can lead to the individual's disability and functional impairment, and has treatment considered challenging. In this context, the search for alternative therapies for OA is essential, among the possible sources of treatment we highlight medicinal plants. *Arrabidaea chica* (Humb. & Bonpl.) Verlot is a plant known more popularly as pariri or crajiru, and is present in the National List of Medicinal Plants of Interest to SUS (RENISUS) due to its use in traditional and popular medicine, especially for to treat skin conditions, but also by anti-inflammatory, healing, anti-hypertensive, hepatoprotective, antiparasitic, anti-tumor actions, among others. Thus, highlighting its great pharmacological potential and interest for new scientific investigations. Thus, the objective of this research was to evaluate the anti-inflammatory, anti-nociceptive and regenerative action of *A. chica* in the control of OA, in addition to its chemical composition. For this, the plant material (leaves of *A. chica*) was collected, dried at 40 ºC, pulverized, and then soaked in 70% ethyl alcohol under maceration, being subsequently filtered and concentrated in a rotary evaporator at 40 ° C, obtaining it if and the hydroethanolic extract, part of this extract was also subjected to liquid-liquid partition, to obtain the fractions (hexane, butanolic and ethyl acetate) of *A. chica*. Subsequently, the extract and the fractions obtained were subjected to in vitro analysis, in an inhibition test for cyclooxygenase 1 (COX – 2) and 2 (COX – 2). The extract and fractions also had their effect observed in in vivo studies, in which *Rattus norvegicus* rats underwent experimental OA induction in the knee, with sodium monoiodoacetate (MIA) - (2 mg / kg), and were treated with hydroethanolic extract of A. chica at doses of 50, 150 and 450 mg / kg, and with hexane, ethyl acetate and butanolic fractions of *A. chica*, at a dose of 5 mg / kg, administered orally / daily, during 26 days. The animals were clinically evaluated every 7 days, through the activity tests: motor (Rotarod), disability / weight distribution in the paws (Weight Bearing), mechanical hyperalgesia (Randall-Selitto) and mechanical allodynia (Von Frey). On the 29th day, the animals were euthanized and the organs (liver, spleen and kidney) and knees were collected for later histopathological analysis. Chemical analyzes were also carried out by HPLC-MS and CG-MS, to identify the compounds present in the extract and in the hexane fraction of *A. chica*, these were subjected to in silico testing of molecular docking, targeting COX-2, the in order to guide the choice of the most promising compounds for the treatment of OA. The results obtained showed that the extract and fractions of *A. chica* inhibit COX – 1 and 2, with the hexane fraction showing the best results, being able to inhibit COX-2 by more than 90%, already at the dose of 10µg / mL and with greater affinity for COX-2 than for COX-1. The extract and fractions of *A. chica*, also induced significant improvements in the parameters of disability, motor activity, hyperalgesia and allodynia resulting from OA. The extract and fractions also produced improvements in the radiological condition of the knees, however in the histopathological evaluations these improvements were only significant in the treatments with the hexane fractions and ethyl acetate, which obtained, respectively, average scores of 2.9 (± 1.8) and 3.7 (± 1.6), being significantly smaller than the saline group (CTL-), with a mean of 6.0 (± 0.5). It should also be noted that the fractions had excellent results in very low doses, of 5mg / kg. Histopathological analyzes of the liver, spleen and kidney also show that the administered doses of the extract and fractions do not produce toxicity to the animals. As for the chemical composition of the extract, 22 compounds were identified, among which 12 are reported for the first time in this species, most of the identified compounds are important flavonoids, which have great anti-inflammatory potential highlighted in the literature. In silico analyzes suggested the existence of very favorable interactions between some of these flavonoids and the COX – 2 enzyme, mainly for amentoflavone (–9.21 kcal/mol free binding energy), quercetin-O-gallate (free energy of

binding of –8.86 kcal / mol) and crisoeriol-O-glucoside (free binding energy of –8.45 kcal/mol). In the hexanic fraction, 20 compounds were identified, being these fatty acids, terpenes and phytosterols, among which we highlight phytol with a major component, which has significant records of analgesic and anti-inflammatory activity. From the molecular docking of these 20 compounds with COX-2, very favorable interactions have also been suggested, between alpha-tocopherol (Vitamin E), scalene and beta-sitosterol, with free-binding energies of -10.4, -10.4 and -9.8 kcal / mol, respectively. From these data, it is concluded that the extract and fractions of *A. chica* have analgesic and anti-inflammatory properties and can be useful for the treatment of OA, and that the activities shown here may be linked to flavonoids, terpenes and phytosterols, which has the potential to intervene in the arachidonic acid pathway, reducing the inflammatory process.

Keywords: pain, inflammation, medicinal plants, *Bignoniaceae*, antinociception, pariri.

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LISTA DE ABREVIATURA E SIGLAS

Å – Angström

AA – Ácido araquidônico

ADMTS – Desintegrina e metaloproteinase com motivos de trombospondina

AE – Extratos aquoso

AH – Ácido hialurônico

AINEs/ NSAIDs – Anti–inflamatórios não esteroidais

AIS – Ações Integradas de Saúde

AP – Anteroposterior

APs – Potenciais de ação

ASU – Mistura de insaponificáveis de abacate e soja

ATP– Adenosina trifosfato

AVISA – Agência Nacional de Vigilância Sanitária

CAM – Membrana corioalantóide

CH_2Cl_2 – Diclorometano

CID – Dissociação induzida por colisão

CIPLAN – Comissão Interministerial de Planejamento e Coordenação

CIS – Comissões Interinstitucionais de Saúde

CLEAN – grupo de animais sem OA induzida e sem tratamento

CTL- – Grupo de animais com OA e tratado com soro fisiológico (NaCl 0,9%)

CTL + Melox – Grupo com OA e tratado com Meloxicam® – 0,5 mg/kg

COX1– Enzima ciclo-oxigenase 1

COX-2 – Enzima ciclo-oxigenase 2

DFT –Teoria da Densidade Funcional

DPPH – 2,2 difenil- 1- picrilhidrazil

EB – Extrato bruto

EDTA – Ácido etilenodiamino tetra–acético

EE – Extrato etanólico

EHA – Extrato hidroetanólico de Arrabidaea chica

ESI –ionização por eletropulverização

ESI–MSn – Fragmentação em múltiplos estágios

FIA – fluxo de injeção direta

FLS – Sinoviócitos semelhantes a fibroblastos

GI – Gastrointestinais

HPLC–MS – Cromatografia líquida acoplada a espectrometria de massa

IBGE – Instituto Brasileiro de Geografia e Estatística

IFN–γ – Interferon gama

IL– Interleucina

iNOS – Óxido nítrico sintase induzível

IT – interface tipo armadilha de íons (*ion trap*)

Jurkat e HL60 – Linhagens de células leucêmicas humanas

LDH – Lactato desidrogenase

LGA – Algoritmo Genético Lamarckiano

LPS – Lipopolissacarídeos

MeOH – Metanol

MIA– Monoiodoacetato de sódio

MIP–1β – Quimiocina inflamatórias de Macrófagos tipo 1 beta

MLS – Condrócitos e sinoviócitos semelhantes a macrófagos

MMPs – Metaloproteinases de matriz

MPO – Mieloperoxidase

MS/MS – Espectrometria de massas em tandem

MT – Medicina Tradicional

NADP– Fosfato de nicotinamida e adenina dinucleotídeo

NF–kB – Fator nuclear kappa beta

NF–κB –IκB – Inibidor do fator nuclear kappa B

NO – Óxido nítrico

NPWT – limiar nociceptivo de retirada da pata

OA – Osteoartrite

OARSI – Sociedad Internacional de Investigação da Osteoartrite

OMS – Organização Mundial da Saúde

One–way ANOVA – Análise univariada de variância

PGE 2 – Prostaglandina E2

PTFE – filtro de politetrafluoroetileno

PVM/MA – Polímero bioadesivo

RANKL – ligante do receptor do fator nuclear kappa B

RENISUS – Relação Nacional de Plantas Medicinais de Interesse ao Sistema Único de

ROS – Espécies reativas de oxigênio

SNC – Sistema Nervoso Central

SUDS – Sistema Unificado e Descentralizado de saúde

SUS – Sistema Único de Saúde

SVS – Secretaria de Vigilância Sanitária

TCD4 + – Linfócitos T auxiliares

TCD8 + – Linfócitos T citotóxicos

TFD – Terapia fotodinâmica

TGF–β – Fator de crescimento transformador beta

TNF–α – Fator de necrose tumoral alfa

TWO–way ANOVA – Análise de variância bivariada

VEGF – Fator de crescimento endotelial vascular

4T1 – Células de adenocarcinoma mamário murino

SUMÁRIO

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1. INTRODUÇÃO

Ao longo da história da humanidade, as plantas têm sido utilizadas como importante recurso terapêutico, visto que são detentoras de uma grande variedade de metabólitos que apresentam atividades biológicas e farmacológicas relevantes, com grande potencial para o desenvolvimento de fármacos (JOLY et al., 2011; LI et al., 2017; AYE et al., 2019).

Dentre as plantas medicinais tradicionalmente utilizadas, encontra–se a espécie vegetal *Arrabidaea chica* (Humb. & Bonpl.) Verlot, nativa da floresta tropical e muito comum em quase todo o Brasil (LINO VON POSER et al., 2000). Conhecida pelas comunidades tradicionais principalmente como crajiru, carajiru ou pariri, vem sendo utilizada pela medicina tradicional e popular para diversos fins terapêuticos, associado principalmente a sua atividade anti–inflamatória, cicatrizante, controle de cólicas intestinais, afecções da pele, disfunções sanguíneas e leucemia (LORENZI; MATOS, 2008).

Nosso grupo de estudo também investigou o potencial analgésico da *A. chica*, e verificou a capacidade do extrato e frações em aliviar a dor neuropática induzida por compressão nervosa (MOREIRA LIMA, 2017). Com base nesses resultados promissores para dor neuropática surgiu à perspectiva de também estudar os efeitos de *A. chica* em um modelo de dor nociceptiva, que já está bem implantado no laboratório, no caso o modelo de osteoartrite (OA) induzida em ratos.

Considerando também a atividade anti-inflamatória desta planta, nossa hipótese é que *A. chica* possa ter efeito positivo no tratamento de doenças em que o processo inflamatório tem um papel crítico, como é o caso da OA. A osteoartrite vem sendo classificada como uma doença inflamatória, com elevação de citocinas pró-inflamatórias, como a interleucina 1β (IL-1β) e o fator de necrose tumoral alfa (TNF-α), que diminuem a síntese de colágeno e promovem o aumento de mediadores catabólicos, como metaloproteinases, que degradam componentes da matriz extracelular e do osso subcondral (ATTUR et al., 2002; REZENDE; CAMPOS; PAILO, 2013; ROBINSON et al., 2016; WOODELL-MAY & SOMMERFELD, 2020).

A doença é multifatorial, incluindo fatores genéticos, metabólicos e traumáticos, levando a alterações morfológicas, bioquímicas, moleculares e biomecânicas, que resultam em ulceração e perda da cartilagem articular, remodelação e esclerose do osso subcondral, com formação de osteófitos. Pode afetar qualquer articulação sinovial, contudo, acomete principalmente joelhos, quadris, mãos e coluna, e pode ocorrer em mais de uma articulação ao mesmo tempo (HASEEB; HAQQI, 2013; O'NEILL; MCCABE; MCBETH, 2018).

A OA é considerada a doença osteoarticular mais comum, que afeta de maneira alarmante a população idosa, e está associada a elevados custos socioeconômicos. Estima–se que os custos associados à OA tenham uma carga socioeconômica entre 1,0 e 2,5% do produto interno bruto nos países desenvolvidos (HILIGSMANN et al., 2013). A OMS estima que 40% dos adultos acima de 70 anos apresentem OA de joelhos, 80% das pessoas com OA apresentem limitação de movimentos, e 25% não consigam desempenhar suas principais atividades de vida diária, ocasionando assim, grandes perdas funcionais e de qualidade de vida (BROOKS, 2002; ROSIS; KAIRALLA, 2010; KAWANO et al., 2015). Dessa forma, a OA constitui–se diretamente como um problema de saúde pública, com meios reduzidos de tratamento e sem cura (JOHNSON; HUNTER, 2014; KAWANO ET AL., 2015; GRÄSSEL & MUSCHTER, 2020).

Os tratamentos disponíveis para OA são baseados principalmente na utilização de analgésicos, inibidores da enzima ciclo-oxigenase - 2 (COX-2) e drogas anti-inflamatórias não esteroidais (AINES), contudo ainda é muito insuficiente, uma vez que não conseguem reverter o avanço da doença e apresentam efeitos colaterais importantes, além da dor ser refratária aos tratamentos atuais (NIELSEN, 2006; YU; HUNTER, 2015). O uso prolongado de AINES, apesar de promover alívio momentâneo, eleva o risco de disfunção renal, complicações gastrointestinais e doenças cardiovasculares (BATLOUNI, 2010; JOSÉ, 2013; ATIQUZZAMAN et al., 2019). Sendo, portanto, de fundamental importância o investimento em pesquisas que busquem novos tratamentos.

Neste contexto, o presente estudo avaliou a resposta anti-inflamatória e anti-nociceptiva do extrato hidroetanólico (EHA) e frações (hexânica, acetato de etila e butanólica) de *Arrabidaea chica* (pariri) em modelo experimental de osteoartrite.

Os resultados desta Tese foram divididos em dois capítulos, sendo no capítulo I apresentados os efeitos do extrato hidroetanólico de *A. chica*, destacando sua capacidade de inibir as enzimas ciclo-oxigenase 1 e 2, e intervir clinicamente na OA induzida em ratos, bem com seus efeitos radiográficos e histopatológicos no joelho dos animais. Neste capitulo são apresentados também as análises químicas, com identificação dos compostos presentes no extrato de *A. chica*, e os resultados dos ensaios *in silico* de docagem molecular. Estes resultados geraram o artigo intitulado: **"***Effects of Extract of Arrabidaea chica Verlot on an Experimental Model of Osteoarthritis*" publicado no *International Journal of Molecular Sciences.*

O capítulo II refere-se à investigação das atividades anti-inflamatória e antinociceptiva *in vitro* e *in vivo*, das frações hexânica, acetato de etila e butanólica de *A. chica*, destacando a

capacidade das frações inibirem as enzimas ciclo-oxigenase 1 e 2, e intervirem clinicamente na OA induzida em ratos, bem com seus efeitos radiográficos e histopatológicos no joelho dos animais. Realizou- se também a identificação da composição química da fração com melhor potencial no tratamento de OA, bem como um estudo *in silico* destes compostos tendo como alvo a COX-2. Os resultados foram apresentados no manuscrito intitulado: "*Arrabidaea chica Verlot fractions reduces MIA-induced osteoarthritis progression in rats knees"*, submetido a revista *Inflammopharmacology*.

2. REFERENCIAL TEÓRICO

2.1 Plantas medicinais

O potencial terapêutico das plantas é conhecido desde os primórdios das civilizações, inicialmente baseados apenas no conhecimento popular empírico, mas com o passar dos anos os vegetais têm sido cada vez mais explorados, e a indústria farmacêutica vem se utilizado de maneira crescente deles para produção de medicamentos (MARTINS et al., 1994; RODRIGUES; CARVALHO, 2001; DIAS, 2005; BRASIL, 2016). A Organização Mundial da Saúde (OMS) reconhece que grande parte da população dos países em desenvolvimento depende da medicina tradicional para sua atenção primária a saúde, seja pela escassez de recurso financeiro das populações para aquisição de produtos comerciais, aceitabilidade ou falta de confiança no Sistema Público de Saúde (CALIXTO, 2000; BRASIL, 2016).

Embora as plantas medicinais tenham uma boa aceitação pela população, e se tenha tido um crescimento no número de estudos, somente um pequeno percentual delas tem dados científicos que comprovem a sua eficácia, e segurança toxicológica (BRASIL, 2006; VILELA, 2009; ZAGO, 2018). Apesar do uso imemorial de plantas medicinais, a fitoterapia com finalidade profilática, curativa, paliativa ou com fins de diagnóstico passou a ser oficialmente reconhecido pela OMS somente 1978, quando houve então a recomendação mundial de difusão e pesquisas dos conhecimentos necessários para o seu uso. A OMS reforçou o compromisso de estimular o desenvolvimento de políticas públicas a fim de inseri-las no sistema oficial de saúde dos seus Estados–membros. Então em 2005, publicou o documento Política Nacional de Medicina Tradicional e Regulamentação de Medicamentos Fitoterápicos, discutindo a situação mundial a respeito das políticas da medicina tradicional (MT) e fitoterápicos, inclusive do Brasil (BRASIL, 2006).

Cabendo destacar, que o Brasil é o país com maior diversidade vegetal do mundo, com mais de 36.000 espécies catalogadas (BFG, 2018), além de possuir ampla tradição no uso das plantas medicinais. Contudo, estima-se que menos de 15% das espécies tenham sido estudadas para fins de utilização na medicina, marcando a insuficiência de estudos científicos acerca do assunto (CONSERVATION INTERNATIONAL, 2010). Portanto, torna–se necessário estimular a realização desses estudos, visando à validação das propriedades medicinais de plantas e garantindo a segurança de seu uso.

Nesta perspectiva de estimular o desenvolvimento de fitoterápicos no Brasil, para uso nos programas de saúde pública, após a década de 1980, diversos instrumentos normativos como resoluções, portarias e relatórios foram elaborados. Destacando-se a Portaria n. º 212, de 11 de setembro de 1981, que define o estudo das plantas medicinais como uma das

prioridades de investigação clínica; a Resolução da Comissão Interministerial de Planejamento e Coordenação (CIPLAN) Nº 08, de 1988, que implanta a prática de Fitoterapia nos serviços de saúde, e orienta através das Comissões Interinstitucionais de saúde (CIS) a busca da inclusão da Fitoterapia nas Ações Integradas de Saúde (AIS), e/ ou programação do Sistema Unificado e Descentralizado de Saúde (SUDS) nas Unidades Federadas, visando colaborar com a prática oficial da medicina moderna, em caráter complementar; a Portaria nº 31/SVS – Secretaria de Vigilância Sanitária, de 1994, que cria o Grupo de Estudos de Produtos Fitoterápicos; a Portaria nº 06/SVS, de 1995, que institui e normatiza o registro de produtos fitoterápicos junto ao Sistema de Vigilância Sanitária; a Resolução da Diretoria Colegiada da Agência Nacional de Vigilância Sanitária (RDC/Anvisa) nº 48 de 2004 que dispõe sobre o registro de medicamentos fitoterápicos.

A Portaria nº 2.960, instituiu em 2008 o Programa Nacional de Plantas Medicinal e Fitoterápico, objetivando inserir com segurança, eficácia e qualidade, plantas medicinais, fitoterápicos e serviços relacionados à fitoterapia no Sistema Único de Saúde (SUS). Além de promover e reconhecer as práticas populares e tradicionais de uso de plantas medicinais. O Ministério da Saúde, também divulgou em 2008 uma Relação Nacional de Plantas Medicinais de Interesse ao Sistema Único de Saúde (RENISUS). Nessa lista constam as plantas medicinais que apresentam potencial para gerar produtos de interesse ao SUS. Dentre as espécies citadas no RENISUS encontra–se *Arrabidaea chica* (Humb & Bonpl.) Verlot (BRASIL, 2009), espécie vegetal investigada no presente estudo.

Na portaria nº 533 de 28 de março de 2012, o Ministério da Saúde estabelece também a lista de medicamentos e insumos da Relação Nacional de Medicamentos Essenciais (RENAME), que compreende a seleção e a padronização de medicamentos indicados para atendimento de doenças ou de agravos no âmbito do SUS. Nesta relação constam apenas 12 fitoterápicos, número que se mantém até a lista 2020 (BRASIL, 2020), demonstrando poucos avanços na recomendação de fitoterápicos no sistema público de saúde e a necessidade de mais incentivos para estudo e utilização desses.

Cabe aqui, destacar também, que além dos benefícios terapêuticos, os fitoterápicos também movimentam um mercado mundial de cerca de US$ 44 bilhões por ano. No Brasil não existem dados oficiais sobre o tamanho desse mercado, e as estimativas variam entre US$ 350 milhões e US$ 550 milhões por ano (BRASIL, 2012). Entretanto, este mercado ainda é bem inferior a capacidade do Brasil. Assim os investimentos e pesquisas na bioprospecção dos ativos da biodiversidade brasileira são fundamentais para que haja crescimento deste mercado e diminuição da dependência externa na produção de fármacos.

2.2 Desenvolvimento de fármacos – abordagens computacionais

O desenvolvimento de novos fármacos é um processo complexo e se tratando de fármacos a base de plantas, deve–se levar em consideração ainda que as espécies vegetais geralmente apresentam muitos compostos químicos o que torna a identificação dos reais responsáveis pela atividade biológica um processo longo e demorado. Nesse sentido, a bioinformática têm contribuído de modo significativo, pois utiliza bases de ciências como bioquímica, farmacologia, biologia molecular, imunologia, farmacocinética, físico–química, métodos de modelagem, entre outras, para a identificação, planejamento e o desenvolvimento de compostos bioativos, que podem ser usados para prevenção, tratamento ou cura de enfermidades (WERMUTH, 2003; GUIDO; ANDRICOPULO; OLIVA, 2010).

O conhecimento dos alvos moleculares (como enzimas, receptores, ácidos nucléicos) é indispensável para aplicação dos protocolos computacionais. Assim o avanço na biologia molecular, engenharia genética e na biologia estrutural tem tornado possível muitos dos estudos de bioinformática, visto que é crescente o número de alvos para os quais se conhece a estrutura tridimensional (3D) e a localização precisa do sítio ativo (GUIDO, ANDRICOPULO, OLIVA, 2010). Contudo, quando não se conhece a estrutura 3D ainda é possível utilizar o método de modelagem por homologia, com base em uma ou mais estruturas já conhecidas, fazendo predições bem-sucedidas de estruturas de alvos moleculares ainda não cristalografadas (WATSON et al., 2007; CAVASOTTO & PHATAK, 2009). Quanto aos ligantes (moléculas potencialmente ativas), estes podem ter origem a partir de produtos naturais, através de síntese orgânica, coleções combinatórias, ou ainda mediante planejamento racional (KORTAGERE & EKINS, 2010; HUANG et al., 2010; GUIDO, ANDRICOPULO, OLIVA, 2010). Tendo o conhecimento sobre essas estruturas é possível aplicar ensaios virtuais para planejamento de fármacos.

A docagem molecular está entre as principais ferramentas em uso pela bioinformática para a identificação e seleção de novos candidatos a fármacos, visto que através dela é possível avaliar as interações dos candidatos a fármacos com seus potenciais alvos. Assim, a docagem molecular consiste na predição do posicionamento (orientação e conformação) de um ligante dentro de um sítio de interação alvo (KITCHEN et al., 2004). Para isso, o programa faz uma busca dos diferentes modos de ligação adquiridos pelas múltiplas conformações do ligante dentro do sítio ativo do receptor e a afinidade de cada situação é uma função matemática denominada de algorítmico genético, que faz a busca

conformacional de encaixe e interação do ligante dentro do receptor (MORRIS et al., 1998; HUEY et al., 2007).

Para avaliar os resultados da docagem utilizam-se os valores da energia de interação do complexo ligante–proteína, da energia intramolecular do ligante ou constante de inibição (MAGALHAES; BARBOSA; DARDENNE, 2004). Para implementação destes protocolos de bioinformática os softwares mais usados são o GOLD, o AutoDock, o DOCK e o EADock, que realizam a docagem automática de um ligante flexível no sítio ativo de uma macromolécula rígida (MORRIS et al., 1998; ZOETE; GROSDIDIER; MICHIELIN, 2009).

A constante evolução nas tecnologias computacionais, a rapidez, e o custo pequeno comparado aos demais métodos são as principais vantagens do uso de métodos de bioinformática (TAKEDA-SHITAKA et al., 2004; MA et- al., 2011). Os estudos computacionais/*in silico* fornecem um importante direcionamento às pesquisas, com resultados que favorecem uma pré-seleção de moléculas potencialmente ativas, que levam a abordagens experimentais mais promissoras (OPREA, 2005; ZHENG et al., 2013; KASERER et al., 2015; TORRES et al., 2019).

Dessa forma, esta ciência tem fornecido um notável direcionamento no estudo e planejamento de fármacos, envolvendo o emprego de *screenings*, cálculos, análises e simulações computacionais (DE MOLFETTA et al., 2009; GURUDEEBAN et al., 2012; DULIN et al., 2014; CALADO et al., 2015; SILVA et al., 2017). Com aplicações a diversas situações, dentre elas as patologias, favorecendo o desenvolvimento de medicamentos pra tratamento de cânceres (AMIN et al., 2013; SABBAH et al., 2015); doenças cardiovasculares (MENA-ULECIA et al., 2015; DONG et al., 2016); condições inflamatórias (FLOWER, 2003; SILVA et al., 2017); tratamento do HIV (VAN DRIE, 2007); e outras doenças virais, como influenza (VONITZSTEIN et al., 1993) e Zika vírus (ZHANG et al., 2016; RAMHARACK & SOLIMAN, 2017); para doenças parasitárias, como: malária (CHAUDHARY & PRASAD, 2014; COBB et al., 2015), tuberculose (VIANNA & AZEVEDO JUNIOR); doença de Chagas (ROCHA, 2010), dentre outras.

Jarapula e colaboradores (2016) observaram correlações significativas entre resultados de seus estudos *in silico* e *in vivo*. Ao avaliarem compostos derivados de isatina, através da atividade anti-inflamatória *in vivo* pelo método de edema de pata induzido por carragenina, observaram que compostos VIIc e VIId exibiram 65% e 63% de redução do edema de pata, respectivamente. Os estudos de docagem molecular realizados com as enzimas COX-1 e COX-2, também evidenciaram que os mesmos compostos: VIIc e VIId exibiram

boas pontuações de afinidade com o sítio ativo de COX-2, e baixa afinidade com COX-1, semelhante ao fármaco celecoxib.

Świątek e colaboradores (2017) comparando dados de ensaios *in vitro* com estudos de docagem molecular, demonstraram também que dentre os derivados de isotiazolopiridina testados, o que apresentou maior percentual de inibição para COX-2 *in vitro*, foi também o que apresentou os melhores parâmetros de afinidade energética nos ensaios *in silico.*

O potencial das ferramentas computacionais para seleção de novas moléculas bioativas, também foi demonstrado por Silva e colaboradores (2017). Neste estudo observaram que dentre os compostos identificados na fração acetato de etila de *Borreria verticilata,* o triterpeno ácido ursólico foi a molécula que apresentou os melhores parâmetros de afinidade, em estudos *in silico,* com as estruturas da enzima COX-2 e receptor N-metil-D-aspartato (NMDA). Os estudos *in vivo* realizados por testes de edema de pata induzido por carragenina (perfil inflamatório mediado pela alta atividade da COX-2) e *tail flick* (mecanismos centrais), demonstraram que o ácido ursólico mostrou-se muito eficiente, sendo até superior que fármacos controle indometacina e memantina, demonstrando que a docagem molecular foi eficiente na seleção dos compostos com maior potencial anti-inflamatório e anti-nociceptivo.

Outro estudo avaliou também o potencial farmacológico de sessenta e oito compostos de *Myrica nagi* Thunb., identificando através de análises *in silico,* o composto miricetina, como o potencialmente mais ativo, por possuir excelentes propriedades metabólicas de distribuição, absorção, excreção e toxicidade (ADMET), além de bons parâmetros de afinidade com a enzima COX-2. Demonstraram também através de estudos *in vitro* a atividade de citotoxicidade da miricetina de *M. nagi*, utilizando o ensaio MTT e, obtiveram uma IC50 de 102 µg/mL. Relatando no geral, que miricetina possui propriedades desejadas para ser um potente anti-inflamatório (Kumar et al., 2019).

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2.3 *Arrabidaea chica* (Humb. & Bonpl.) **Verlot**

Arrabidaea chica (figura 1) é uma planta pertencente à família Bignoniaceae, que compreende aproximadamente 830 espécies de plantas arbustivas, arbóreas e trepadeiras distribuídas em 120 gêneros. A família Bignoniaceae é dividida em sete tribos, sendo que *Arrabidaea chica* encontra-se na tribo Bignonieae, que constitui um clado de lianas neotropicais grande e morfologicamente diverso (LOHMANN, 2006; BEHRENS; TELLIS; CHAGAS, 2012).

Taxonomicamente a espécie *Arrabidaea chica* foi classificada por Croquist (1981) como pertencente:

Divisão: Magnoliophyta

Classe: Magnoliopsida

Subclasse: Asteridae

Ordem: Scrophulariales

Família: Bignoniaceae

Gênero: *Arrabidaea*

Figura 1 - Aspectos gerais da *Arrabidaea chica* (Humb. & Bonpl.) Verlot. Fonte: autor

Sendo as espécies deste gênero encontradas em sua maioria em regiões tropicais e subtropicais, *Arrabidaea chica* têm como principais centros de distribuição o continente africano e as Américas (Figura 2), Central e do Sul, destacando o Peru e Brasil Central, com prevalência desde a região amazônica até o Rio Grande do Sul, incluindo regiões de cerrado e Mata atlântica, não possuindo um hábitat único (ALVES et al., 2010; BEHRENS; TELLIS; CHAGAS, 2012).

Arrabidaea chica é facilmente cultivada e tem crescimento rápido (BARROS; POHLIT; CHAVES, 2008). Na região Amazônica, por exemplo, a espécie é facilmente multiplicada por estacas (CORRÊA,1984).

Figura 2 – Distribuição da espécie *Arrabidaea chica* nas Américas. Fonte: Smithsonian Tropical Research Institute: http://biogeodb.stri.si.edu/herbarium/species/15406/?search_key=Arrabidaea+chica

Arrabidaea chica é popularmente conhecida como carajiru, crajiru, cajuru, carajuru, chica, pariri, crejeru, guajuru–piranga, guarajuru, oajuru, oajuru–piranga, paripari, crejer. (VAN DEN BERG, 1982; MORS; RIZZINI; PEREIRA, 2000; VON POSER et al., 2000). Apresenta como sinonímias *Adenocalyma portocalymma* A. Stahl; *Arrabidaea acutifolia* A. D C; *Arrabidaea cuprea* (Charm.) Bornm.; *Arrabidaea larensis* Pittier; *Arrabidaea rosea* D C; *Bignonia chica* Humb. & Bonpl.; *Bignonia cuprea* Cham.; *Bignonia erubescens* S. Moore; *Bignonia triphylla* Willd. Ex D C; *Lundia chica* (Humb. & Bonpl.) Seem.; *Temnocydia carajura* Mart. Ex D C.; *Vasconcellia acutifolia* C. Mart. Ex D C e mais recentemente *Fridericia chica* (Bonpl.) L.G. Lohmann (BEHRENS; TELLIS; CHAGAS, 2012).

A espécie apresenta também diversas variedades: acutifolia de folhas maiores (até 15 cm de comprimento), com reticulado purpúreo e corola menor (até 2cm); angustifólia, de porte menor, folhas lanceoladas e menores (até 5cm de comprimento); cuprea, de folhas menores, estreitas, curto–obtuso–acuminadas, com reticulado cor de cobre na parte inferior; thyrsoidea, de folhas maiores, agudíssimas (até 10 cm de comprimento e 6 cm de largura), panícula maior e corola de 3cm (CORRÊA, 1984).

Devido aos seus pigmentos carajurina e carajurona, *A. chica* é bastante usada no Brasil, pelos índios, para fazer tatuagens. Em algumas tribos, é preparada uma pasta com essa planta, que funciona como repelente de insetos, além de também ser utilizada como protetor solar. Vários cosméticos e produtos têm sido comercializados, principalmente na região Norte do Brasil, graças a esses pigmentos, que são obtidos a partir da fermentação de suas folhas (CHAPMAN; PERKIN; ROBINSON, 1927; BARBOSA et al., 2008; SCHIOZER et al., 2012).

As folhas de *A. chica* têm sido também empregadas popularmente no tratamento de enfermidades da pele, como: psoríase, impingem, úlceras e piodermites; têm sido utilizados também por suas propriedades adstringentes, anti–inflamatórias, para tratar cólica intestinal, diarreia com sangue, corrimento vaginal, como agente anti–anêmico, para diabetes, leucemia e outros tipos de câncer. Segundo relatos, algumas tribos indígenas também preparavam infusões pra tratar conjuntivite aguda, infecções fúngicas e herpes (CHAPMAN; PERKIN; ROBINSON, 1927; CORRÊA, 1984; KALIL FILHO ET AL., 2000; BORRÁS, 2003; LORENZI; MATOS, 2008; OLIVEIRA et al., 2009).

No Maranhão, também é usada no controle da pressão arterial e cálculos renais (RÊGO, 1995). Além disso, os extratos de folhas de *A. chica* começaram a ser utilizados na indústria de cosméticos do Brasil na formulação de xampus, cremes e sabonetes (SCHIOZER et al., 2012).

O gênero *Arrabidaea* é conhecido como fonte de flavonoides, particularmente antocianinas. O primeiro estudo fitoquímico de *A. chica,* realizado com as folhas, possibilitou o isolamento de dois pigmentos avermelhados chamados de carajurina (6,7– diidroxi–5,4'–dimetoxiflavílio) e carajurona (6,7,4'–triidroxi–5–metoxi–flavílio) (CHAPMAN; PERKIN; ROBINSON, 1927), cuja estrutura só foi completamente elucidada por Zorn e colaboradores (2001). Estudos realizados por Scogin (1980) e Harborne & Williams (2001) afirmaram que a ocorrência desse pigmento estava restrita a essa espécie, dentro da família Bignoniaceae.

Outros compostos foram também sendo identificados e isolados de folhas da espécie, como taninos, 7,4–dihidroxi– 5–metoxiflavona, fitosteróis, flavonoides, alguns compostos derivados das 3–deoxiantocianidinas como antocianinas, o pigmento 1, o pigmento 2 e a luteolina, a flavona carajuruflavona e outros compostos fenólicos como antraquinonas, triterpenos, saponinas e esteroides (TAKEMURA et al., 1995; ZORN et al., 2001; DEVIA et al., 2002; CORTEZ DE SÁ et al., 2016).

ZORN e colaboradores (2001) isolaram das folhas de *A. chica* quatro antocianinas: carajurina, carajurona, 6,7,3′–triidroxi–5,4′–dimetoxiflavílio e 6,7,3′,4′–tetraidroxi–5–metoxiflavílio, e a flavona acacetina. Barbosa e colaboradores (2008) relataram o isolamento de 4′–hidroxi–3,7–dimetoxi–flavona, vicenina e do flavonol canferol de folhas de *A. chica*. SIRAICHI e colaboradores (2013) descreveram pela primeira vez a presença das flavonas escutelareína e isoescutelareína em extratos de folhas *A. chica*, além da 6–hidroxi–luteolina e hispidulina.

Gemelli e colaboradores (2015) avaliaram também a composição do extrato aquoso e fração butanólica de folhas de *A. chica* por cromatografia líquida acoplado à detecção UV, e sugeriram com auxílio de padrões a presença de ácidos fenólicos e flavonóides: Ácido gálico, ácido elágico, rutina e luteolina, sendo estes dois últimos os principais compostos fenólicos da fração butanólica.

Estudos de frações do extrato hexânico de folhas *A. chica*, realizados por cromatografia gasosa acoplada a espectrometria massa, também permitiram identificação de ácidos graxos – n–hexadecanoico, linoleico, linolênico, octadecanoico, eicosanóico; esteroides – campesterol, estigmasterol, gama–sitosterol; além do fitol e vitamina E (RODRIGUES et al., 2014). Mota (2011) também identificou triterpenos pentacíclicos: ácido oleanoico e ácido ursólico em frações derivadas de extrato clorofórmico de *A. chica*. Miranda e colaboradores (2017) isolaram um composto fotossensibilizador, fotoforbide A, de folhas de *A. chica*.

De modo geral, os estudos apontam uma grande abundância de flavonoides nas folhas de *A. chica,* que tem sido utilizada para explicar o extenso número de ações terapêuticas atribuídas a esta espécie como: anti–inflamatória, antioxidante, antimicrobiana, anti–hipertensiva, analgésica, dentre outras (NAIR et al., 2006; CARTAGENES et al., 2010; BRUNETTI et al., 2013; DE QUEIROZ et al., 2014).

Jorge (2008) avaliou a atividade antioxidante do extrato metanólico das folhas de *A. chica,* e afirmaram que este apresentou moderado efeito antioxidante pelo método de sequestro do radical livre (DPPH) e também moderado efeito redutor de agentes oxidantes. Taffarello e colaboradores (2013) observaram também uma moderada atividade antioxidante, pelo ensaio de DPPH para extrato metanólico de folhas *A. chica*, contudo destacaram que esta atividade teve um aumento quando o extrato foi tratado previamente com xilanases de *Bacillus pumilus*, e sugeriram que tal atividade foi favorecida devido após o processo de fermentação da planta ocorrer a liberação das agliconas, aumentando a atividade antioxidante.

Cartágenes e colaboradores (2014) investigaram o efeito do extrato hidroetanólico de folhas *A. chica* no controle do tônus vascular e pressão arterial, e demonstraram que o tratamento com o extrato nas doses de 0,1, 0,25 e 0,5 g/Kg/dia, por 60 dias, causou uma redução significativa nos níveis médios da pressão arterial sistólica, bem como reduziu a reatividade da musculatura vascular das artérias mesentéricas dos animais tratados.

Na avaliação do potencial cicatrizante, um estudo utilizando modelo de úlcera gástrica induzida por etanol em ratos, demonstrou que o extrato etanólico de folhas *A. chica* administrado nas doses de 100, 300 e 1000 mg/Kg reduziu o índice de lesões ulcerativas.

Neste mesmo estudo, observaram também os efeitos cicatrizantes do extrato etanólico de *A. chica* (EB) e extrato etanólico micro encapsulado (EBM), os quais diminuíram significantemente a área ulcerada (85% e 86% de contração) quando comparados com o grupo salina (57% de contração), e os extratos também estimularam o crescimento de fibroblastos e a produção de colágeno, bem como a neovascularização (JORGE, 2013).

Em modelo animal de lesão do tendão calcâneo o extrato etanólico de folhas *A. chica* também favoreceu a cicatrização, através da melhora na organização das fibras de colágeno e aumento na quantidade de dermatan sulfato (ARO et al., 2013).

Quantos aos efeitos angiogênicos, o extrato etanólico de folhas da *A. chica* e sua fração aquosa foram avaliados em modelo de implante de esponja murínico. E verificou–se que os tratamentos com o extrato etanólico (30 a 300 mg/Kg/dia) e fração aquosa (300 mg/Kg/dia), administrados por 10 dias, por via oral, reduziram a neoformação vascular no implante de esponja sem alterar os valores do fator de crescimento endotelial vascular (VEGF) (RIBEIRO, 2012).

Michel e colaboradores (2015) também observaram uma diminuição na angiogênese, determinada pela diminuição na concentração de hemoglobina, em camundongos *Swiss* tratados com extratos de folhas *A. chica*, quando comparados com o controle. Contrariamente, o estudo realizado por Jorge (2013) em membrana corioalantóide (CAM) de ovos e tegumento do dorso de camundongos, demonstrou que os grupos tratados com extrato etanólico de folhas *A. chica* tiveram aumento na vascularização, bem como aumento no calibre dos vasos sanguíneos, que indicaram ação pró–angiogênica.

Considerando ainda, as atividades biológicas da *A. chica*, Ribeiro (2012) avaliou a atividade citotóxica dos extratos etanólico, metanólico e aquosos de folhas *A. chica* e de frações destes extratos em linhagens de células tumorais e, observou *in vitro* que o extrato etanólico apresentou atividade citotóxica contras células leucêmicas humanas (Jurkat e HL60). Em avaliações *in vivo*, conduzidas em camundongos com tumor sólido de *Ehrlich*, os extratos etanólicos e fração aquosa, causaram redução significativa no volume do tumor sólido de *Ehrlich*, e no percentual de linfócitos $TCD4^+$, a fração também levou à redução das células NK e de linfócitos $TCD3^+$, associada à diminuição de linfócitos $TCD8^+$, mostrando que o extrato etanólico e a fração aquosa da *A. chica* apresentam ação imunomoduladora, além de efeito antitumoral.

Silva (2013) avaliou o efeito do extrato clorofórmico de partes aéreas de *A. chica*, incorporado ou não em nano emulsão polimérica PVM/MA (polímero bioadesivo) contra células de adenocarcinoma mamário murino (4T1) *in vitro*, e observou uma intensa redução

na viabilidade celular em comparação ao controle, mostrando o potencial que essa planta tem em ser um FS (fármaco fotossensibilizante), para uso em terapia fotodinâmica (TFD), que constitui uma importante abordagem alternativa no tratamento de câncer, por ser altamente tóxico para as células de interesse.

Michel e colaboradores (2015) também avaliaram o efeito das folhas *A. chica* sobre células tumorais. Testaram extratos etanólicos, aquosos e frações em três concentrações (25, 50 e 100 μg mL^{-1}), e observaram que extrato etanólico apresentou atividade antiproliferativa contra células HL60 e Jurkat e atividade pró–apoptótica em células HL60 e que a fração também demonstrou atividade antiproliferativa significativa e atividade pró–apoptótica contra células HL60.

Vários estudos avaliaram também a toxicidade da *A. chica* (SAMPAIO et al., 1998; CUNHA et al., 2000; MENDES et al., 2001; CARTÁGENES, 2009; AMARAL, 2012; SÁ et al, 2016). O extrato em administrações via oral, na dose de até 3.5 g/kg não provocou sinais de toxicidade em ratos, e mesmo em dose de 5g/kg provocou apenas diarreia nos primeiros dias de administração (CARTAGENES, 2009).

Amaral e colaboradores (2012) analisaram as propriedades diuréticas do extrato do etanólico de folhas de *A. chica*, bem como de suas frações: acetato de etila, diclorometano, hexano e n–butanol, além do composto luteolina isolado. Os testes realizados *in vivo,* demonstraram que a fração hexânica e a luteolina em concentrações de 100 mg Kg^{-1} aumentaram em 79% e 94% o volume urinário, respectivamente.

Na ação antiprotozoária, Rodrigues e colaboradores (2014) destacaram o efeito do extrato hexânico de folhas de *A. chica* e suas frações contra duas espécies de leishmania*: L. amazonensis* e *L. infantum*. A atividade leismanicida de *A. chica*, foi também comprovada para o extrato etanólico das folhas, sendo capaz de reduz 50% na viabilidade de promastigotas *L. amazonensis* com uma concentração de 125 μg mL^{-1}, as frações clorofórmio, metanol e acetato de etila de *A. chica* também foram eficientes contra as formas promastigotas de *L. amazonensis* (SÁ et al., 2016).

O extrato etanólico e frações de folhas de *A. chica* foram também foram avaliados quanto ao potencial tripanocida, e demonstraram atividade significativa contra formas tripomastigotas de *T. cruzi* (cepa Y), induzindo entre 20 e 71% de lise celular. Sendo o melhor resultado obtido com a fração eluída com diclorometano (CH_2Cl_2) e metanol (MeOH) (50: 50), que produziu 71% de lise das células de parasitas (BARBOSA et al., 2008).

Vários estudos têm comprovado a ação antifúngica e antibacteriana de *A. chica.* Berla (2008) avaliou a atividade inibitória *in vitro* do extrato aquoso e hidroetanólico de

folhas, autoclavados e filtrados, sobre vinte e quatro cepas de *Candida albicans* e mostrou que o extrato hidroetanólico autoclavado e o filtrado, apresentaram atividade fungistática sobre todas as cepas de *C. albicans* na concentração de 20% (200 mg mL^{-1}). Barbosa e colaboradores (2008) demonstraram a atividade antifúngica do extrato de folhas de *A. chica* sobre o fungo patogênico *T. mentagrophytes* em uma concentração de inibição mínima de 3,125 mg mL^{-1}. Ferreira (2013) observaram também que o extrato etanólico de folhas foi eficácia sobre várias cepas bacterianas, com concentrações inibitórias mínimas de 15,625 mg mL^{-1} para *Salmonella typhimurium* e *Shigella sonnei*, 62,5 mg mL^{-1} para *Escherichia coli* e 31,25 mg mL^{-1} para *Lactobacillus acidophilus*.

O efeito do extrato hidroetanólico de folhas de *A. chica* também foi avaliado em modelo *in vivo* de intoxicação hepática de ratos *Wista*r, induzida por tetracloreto de carbono (CCI4). Foi observado que a administração de 300, 500 ou 600 mg/kg do extrato resultou na redução dos níveis transaminase glutâmica pirúvica, e na supressão dos níveis de transaminase oxalacética glutâmico e de bilirrubina no plasma, quando comparado com o controle. Segundo os autores estes resultados demonstram o caráter protetor do extrato e a sua capacidade para manter a integridade funcional das células hepáticas (MEDEIROS et al., 2011).

A atividade anti-inflamatória de *A. chica* também tem sido bastante estudada e vem sendo comprovada em diversas abordagens. Zorn e colaboradores (2001), demonstraram que extrato lipofílico (200 μg mL^{-1}) de folhas de *A. chica* e a carajurina possuem efeitos anti–infamatório *in vitro*, inibindo a ativação do fator nuclear–κB (NF–κB), que regula a transcrição de genes de várias citocinas inflamatórias, quimiocinas, moléculas de adesão e enzimas inflamatórias como iNOS, fosfolipase A2 citosólica e COX–2, sendo que a cajurina (500 μM) inibiu completamente o fator nuclear–κB.

Investigações sobre o efeito anti-inflamatório do extrato aquoso de folhas de *A. chica* foram realizadas também, em edemas induzidos por venenos de serpentes dos gêneros *Brothrops* e *Crotalus*, em camundongos, e mostram que o extrato reduziu o edema provocado pelo veneno de ambas as serpentes. A análise histopatológica, também evidenciou o efeito anti-inflamatório, com a inibição do infiltrado de granulócitos e a miocitólise (OLIVEIRA et al., 2008).

Michel e colaboradores (2015) também avaliaram o efeito anti-inflamatório de extrato aquoso e etanólico de folhas de *A. chica* (na concentração de 300 mg/Kg/dia), administrados por via oral, durante 8 dias, em camundongos previamente submetidos a processo inflamatório por implante de discos de esponja. Nesta situação verificaram que ambos os

extratos, etanólico e aquoso foram capazes de diminuir acentuadamente a acumulação de neutrófilos, no entanto sem alterar o nível de citocinas (IL–2, IL–4, IL–5, IFN–γ, TNF–α e VEGF) dos animais tratados.

Lima e colaboradores (2018) observaram também que em peritonite induzida por lipopolissacarídeos em camundongos, o pré–tratamento oral com extrato hidroetanólico das folhas ou com o composto isolado 4 ', 6,7–tri–hidroxi–5–metoxiflavona (5–O–metil–scutellarein) levaram à diminuição da migração de leucócitos para a cavidade peritoneal, além da redução das concentrações de citocinas pró–inflamatórias (TNFα e IL–1β). MOREIRA LIMA (2017) também observou que a administração por via oral das frações hexânica, butanólica, acetato de etila e clorofórmica (1 mg/ kg) de folhas de *A. chica* reduziram a concentração da citocina pró–inflamatória IL1 após indução da dor neuropática por compressão do nervo ciático.

A atividade anti-inflamatória desta planta, pode ainda ser explorada para o tratamento de várias outras patológicas, em que o processo inflamatório tem um papel crítico, como é o caso da OA. (ATTUR et al., 2002; ROBINSON et al., 2016; WOODELL-MAY & SOMMERFELD, 2020).

2.4 Osteoartrite

A osteoartrite (OA) é a forma de artrite mais comum na população mundial, e gera grandes impacto socioeconômico, uma vez que pode levar a incapacidade funcional dos indivíduos, afetando a qualidade de vida, além de produzir altos custos para os serviços de saúde, relacionados principalmente ao controle do quadro álgico e a realização de artroplastias (SHARMA; KAPOOR; ISSA, 2006; ROSIS; KAIRALLA, 2010; KAWANO et al., 2015).

Constitui–se também como uma importante questão de saúde pública relacionada ao envelhecimento e tende a se tornar cada vez mais importante com o maior envelhecimento da população mundial. No Brasil projeta–se que a população idosa (acima de 60 anos) deve dobrar até o ano de 2042, na comparação com os números de 2017, cuja população idosa era de 28 milhões de idosos. Em 2042, poderá chegar a 57 milhões de idosos (24,5%) (IBGE, 2018).

No Brasil, os estudos epidemiológicos são escassos e não mostram a amplitude do impacto da OA na sociedade, além disso, as estimativas de ocorrência de OA variam com a definição usada para categorizar a doença e também características da população do estudo incluindo a idade (PEREIRA et al., 2011). Contudo, o Ministério da Saúde aponta que as doenças reumáticas já afetam aproximadamente 12 milhões de brasileiros (Brasil, 2011). E a

Organização Mundial de Saúde estima que no mundo haja 9,6% dos homens e 18% das mulheres acima de 60 anos com OA (WHO, 2018).

De forma geral, as articulações mais comumente afetadas são quadril, joelho e mão (HEBERT; XAVIER, 2003). Estima–se que a OA sintomática do joelho afeta aproximadamente 12% da população idosa (≥ 60 anos), a OA sintomática de mão (6,8% ≥ 26 anos) e quadril (9,2%, ≥45 anos) (KAWANO et al., 2015; MAKSIMOVIĆ; SAMARDŽIĆ, 2018). A OA pode acometer mais de uma articulação, mas sua manifestação não é sistêmica. A dor, a rigidez articular e a limitação ao movimento são os sintomas mais significativos da doença (WHO, 2018; LITWIC et al., 2013).

A OA é uma doença multifatorial, que envolve uma interação complexa entre fatores mecânicos, celulares e bioquímicos. Incluindo idade, sexo, genética, fatores metabólicos e bioquímicos, etnia, estrutura/alinhamento da articulação, trauma, atividade física e também ocupação (Figura 3) (HASEEB; HAQQI, 2013; JOHNSON; HUNTER, 2014; O'NEILL; MCCABE; MCBETH, 2018). Dentre os fatores de risco, a obesidade e a idade são os mais fortemente associados ao desenvolvimento e a progressão da OA (MAKSIMOVIĆ; SAMARDŽIĆ, 2018)

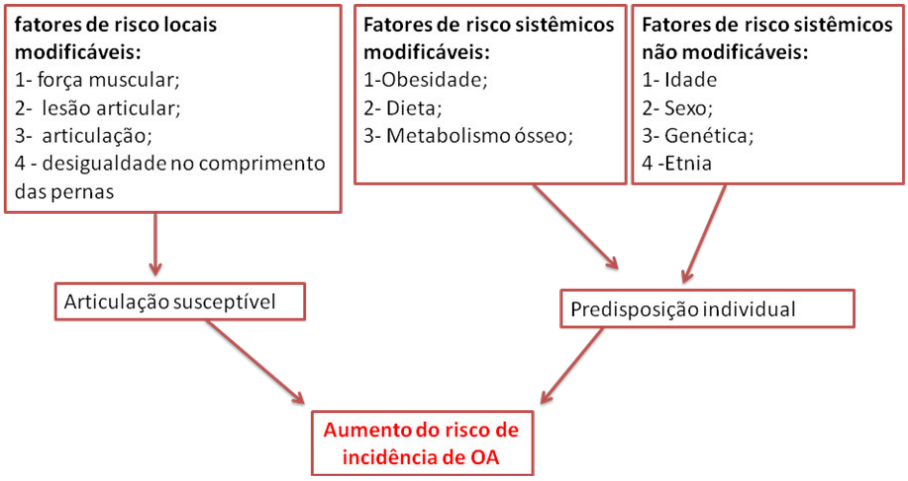

Figura 3 – Associação de fatores de risco para incidência de OA. **Fonte**: adaptado de JOHNSON & HUNTER (2014).

Embora vários fatores de risco estejam associados à OA, a etiologia exata da doença permanece em grande parte desconhecida. Contudo, a compreensão da etiologia da doença parece ser determinante para caracterizar a sequência das alterações patológicas na OA. Em indivíduo obeso, por exemplo, a alteração inicial pode ser a formação óssea aumentada, seguida pela destruição articular e sinovite. No caso de lesões traumáticas, tem–se a inflamação articular aguda seguida por sinovite e destruição da cartilagem e por último,

alterações ósseas (FELSON, 2006; KRASNOKUTSKY; SAMUELS; ABRAMSON, 2007; LOESER, 2009; ROBINSON et al., 2016).

A osteoartrite é uma doença articular caracterizada pela destruição progressiva da cartilagem articular, inflamação sinovial, alterações no osso subcondral e músculo peri–articular e dor, ou seja, é um distúrbio da articulação como um todo (Figura 4), cujas vias de degradação articular parecem ser significativamente influenciadas por mediadores inflamatórios liberados do osso subcondral (STEWART; KAWCAK, 2018). Sendo a inflamação responsável por muitas alterações patológicas e de progressão geralmente lenta, que, no entanto, podem levar à incapacidade articular devido à fraca capacidade de reparação da cartilagem (MORTELLARO, 2003; FELLET; FELLET; FELLET, 2007; XIMENES; MELO; CUSTODIO, 2009; YUAN et al., 2014; ROBINSON et al., 2016)

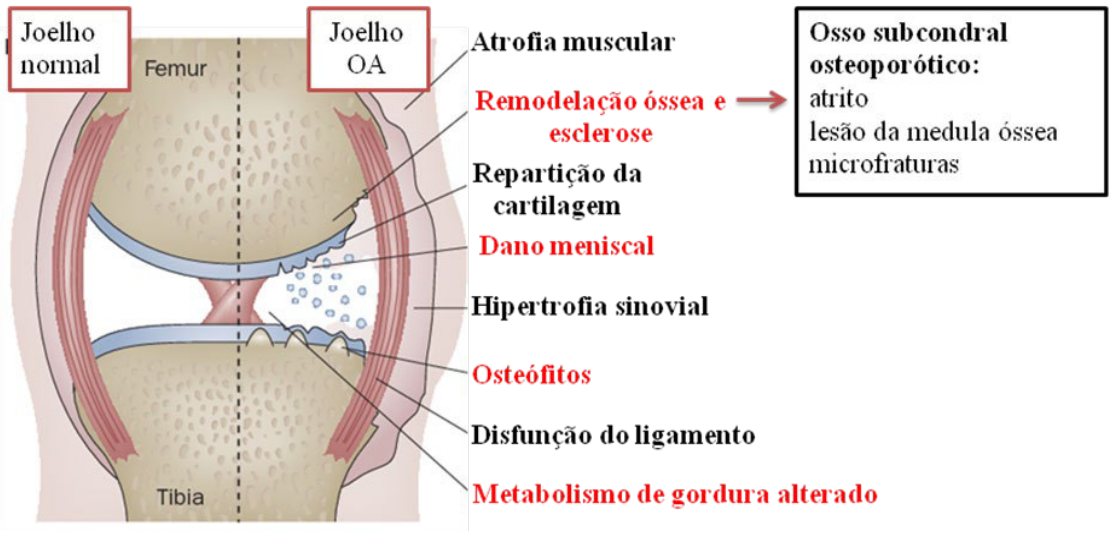

Figura 4 – Representação esquemática da articulação do joelho – comparando articulação normal (lado esquerdo da figura) e com osteoartrite (lado direito da figura). **Fonte**: adaptado de Hunter (2011)

Vários processos celulares e moleculares estão envolvidos nessas alterações patológicas. Quando o mecanismo fisiológico normal que mantém o equilíbrio da matriz extracelular falha, os componentes da matriz extracelular são perdidos, os condrócitos expandidos se aglomeram nas regiões depletadas, um estado oxidativo é induzido no ambiente celular estressado e, por fim, ocorre a apoptose de condrócitos (LEE et al., 2013; NELSON et al., 2014).

A falha no equilíbrio da matriz é devida ao aumento da expressão de enzimas degradadoras de matriz, inibição da síntese de matriz e produção excessiva de mediadores pró–inflamatórios, incluindo citocinas, quimiocinas e produtos de degradação de matriz (IM et al., 2010; MALDONADO; NAM, 2013; REZENDE; CAMPOS; PAILO, 2013). As

alterações ósseas subcondrais levam à formação de osteófitos e esclerose; afrouxamento e fraqueza dos músculos peri–articulares acompanham a destruição da cartilagem articular (VAN DER KRAAN; VAN DEN BERG, 2007; GLYN-JONES et al., 2015).

Avanços na compreensão da fisiopatologia da OA têm indicado ainda, que a inflamação tem um papel crítico na sua patogênese. A inflamação na OA é distinta da artrite reumatoide e de outras doenças autoimunes, é crônica, comparativamente baixa, e mediada principalmente pelo sistema imune inato (ROBINSON et al., 2016). Proteínas plasmáticas inflamatórias estão presentes em níveis anormalmente elevados no sangue e no líquido sinovial de pacientes com OA. Bem como, componentes do sistema complemento e citocinas. Os condrócitos e as células sinoviais na OA produzem muitos dos mediadores inflamatórios, como: IL–1β, TNF e óxido nítrico (NO) (PELLETIER; MARTEL-PELLETIER; ABRAMSON, 2001; GOBEZIE et al., 2007; SOHN et al., 2012).

Após uma lesão tecidual o TNF–α, é a primeira citocina liberada e desencadeia a liberação de outras citocinas, como IL–6 e IL–1β, as quais são responsáveis pela estimulação da síntese de prostaglandinas e liberação de aminas simpáticas. Estas citocinas teriam efeito catabólico, levando a destruição da cartilagem articular pela indução da liberação de enzimas líticas zinco–dependentes, ou seja, metaloproteases (colagenase, gelatinase, estromelisina), além da diminuição de produção de agentes inibitórios teciduais das metaloproteases e dos inibidores do plasminogênio (CUNHA et al., 2005; CHOI et al., 2019).

Os níveis de IL– 1β e TNF–α são aumentados pela secreção por condrócitos e sinoviócitos semelhantes a macrófagos (MLS). Estas citocinas afetam também os condrócitos e sinoviócitos semelhantes a fibroblastos (FLS), aumentando a produção de citocinas e enzimas proteolíticas, e inibem a síntese de componentes da matriz extracelular, sendo que a IL–1 inibiria a síntese de agrecanos e suprimiria a síntese dos colágenos II e IX, que são constituintes próprios da cartilagem, e aumentaria a produção dos colágenos I e III, resultando numa reparação tecidual deficiente (Figura 5) (ROBINSON et al., 2016; SIEBUHR et al., 2016).

Além disso, o MLS produz IL–18, que estimula o próprio MLS e o FLS a produzir quimioatraentes e moléculas de adesão de neutrófilos. O aumento do nível de IL–6 aumenta o nível de proteína C–reativa (PCR) na circulação. A vascularização da membrana sinovial aumenta com a inflamação, aumentando o nível de células inflamatórias infiltrantes. Os osteoclastos produzem IL–1β, IL–6, TNF–α e fator de crescimento transformador (TGF)–β, aumentando o nível dessas citocinas na articulação afetada pela OA e, em resposta, secretam níveis elevados de metaloproteinases de matriz (MMPs) e catepsinas. O aumento do nível das

citocinas também é uma resposta a um nível elevado do ligante do receptor ativador do fator nuclear kappa B (NF–κB) ligante (RANKL) produzido pelos osteoblastos, como está representado na Figura 5 (SIEBUHR et al., 2016).

Na cartilagem de indivíduos com OA, a atividade de fatores de transcrição redox–sensíveis, como o NF–κB, pode ser regulada positiva e negativamente pelas espécies reativas de oxigênio (ROS). A oxidação da cisteína reduz o potencial de ligação NF–κB ao DNA, enquanto a degradação do inibidor de NF–κB – IκB pode ser desencadeada por um aumento das ROS, levando à ativação do NF–κB. Níveis elevados de ROS resultam na regulação positiva da sinalização de NF–κB, contribuindo para alterações fenotípicas pró–inflamatórias no tecido da OA, incluindo a indução da produção de iNOS, IL–8 e COX–2, como se pode observar na Figura 6 (GLINEUR; DAVIOUD-CHARVET; VANDENBUNDER, 2000; JANSSEN-HEININGER; POYNTER; BAEUERLE, 2000).

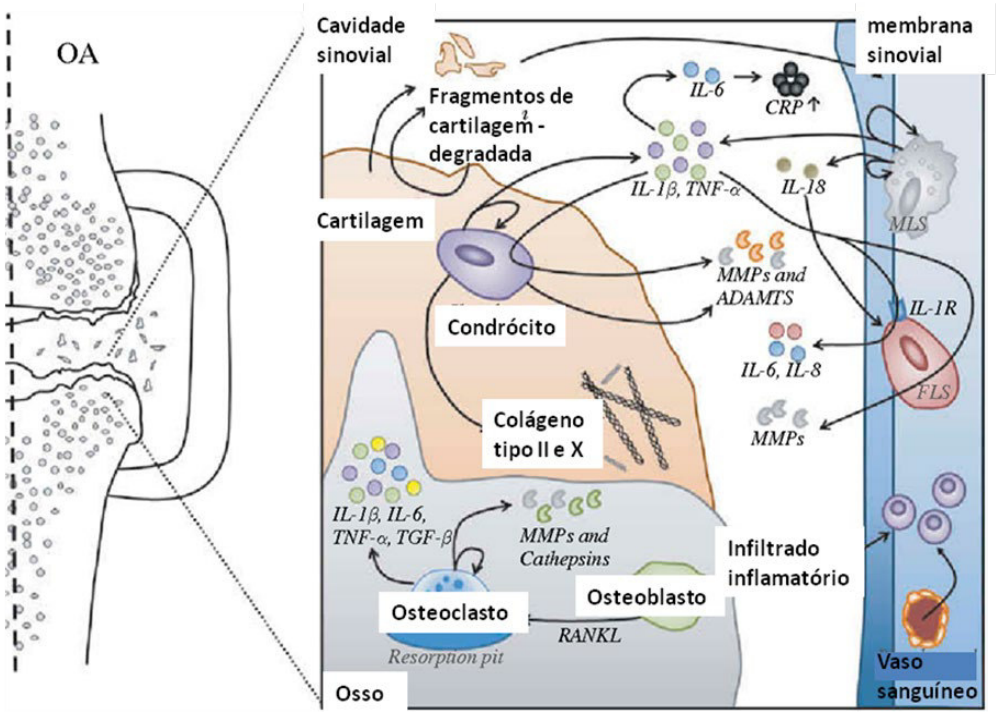

Figura 5 – Vias de sinalização e mudanças estruturais no desenvolvimento da osteoartrite. Fonte: adaptado de Siebuhr et al. (2016).

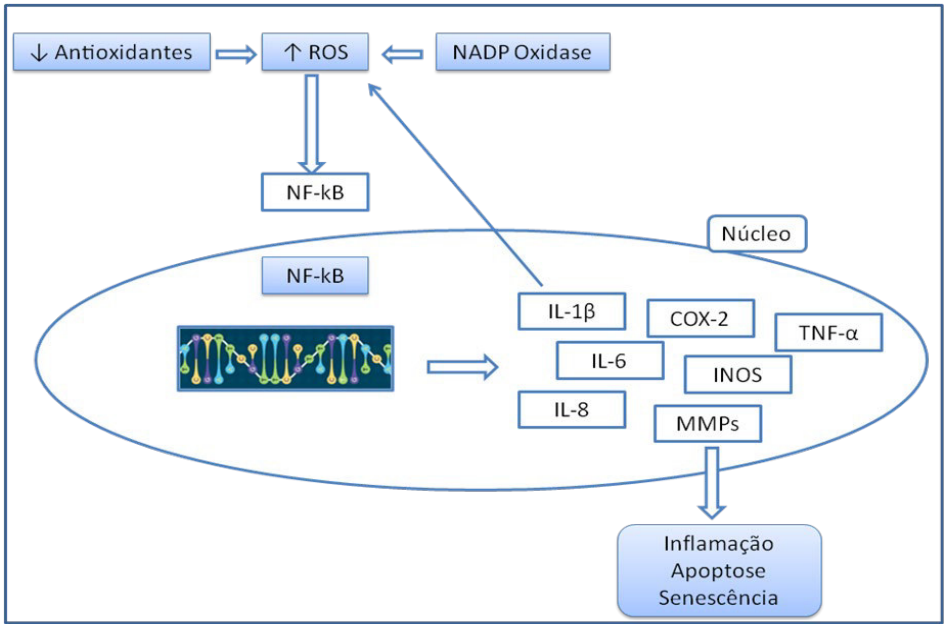

Figura 6 – Interação entre o ROS e o NF–κB – nas vias de sinalização da cartilagem da osteoartrite (OA). **Fonte**: adaptado LEPETSOS et al. (2019).

A expressões de iNOS e COX-2, e consequentemente a produção de NO e PGE2 pode ser induzida pela citocina IL-1β, e níveis elevados de NO e PGE2 tem significativa importância em indivíduos com OA (KOCH, et.al., 1988). Pois, a PGE2 é um importante mediador do processo inflamatório e está intimamente relacionada à produção de NO (AHMAD, et al., 2002; CHANG, et al., 2007). Estudos mostram que cartilagens afetadas pela OA, bem como tecido sinovial retirado de pacientes afetados pela artrite reumatoide tiveram indução de ciclooxigenase-2 (MARTEL-PELLETIER; PELLETIER; FAHMI, 2003; KOJIMA; KATO; KAWA, 2005; BENITO, et al., 2005). A COX-2 é considerada um importante mediador da destruição tecidual em doenças inflamatórias ósseas (KOJIMA; KATO; KAWAI, 2005; VUOLTEENAHO; MOILANEN; MOILANEN, 2008).

A ciclo-oxigenase (COX) é uma proteína com função enzimática que catalisa a biossíntese das prostaglandinas, através da conversão do ácido araquidônico. A COX, também conhecida como prostaglandina H sintetase (PGHS) tem uma ação bivalente, pois catalisam duas reações separadas, incluindo uma reação de COX, que permite a formação da prostaglandina G_2 (PGG_2) a partir do ácido araquidônico (AA) e uma reação de peroxidase, em que este componente da mesma enzima, permite a PGG_2 formada sofre uma redução em dois elétrons para formar a Prostaglandina H_2 (PGH2). Após a formação da PGH_2 podem ser produzidas 4 prostaglandinas: Prostaglandina D_2 (PGD_2), Prostaglandina E_2 (PGE_2), Prostaglandina F_2 (PGF_2) e a Prostaglandina I_2 (PGI_2 ou prostaciclina); e tromboxanos (TX). Várias enzimas atuam na síntese destes mediadores, que variam de acordo com o tecido,

determinando certa especificidade na sua regulação (Figura 7) (GARAVITO & DEWITT, 1999; CARVALHO; CARVALHO; RIOS-SANTOS, 2004; CHANDRASEKHARAN & SIMMONS, 2004).

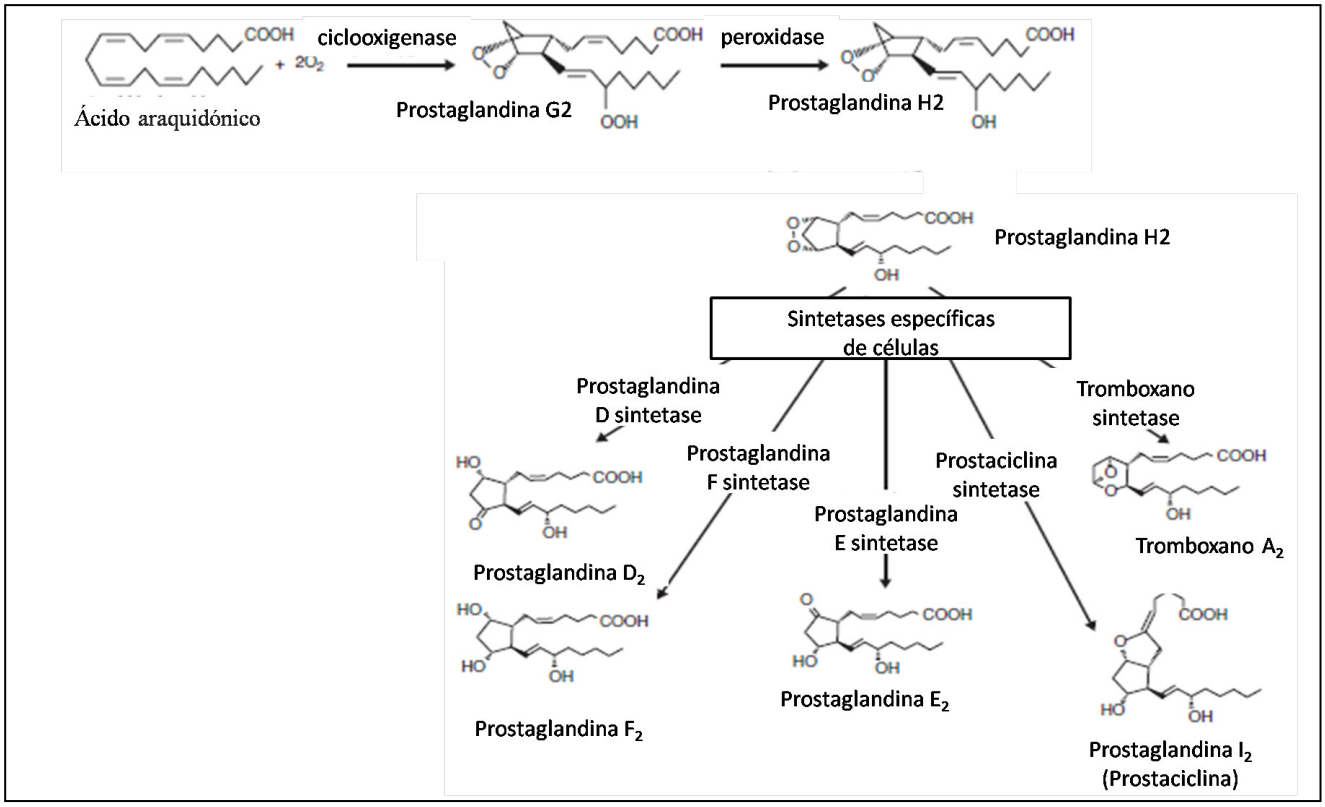

Figura 7 - Biossíntese das Prostaglandinas por COXs. Fonte: Adaptado de CHANDRASEKHARAN & SIMMONS (2004)

Existem três isoformas conhecidas da COX, definidas como COX-1, COX-2 e COX-3 (DUBOIS et al., 1998; WILLOUGHBY; MOORE; COLVILLE-NASH, 2000). A primeira a ser caracterizada foi a COX-1, que é constitutivamente expressa na maioria dos tecidos, sendo responsável pela produção de prostaglandinas, que são vitais para processos fisiológicos normais. A Cox-2 é uma forma principalmente induzível da enzima, sendo expressa caracteristicamente por células envolvidas no processo inflamatório, assim tem sido implicada em estados patológicos. A COX-3 é considerada como uma variante da COX-1, que se encontra distribuída principalmente no córtex cerebral, medula espinhal e coração (CROFFORD, 1997; VANE; BAKHLE; BOTTING, 1998; CHANDRASEKHARAN et al., 2002; LÚCIO et al., 2008). Apesar da COX-1 e 2 assumirem prioritariamente as condições descritas anteriormente, não se pode simplificar a atuação destas isoformas de COX, pois a COX-1 pode também contribuir para os processos inflamatórios, enquanto a COX-2 também se expressa constitutivamente em vários tecidos e órgãos, como cérebro, rins e trato reprodutivo (PATRIGNANI et al., 2005; ZARGHI & ARFAEI, 2011). Assim, apesar da

inibição seletiva da COX-2 ser frequentemente explorada como perspectiva terapêutica para o desenvolvimento de drogas mais seletivas e com menos efeitos adversos (VANE, 1994; FITZGERALD & PATRONO, 2001; MASTBERGEN, S. C.; LAFEBER, F. P. J. G.; BIJLSMA, 2002) existem importantes ressalvas, dependendo das condições clínicas do indivíduo que fará uso do tratamento (BERTOLINI; OTTANI; SANDRINI, 2001; PARENTE, 2001; GAETANO G, DONATI MB, CERLETTI, 2003).

2.5 Dor e nocicepção

No que diz respeito as manifestações clínica da OA a dor é a principal queixa e razão pela qual os indivíduos afetados buscam os serviços de saúde. A definição de dor promulgada pela Associação Internacional para o Estudo Dor (*International Association for the Study of Pain* - IASP), desde 1979, descreve a dor como: "Uma experiência sensorial e emocional desagradável associada a um potencial real ou potencial danos nos tecidos, ou descritos em termos de tais danos". Contudo, avanços substanciais na compreensão da dor, na avaliação e tratamento, a luz de perspectivas multidisciplinares e surgimento de modelos de doença, levaram Williams & Craig, (2016) a propor uma nova definição de dor, afirmando que a dor é "uma experiência angustiante, associada a um potencial dano tecidual com sensorial, emocional, cognitivo, e componentes sociais". A dor é, portanto, uma experiência subjetiva, que envolve muitos aspectos, que vão além do dano real, mas levam em consideração todo o estado do indivíduo, desde o físico até o emocional e social.

Vale lembrar ainda, que a dor é um importante mecanismo de sinalização para o organismo quanto à ocorrência de estímulos nocivos, com função de restaurar o equilíbrio do corpo, através da ativação de reações e/ou comportamentos de prevenção na tentativa de inibir as causas da dor, e corrigir ou limitar os danos. Contudo, a ação prolongada desde estímulo doloroso pode ser prejudicial ao organismo, e merece atenção (LENT, 2010; CLAUW; HASSETT, 2017).

Quanto à classificação da dor, esta pode ser feita de muitas maneiras, que podem ainda se sobrepor, sendo que os tipos mais comuns incluem: dor nociceptiva (frequentemente inflamatória) resultante de uma resposta normal a estímulos nocivos ou lesão de tecidos como pele, músculos, órgãos viscerais, articulações, tendões ou ossos; dor neuropática, iniciada ou causada por uma lesão primária ou doença no sistema nervoso somatossensorial; e dor idiopática, que não tem causa identificada (THAKUR; DICKENSON; BARON, 2014). A dor pode ser classificada ainda quanto à duração, em aguda ou crônica. A dor aguda ou rápida é caracterizada por ser pontual, e desaparece após a remoção do agente causador. A dor crônica

ou lenta, típica da OA, é persistente e está associada a alterações fisiológicas e mudanças no padrão de transmissão neuronal (MERSKEY & BOGDUK, 1994; RODITI; ROBINSON, 2011). A dor na OA é mais frequentemente caracterizada como uma dor nociceptiva inflamatória, resultante da ativação e sensibilização da via nociceptiva. Contudo, cada vez mais estudos apoiam a hipótese de que a osteoartrite seja um estado de dor mista e que, em alguns indivíduos, os fatores do SNC desempenham um papel muito importante (ZHANG; JORDAN, 2008; THAKUR; DICKENSON; BARON, 2014; CLAUW; HASSETT, 2017).

A nocicepção consiste então na percepção dos estímulos pelos receptores da dor (nociceptores). Os nociceptores são terminações nervosas livres, que podem ser classificados em três grandes grupos principais, de acordo com sua velocidade de condução e principais estímulos de ativação. O primeiro grupo é formado pelas as fibras Aβ mielinizadas de condução rápida, que têm um limiar de resposta baixo, conduzem principalmente estímulos táteis inócuos, como estímulos mecânicos e térmicos de baixa intensidade e estão envolvidas em respostas reflexas. Os outros dois subtipos, as fibras Aδ e C (nociceptores) são capazes de traduzir um estímulo agressivo de natureza térmica, química ou mecânica, em estímulo elétrico que será transmitido até o sistema nervoso central e interpretado no córtex cerebral como dor. As fibras Aδ são mielinizadas e, em função disso transmitem o estímulo doloroso de forma rápida, e são responsáveis pela primeira fase da dor, rápida e forte, do tipo picada ou ferroada. As fibras C não são mielinizadas, e por isso tem uma velocidade de condução lenta, e são responsáveis pela segunda fase da dor ou dor difusa, com sensação de queimação persistente (GRUBB, 2004; MCCABE, 2004; FELSON, 2005; KLAUMANN, WOUK, SILLAS, 2008).

Diferentes mediadores, como citocinas pró–inflamatórias (IL–1–alfa, IL–1–beta, IL–6 e TNF–alfa), quimiocinas, bradicininas, prostaglandinas, espécies reativas de oxigênio, aminas vasoativas, lipídios, ATP e outros fatores liberados por leucócitos infiltrantes, células endoteliais vasculares ou mastócitos residentes em tecidos, têm sido implicados como componentes responsáveis pela sensibilização de nociceptores (Figura 8) (VERRI et al., 2006; BINGHAM et al., 2009).

Após a sensibilização, ocorre a tradução dos estímulos nocivos em atividade eletroquímica – pela despolarização dos terminais periféricos de neurônios sensoriais primários de alto limiar, com os potenciais de ação (APs) resultantes conduzidos ao sistema nervoso central (SNC) pelos axônios sensitivos aferentes primários. Os neurônios de projeção secundária no corno dorsal transmitem informações para o tronco cerebral e o tálamo, que

transmitem o sinal para o córtex, hipotálamo e sistema límbico. A partir destes iniciam–se as vias descendentes, que possuem um papel importante na modulação da dor. Elas são projetadas a uma região mesocefálica denominada de sustância cinzenta, e destas para diferentes núcleos bulbares, e destes ao corno dorsal da medula. Estimulações farmacológicas ou elétricas desses núcleos podem inibir a transmissão sináptica, provocando o bloqueio da dor (Figura 8) (FIELDS & MARTIN, 1998; GOTTSCHALK; SMITH, 2001; WOOLF; MA, 2007; GUYTON & HALL, 2017).

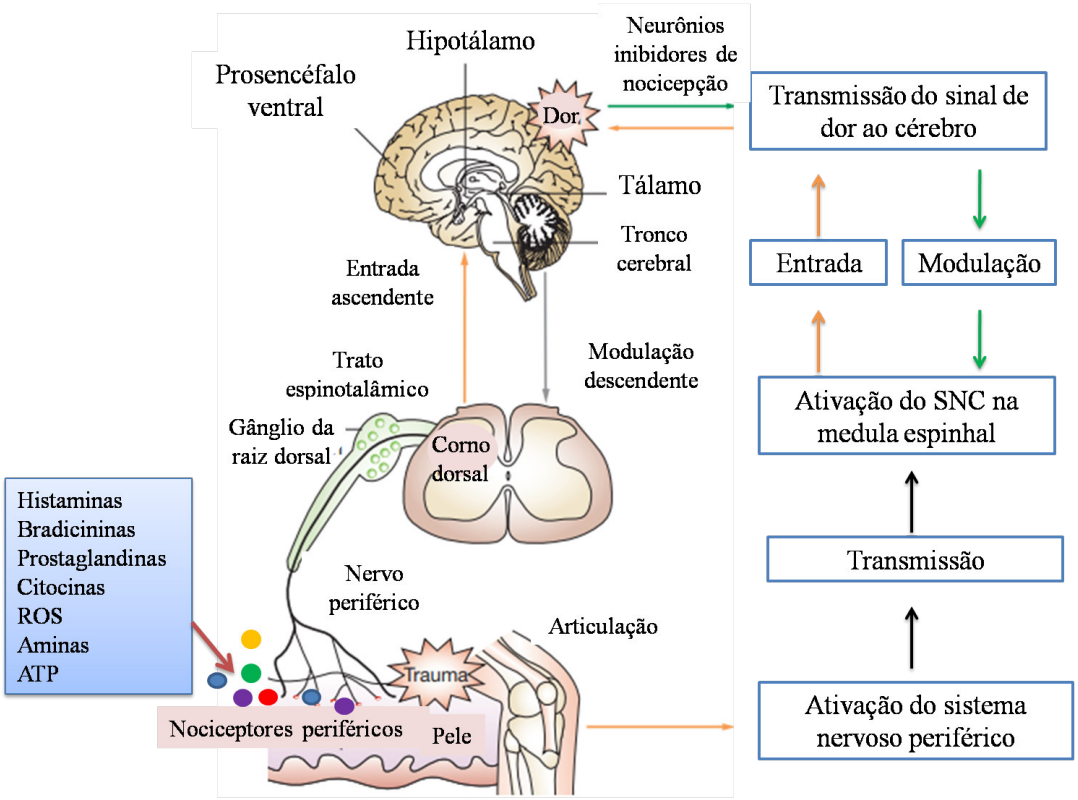

Figura 8 – Representação esquemática das vias da dor nociceptiva. Fonte: adaptado BINGHAM et al. (2009).

2.6 Modelo experimental de OA induzida por Monoiodoacetato (MIA)

Apesar da grande evolução tecnológica que o mundo tem visto ao longo das últimas décadas, ainda não existem alternativas válidas em relação ao uso de animais experimentais para todas as pesquisas que precisam ser realizados. Assim o uso de animais tem sido uma parte integrante e imprescindível do desenvolvimento da medicina, pois ajuda na compreensão dos fenômenos fisiopatológicos, bem como na avaliação da eficácia de potenciais medicamentos. Desse modo, tem evitado a mortalidade de um grande número de seres humanos, bem como de outros organismos, e prevenido ou tratado a dor decorrente de

diversas patologias (CASTRO–COSTA, SANTOS, CASTRO–COSTA, 2009; ANDERSEN; WINTER, 2019).

Apesar dos animais não serem capazes de verbalizar a ocorrência de dor, quando são submetidos a um estímulo doloroso, eles exibem respostas comportamentais, motoras e fisiológicas semelhantes às humanas e a partir da observação desses fatores pode–se inferir que um animal está experimentando uma resposta álgica (LAPA et al., 2007).

Os modelos animais de OA pertencem a três categorias gerais *in vivo*: modelos OA de ocorrência natural, incluindo animais geneticamente modificados; modelos de iniciação ou aceleração da degeneração articular desenvolvidos após cirurgia ou outro trauma; e modelos químicos desenvolvidos através da injeção intra–articular de substâncias condrotóxicas ou pró–inflamatórias (TEEPLE et al., 2013; KUYINU et al., 2016).

De todos os modelos químicos de OA, o monoiodoacetato de sódio (MIA) é o mais usado, particularmente para testar a eficácia de agentes farmacológicos no tratamento da dor, por ser mais preditivo da eficácia de drogas. Visto que, este modelo gera dados reprodutíveis, robustos e rápidos, que assemelhar–se à OA em humanos, em termos tanto de características histológicos como relacionados com a dor (BOVE et al., 2003; POMONIS et al., 2005; SCHUELERT; MCDOUGALL, 2009; IM et al., 2010; KELLY et al., 2012; LAMPROPOULOU-ADAMIDOU et al., 2014; TAKAHASHI et al., 2018).

O MIA é um inibidor da gliceraldeído–3–fosfato desidrogenase, que interrompe a glicólise, provocando a morte de condrócitos, perda de proteoglicanos, fibrilação, assim degradando a cartilagem articular e formando cistos e grandes osteófitos (BENDELE, 2001; MARKER; POMONIS, 2012).

Cronologicamente, as lesões histológicas na osteoartrite, induzida por MIA, tem inicialmente encolhimento, degeneração e morte de condrócitos, com edema sinovial e moderado infiltrado de células mononucleares, resultando em uma rápida inflamação e dor que se desenvolve até 7 dias. Após este período os osteoblastos são agrupados e ocorre aumento da ativação dos osteoclastos, indicando aumento da remodelação óssea. Seguida da fragmentação e erosão da cartilagem, intimamente ligada com colapso e fragmentação das trabéculas ósseas subjacentes, e a partir do 10º dia após indução instala–se uma dor musculoesquelética crônica (BOVE et al., 2003; FERNIHOUGH et al., 2004).

2.7 Tratamentos para OA

Apesar da alta e crescente prevalência da OA as opções terapêuticas ainda são muito limitadas. Os tratamentos devem ser multidisciplinares, pois as prescrições medicamentosas

isoladas não são suficientes para o controle ideal da OA, que promova melhora funcional, mecânica e clínica (COIMBRA et al., 2002).

Assim o tratamento deve adotar diversas abordagens desde educar os pacientes sobre os processos da doença, com tratamentos personalizados que inclui mudanças no estilo de vida e exercícios físicos; tratamentos farmacológicos orais; injeções intra-articulares, até intervenções cirúrgicas (JORDAN et al., 2003), podendo assim, ser divididos em tratamento farmacológicos e não–farmacológicos (COIMBRA et al., 2002).

Quando se trata do manejo não–farmacológico, ele visa melhorar a dor do paciente através de intervenções não medicamentosas, de modo que diminua os efeitos adversos oriundos dos fármacos utilizados. Deste modo, as intervenções compreendem principalmente orientações ao paciente e acesso à informação sobre a OA; intervenções e medidas dietéticas para perda de peso e referenciação a ajuda especializada de doentes com sobrepeso ou obesos; exercício físico, acupuntura e reabilitação funcional (fisioterapia) e procedimentos cirúrgicos. Esse conjunto de estratégias constitui-se como uma importante linha no tratamento, sendo tão fundamental quanto a analgesia, para recuperação e/ou manutenção do estado funcional e a readaptação funcional (FERREIRA et al., 2012; MCALINDON et al., 2014; NICE, 2019).

No que diz respeito às intervenções farmacológicas da OA estas podem ser de uso sistêmico, tópico ou intra–articular, e quanto a ação podem ser divididas em analgésicas e/ou anti–inflamatórias e drogas modificadoras dos sintomas e/ou da evolução da doença e, na maioria dos casos devem estar associadas com o tratamento não farmacológico (JOSÉ, 2013).

Com base na eficácia e custos, o paracetamol tem sido o analgésico geral de escolha para dor leve a moderada na OA, até uma dose máxima diária de 4 g, para o alívio da dor (GODWIN; DAWES, 2004; BELLAMY et al., 2006). Contudo, crescentes preocupações com a segurança do paracetamol estão surgindo, especialmente pelo risco de hepatotoxicidade com doses supraterapêuticas do paracetamol (CRAIG et al., 2012). Além disso, o uso continuado de paracetamol (> 22 dias/ mês) está associada a um aumento do risco de eventos cardiovasculares, semelhante ao uso crônico de anti–inflamatórios não esteroidais (AINEs) (CHAN et al., 2006).

Os AINEs são frequentemente considerados como o tratamento farmacológico de primeira linha, sendo utilizados preferencialmente para a osteoartrite (YU; HUNTER, 2015). Entretanto, os pacientes em uso de AINEs devem ser alertados sobre os efeitos adversos, que podem incluir sangramento gastrointestinal, disfunção renal e elevação da pressão arterial. O que torna relevante uma monitorização constante de parâmetros relacionados com estas funções. Como estratégia para redução da toxicidade, recomenda–se a adição de misoprostol,

de um inibidor da bomba de prótons ou a substituição por salicilatos ou inibidores seletivos da COX–2 (PAVELKA, 2000; SEED; DUNICAN; LYNCH, 2009; WENHAM; CONAGHAN, 2010, JOSÉ, 2013).

Os inibidores seletivos da COX–2 possuem propriedades analgésicas semelhantes aos AINEs não seletivos, mas têm um perfil de segurança melhorado para efeitos adversos gastrointestinais, contudo são caros e conferem aumento do risco cardiovascular (NIELSEN et al., 2006).

Podem ainda ser consideradas outras opções terapêuticas no tratamento da dor na OA, que incluem: AINEs de aplicação tópica, analgésicos opioides, glucosamina e sulfato de condroitina e injeções intra–articulares de glicocorticoides e ácido hialurônico (AH) (YU & HUNTER, 2015; FERREIRA et al., 2013; (BARRETT; SIVIERO, 2002; ALTMAN; SMITH, 2010).

Os produtos de aplicação tópica são uma alternativa para doentes com contra indicações para o uso de terapêutica sistêmica e um adjuvante dos tratamentos convencionais com resposta inadequada. Os benefícios dos AINES tópicos e a capsaicina são alcançados através do uso regular, com aplicação recomendada de 3 a 4 vezes / dia. Contudo, também existem efeitos adversos locais associados, incluindo erupção cutânea, ardor e prurido (BOLTEN, 2004; YU & HUNTER, 2015).

As injeções intra-articulares de glicocorticoides, como betametasona ou prednisolona são utilizadas em caso de inflamação local ou derrame articular. Seu uso intra-articulares fornece principalmente alívio de curto prazo com duração de quatro a oito semanas. E a aplicação intra-articular não deve ser feita mais de uma vez, a cada quatro meses, devido à possível progressão da OA. Os efeitos adversos destas injeções podem ser locais, com infeção da articulação afetada, osteonecrose, ruptura do tendão e atrofia no local de injeção, ou sistêmicos com quadros de hiperglicemia, edema, aumento da pressão arterial, dispepsia e risco de supressão adrenal (ARROLL; GOODYEAR-SMITH, 2004; FERREIRA, 2012).

O ácido hialurônico é uma componente normal da cartilagem, que confere propriedades viscoelásticas que permitem o movimento eficiente das articulações articulares. Na OA, ele encontra–se reduzido. Assim a administração intra-articular de AH tem sido estudada, de modo a avaliar o seu papel na reconstituição do líquido sinovial e na redução dos sintomas. Os estudos mostraram que o AH produz melhoras ao fim de três a quatro semanas e o efeito mantém–se durante meses. Contudo, podem ocorrer efeitos adversos, como edema

agudo da articulação, reações cutâneas locais, além de ser uma terapia relativamente cara, sendo assim pouco acessível (ALTMAN; MOSKOWITZ, 1998; BARRETT, 2002).

A glucosamina e condroitina são os suplementos mais utilizados para a osteoartrite e estão envolvidas na formação da cartilagem articular, por isso, são classificadas como drogas modificadoras dos sintomas e/ou da evolução da doença (BRIEF; MAURER; DI CESARE, 2001). Os condroprotetores têm composição semelhante aos componentes da matriz cartilaginosa. Acredita-se que tenham ação anti-inflamatória e possam reduzir a perda/ e ao mesmo tempo estimular a síntese de proteoglicanos e de colágeno, através da inibição de enzimas degradadoras de cartilagem (ELEOTÉRIO et al., 2015). Os efeitos adversos desses suplementos são mínimos, incluem inchaço, flatulência e cólicas. Contudo ainda existem controvérsias quanto aos seus benefícios (JOSÉ, 2013; FERREIRA, 2012).

O colágeno tipo II ou colágeno hidrolisado, também tem sido uma opção investigada para o tratamento de doenças articulares. Ruiz- Benito (2009) estudou o efeito do colágeno em 250 pessoas com OA no joelho, fornecendo 10g de colágeno hidrolisado, diariamente por 6 meses e, observou que o uso do colágeno promoveu uma redução da dor, apesar de produzir alguns efeitos colaterais, como dores de cabeça, enxaquecas e distúrbios gastrointestinais. Mclindon e colaboradores (2011) avaliaram também o potencial do colágeno tipo II no tratamento de OA, em um estudo clínico com 29 pacientes com OA no joelho. Os participantes receberam doses diárias de 10g de colágeno hidrolisado ou um placebo (sem colágeno), por 24 semanas. Contudo, não foram observadas diferenças entre os pacientes que receberam e não receberam o colágeno, nos aspectos avaliados: dor, rigidez, funcionalidade e locomoção. Entretanto, Prata e colaboradores (2018) observaram que o fornecimento diário de 10 mg do colágeno tipo II para cães produziu melhora significativa nos sinais clínicos da OA. Contudo, 30 dias após o tratamento os sinais reapareceram, indicando a necessidade de um tratamento contínuo. Dessa forma, o papel do colágeno no tratamento de OA, ainda apresenta resultados conflitantes e requer mais estudo.

Quanto aos analgésicos opioides, devido ao potencial abuso de seu uso, eles só devem ser uma opção em caso de insucesso de outras opções farmacológicas, como paracetamol, AINEs, injeções intra–articulares ou terapia tópicas, ou em casos de contra–indicação, como por insuficiência renal ou problemas gastrointestinais (GI), ou ainda de alto risco cirúrgico para a realização de artroplastia (ALTMAN & SMITH, 2010; LANG et al., 2010). Devem ser prescritos primeiro em dosagens baixas e cuidadosamente monitorados para avaliar a dependência potencial. Além de também poderem causar constipação crônica e

poder colocar pacientes idosos em risco de quedas (MANEK; LANE, 2000; HUNTER; LO, 2008).

Além das terapias já citadas, destacamos também o crescente uso de agente biológicos no tratamento da OA, entre os quais podemos citar os anticorpos monoclonais, que tem capacidade de agir bloqueando as moléculas que dão origem à inflamação, como o Tanezumab que agem contra o fator de crescimento do nervo (NAGASHIMA et al., 2011) e o Adalimumab, que é um anticorpo monoclonal que bloqueia o fator de necrose tumoral alfa (TNF-α) (VERBRUGGEN et al., 2012).

Outra forma de tratamento que tem alcançado um amplo espaço e atenção como terapia alternativa é o uso de fitoterápicos que ajudam no tratamento dos sintomas da OA. Neste contexto, vários estudos têm sido realizados (GARBACKI et al., 2002; SCHULZE-TANZIL; HANSEN; SHAKIBAEI, 2004; SENGUPTA et al., 2008; HASEEB; ANSARI; HAQQI, 2017; MULEK et al., 2017), contudo ainda muito aquém do potencial da flora mundial (BRASIL 2006; BATISTA, 2009). Christiansen e colaboradoes (2015) descrevem os efeitos de misturas insaponificáveis de abacate (*Persea gratissima*) e soja (*Glycine max*), tendo como principais componentes os fitoesteróis: β-sitosterol, campesterol e estigmasterol e destacam a ação anabólica e anticatabólica da mistura de insaponificáveis na cartilagem, sendo esta capaz de estimular a síntese de componentes da matriz extracelular (colágeno e agrecan) e inibir a produção de moléculas pró–inflamatórias (TNF–α, IL–1β, IL–6, IL–8, MIP–1β, NO e PGE2), provavelmente por interferência na sinalização do fator de transcrição NF–kB.

Investigações com preparações de raiz de *Harpagophyti radix* também evidenciaram atividade anti-inflamatória *in vitro* pela diminuição da produção de citocinas pró–inflamatórias (TNF–α, IL–1β e IL–6) e PGE2 em monócitos humanos estimulados por lipopolissacarídeos (LPS) (FIEBICH et al., 2012) e pela redução dos níveis de metaloproteinases de matriz (MMP–1, MMP–3, MMP–9) em condrócitos humanos estimulados por IL–1β (SCHULZE–TANZIL et al, 2004). O extrato aquoso de *Harpagophyti procumbens* diminuiu a expressão de COX–2 e iNOS nos fibroblastos de camundongos e, como resultado, diminuiu a geração de PGE2 e NO (JANG, 2003).

Um estudo de revisão realizado por Laslett & Jones (2014) avaliou sessenta e três artigos, que investigaram terapias derivadas de plantas no tratamento da dor, sendo em sua maioria eficazes no tratamento de dores osteoartríticas e limitação funcional quando comparadas ao placebo, e igualmente eficazes quando comparadas com fármacos padrões. Além de também apresentaram perfil de segurança semelhante ao placebo e melhor do que os

fármacos padrões. No entanto, os autores afirmam que faltam estudos de qualidade e dados de longo prazo, e o número de testes para cada terapia é reduzido, e os ensaios necessitam ser mais bem padronizados.

Li e colaboradores (2017) também realizaram uma revisão sobre as plantas úteis para o tratamento da OA, focando nas plantas da medicina tradicional chinesa. Nesta revisão foram discutidos os mecanismos de ação dos compostos presentes nas plantas, capazes de interferir na progressão da OA. Em termos de atividade anti-inflamatória, destacaram as moléculas: aucubina, piperina; Bavachin – fitoestrógeno, astragalin (isolado de *Rosa agrestis*), delfinidina – antocianidina, tetrandrina – alcalóide, resveratrol – fitoalexina, epigalocatequina-3-galato – catequina e berberina – alcaloide presentes em diversas plantas, sendo estas moléculas capazes de diminuir a produção de citocinas inflamatórias, como IL-6, IL-1 β e TNF α, além de inibir a expressão de COX-2 e iNOS, gerando assim uma ação condroprotetora, pela proteção da cartilagem de danos mediados pela inflamação.

Na atividade antiapoptóticas e antioxidante, Li e colaboradores (2017) também destacaram a berberina e o resveratrol, além das substâncias: ginsenosídeo, baicaleína , tetrametilpirazina, 5,7,3 ′, 4′-tetrametoxifavona, como sendo capazes de diminuir a produção de NO (óxido nítrico) e inibir a apoptose de condrócitos. Na atividade anticatabólica, destacaram: biochanina A, crocina, Sinomenine – alcalóide, monotropeina, gentiopicrosídeo, morin - 3,5,7,2 ′, 4′-penta-hidroxifavona, ácido ferúlico, além gerberina e baicaleína, e das fórmulas Sanmiao e Juanbi, como capazes de inibir o aumento da expressão de metaloproteinases da matriz, que desempenham um papel central na destruição catabólica da cartilagem na OA.

Na atividade proliferativa, Li e colaboradores (2017) citam alguns polissacarídeos, o ácido protocatecuico e o psoralen, como importantes para induzir a proliferação de condrócitos e auxiliar no tratamento da OA. Os autores concluem que os dados coletados indicam um efeito promissor dos vegetais na OA, embora os ensaios clínicos e pré-clínicos destas plantas estejam na etapa inicial, e os mecanismos de ação na OA ainda não estejam totalmente formulados ou sob investigação. Soma-se a isso o grande número de componentes presentes nas plantas que dificultam a especificação do alvo.

O estudo de revisão de Maksimović & Samardžić (2018) afirmam que a maioria dos dados atualmente disponíveis sobre as terapias a base de plantas para o tratamento da OA apresentam limitações, com frequentes falhas no desenho da pesquisa, critérios de seleção pouco claros e incompletos, e definição inadequada das intervenções fitoterápicas, sendo

assim insuficientes para reconhecer seu uso no tratamento da OA, com plenas garantias de eficácia e segurança. Neste contexto, investigações mais robustas e melhor padronizadas de produtos naturais são essenciais para o desenvolvimento de novos medicamentos para o tratamento de OA.

3. OBJETIVOS

3.1 GERAL

Avaliar a resposta anti-inflamatória e anti-nociceptiva do extrato hidroetanólico (EHA) e frações (hexânica, acetato de etila e butanólica) de *Arrabidaea chica* (pariri) em modelo experimental de osteoartrite (OA) e elucidar a composição química desse extrato e frações.

3.2 ESPECÍFICOS

- ✓ Verificar o efeito *in vitro* do extrato do extrato hidroetanólico e frações (hexânica, acetato de etila e butanólica) de *A. chica* sobre a inibição da ciclo-oxigenase 1e 2;
- ✓ Avaliar os efeitos do extrato hidroetanólico e frações (hexânica, acetato de etila e butanólica) de *A. chica* sobre o grau de incapacitação articular, através de parâmetros clínicos;
- ✓ Analisar os efeitos do extrato hidroetanólico e frações (hexânica, acetato de etila e butanólica) de *A. chica* sobre a lesão articular, através de métodos radiográficos e ensaios histopatológicos;
- ✓ Identificar a composição química do extrato hidroetanólico e frações (hexânica, acetato de etila e butanólica) de *A. chica.*
- ✓ Identificar através de ensaios *in silico* as substâncias químicas de *A. chica* que podem estar associados à ação antinociceptiva e anti-inflamatória.

4. RESULTADOS

4.1 CAPÍTULO I:

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Article

Effects of Extract of *Arrabidaea chica* Verlot on an Experimental Model of Osteoarthritis

Cleydlenne Costa Vasconcelos [1,*], Alberto Jorge Oliveira Lopes [1], Emerson Lucas Frazão Sousa [1], Darleno Sousa Camelo [1], Fernando César Vilhena Moreira Lima [1], Cláudia Quintino da Rocha [2], Gyl Eanes Barros Silva [1], João Batista Santos Garcia [1] and Maria do Socorro de Sousa Cartágenes [1,*]

[1] Biological and Health Sciences Center, Federal University of Maranhão, Av. dos Portugueses 1966, São Luís, MA 65085-580, Brazil; lopesajo@gmail.com (A.J.O.L.); e.lucasfrazao@gmail.com (E.L.F.S.); leno.camelo@gmail.com (D.S.C.); fernandovilhena15@gmail.com (F.C.V.M.L.); gyleanes@fmrp.usp.br (G.E.B.S.); jbgarcia@uol.com.br (J.B.S.G.)

[2] Department of Chemistry, Federal University of Maranhão, São Luís, MA 65085-580, Brazil; claudiarocha3@yahoo.com.br

* Correspondence: cleydlenne@yahoo.com.br (C.C.V.); socartagenes@ufma.br (M.d.S.d.S.C.)

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Abstract: The aim of this study was to analyze the analgesic potential of *Arrabidaea chica* extract (EHA) as an alternative to osteoarthritis (OA) treatment. Thus, the extract was initially evaluated by the cyclooxygenase inhibition test. The analgesic effect of the extract, in vivo, was also verified in a model of OA induced by sodium monoiodoacetate (2 mg). EHA was administered to rats at doses of 50, 150, and 450 mg/kg between 3 and 25 days after OA induction. The animals were clinically evaluated every 7 days, euthanized at 29 days, and the liver, spleen, kidney and knee collected for histopathological analysis. The chemical composition of EHA was identified by HPLC-MS and the identified compounds submitted to molecular docking study. The results showed that the extract promoted cyclooxygenase inhibition and produced significant improvements in disability, motor activity, hyperalgesia, and OA-induced allodynia parameters, in addition to improvements in the radiological condition of the knees (but not observed in the histopathological study). Chemically the extract is rich in flavonoids. Among them, we evidence that amentoflavone showed very favorable interactions with the enzyme COX-2 in the in silico analysis. Thus, it is concluded that *A. chica* has important analgesic properties for the treatment of OA.

Keywords: pain; inflammation; phytotherapy; antinociception; pariri; bioinformatics; molecular docking; plants

1. Introduction

The plants have contributed significantly to the development of new therapeutic strategies for the treatment of several diseases, mainly through the secondary metabolites, that can interfere in the performance of mediators of the inflammatory process, second messenger production, and expression of transcription factors [1–3]. The *Arrabidaea chica* (Humb. And Bonpl.) B.Verlot (Bignoniaceae) plant species used in this study has been distinguished by its pharmacological potential, associated mainly to its antioxidant, astringent, antimicrobial, antitumor, and anti-inflammatory activities [4,5].

Osteoarthritis (OA) is the disease in which the inflammatory process plays a critical role in its pathogenesis [6]. Thus, *A. chica* could represent a potential alternative to treatment. OA is characterized by progressive destruction of articular cartilage, synovial inflammation, changes in the subchondral bone and peri-articular muscle, pain and has a generally slow progression [7,8]. As for the inflammatory

process, increased levels of pro-inflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor alpha (TNF-α) decrease collagen synthesis and increase catabolic mediators such as metalloproteinases and other substances inflammatory [8,9]. As for existing pharmacological interventions for OA pain, these are often insufficient or poorly tolerated, and OA pain generally remains refractory to available treatments [10,11]. In addition to adverse drug effects resulting from long-term use, non-steroidal anti-inflammatory drugs (NSAIDs) provide temporary relief but can cause gastrointestinal problems, renal dysfunction and elevated blood pressure, among others [12,13].

OA is also an important public health issue related to aging, which affects about 40% of adults over 70 years of age and generates great socioeconomic impact since it can lead to functional disability of the individuals, affecting the quality of life in addition to producing high costs for health services [14–16]. Thus, the present study aimed to investigate the potential of *A. chica* extract as an alternative to the treatment of OA.

2. Results

2.1. Inhibition of Cyclooxygenase 1 and 2

In vitro inhibition assays of cyclooxygenase-1 and 2 (COX-1 and COX-2) were performed in order to investigate whether the hydroethanolic extract of *A. chica* has inhibitory effects on these enzymes. The results of the assay showed that the extract has an inhibitory effect, inhibiting up to 30% of COX-1 and COX-2 in the highest concentration tested, 50 μg/mL. However, concentrations of 2 and 10 μg/mL failed to produce any inhibitory effect on the enzymes, or the effect was very low. When comparing the effects of the extract on COX-1 and COX-2, it was possible to verify that the extract does not appear to be selective for COX-1 or COX-2, since at 50 μg/mL the extract inhibited around 20 to 30% in both the isoforms (Figure 1A,B).

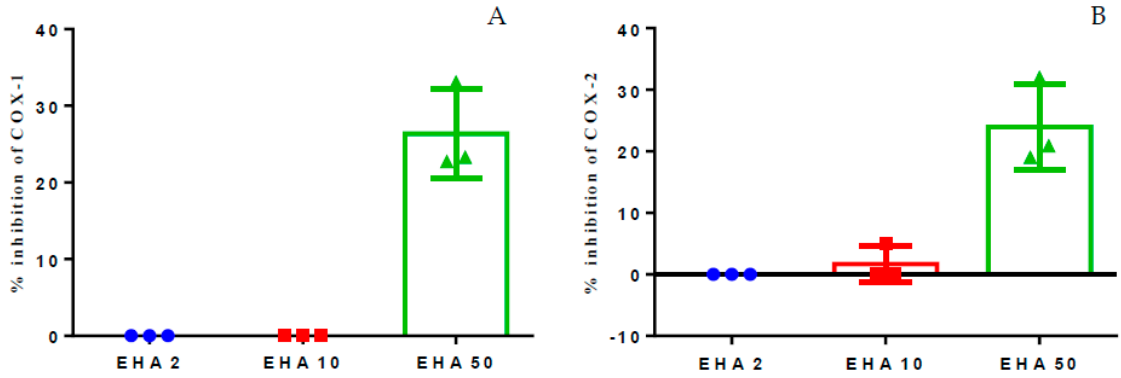

Figure 1. Percent inhibition of in vitro COX-1 (**A**) and COX-2 (**B**), induced by the hydroethanolic extract of *A. chica*, tested in three concentrations: 2, 10, and 50 μg/mL.

2.2. Clinical Evaluations

2.2.1. Evaluation of Motor Activity/Forced Deambulation (Rotarod Test)

The evaluation of the motor activity initially showed that the induction of OA was effective, since the first evaluation after induction (D7) showed a significant difference between the induced groups (EHA50, EHA150, EHA450, CTL + Melox, CTL−, and healthy control), marked by decreased motor activity of animals with induced OA. This decrease was also observed on day 14, demonstrating that, until this period, the treatments did not produce any significant improvement in motor activity (Figure 2). On day 21 after the induction of OA, all treated groups, namely, EHA50 ($p = 0.026$), EHA150 ($p < 0.0001$) EHA450 ($p = 0.0004$), and CTL + Melox ($p = 0.026$) showed a significant difference in relation to the saline group, demonstrating that the extract at this concentration is able to promote

improvement in motor activity. On day 28, these significant differences in relation to the saline group were also observed in relation to the extract EHA450 ($p = 0.028$) and EHA150 ($p = 0.028$) (Figure 2).

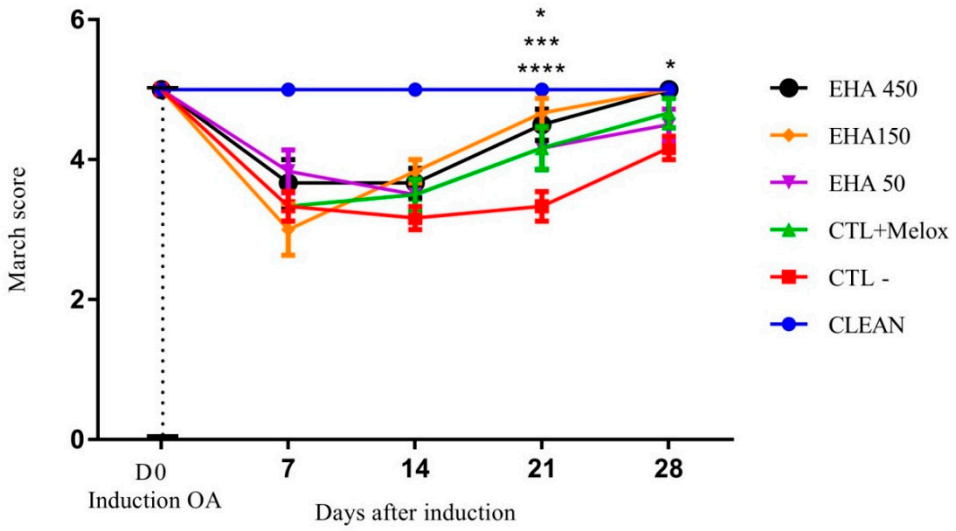

Figure 2. Effects of the *A. chica* extract on the motor activity/march score of rats with induced osteoarthritis (OA). The induction of OA was done by the injection of MIA (2 mg) in the knee of Wistar rats. After 3 days, the animals received oral saline (CTL−), Meloxicam (CTL + Melox), and *A. chica* extract at doses 50, 150, and 450 mg/kg (EHA50, EHA150, and EHA450). After 7, 14, 21, and 28 days of induction, the motor activity of the animals was measured to evaluate the rotarod score. The data are represented in mean ± standard deviation of the means. The healthy group is represented by animals without osteoarthritis and without treatment (CLEAN). * represents significant differences, with $p < 0.05$; *** with $p < 0.0005$; **** with $p < 0.0001$ comparing the treatments to the saline group.

2.2.2. Incapacitation/Weight Distribution Test on Hind Legs (Weight Bearing)

The analysis of the data related to joint incapacitation, evaluated using the percentage of weight distribution in the legs, showed that the induction of OA was effective, since on the seventh day after induction we observed a significant difference between the induced OA group and the control group (CLEAN). On days 14, 21, and 28 post-induction, all treated groups (EHA50, EHA150, EHA450, CTL + Melox) showed significant improvement in disability, and differed significantly from the saline group (CTL−) ($p < 0.0001$) (Figure 3).

2.2.3. Mechanical Hyperalgesia (Randall Selitto Test)

The animals, when evaluated, for mechanical hyperalgesia showed a decrease in the nociceptive paw withdrawal threshold on day 7 after MIA injection. This decrease occurred in all induced groups (EHA50, EHA150, EHA450, CTL + Melox, and CTL−), as expected, confirming the success of OA induction. On day 14 of the experiment, all treated groups EHA450, EHA150, EHA50 ($p < 0.0001$), and CTL + Melox ($p = 0.003$) showed nociceptive threshold increase, with significant improvement in mechanical hyperalgesia when compared to the saline group; the EHA450 extract being statistically better ($p = 0.02$) until drug meloxicam. Also, observations on day 21 and 28 of the analysis, showed that the EHA450, EHA150, EHA50 and CTL + Melox ($p < 0.0001$) treated groups continued to differ significantly from the saline group (Figure 4). Thus, the extract was able to induce significant improvements in hyperalgesia by induced OA from day 14 of the analysis, demonstrating its nociceptive potential.

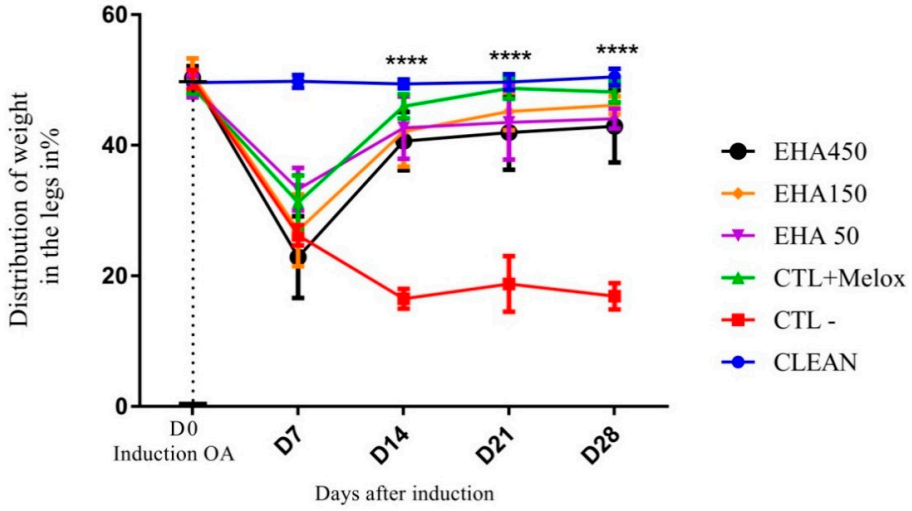

Figure 3. Effects of the extract *A. chica* on the degree of incapacitation of rats with induced osteoarthritis. The induction of osteoarthritis was done by the injection of MIA (2 mg) in the knee of Wistar rats. After 3 days, the animals received oral saline (CTL−), Meloxicam (CTL + Melox) and extract of *A. chica* at doses 50, 150, and 450 mg/kg (EHA50, EHA150, and EHA450). After 7, 14, 21, and 28 days of induction, the weight distribution on the hind legs of the animals was measured for the assessment of the incapacity by means of the Weight Bearing test. The data are represented in mean ± standard deviation of the means. The healthy group is represented by animals without osteoarthritis and no treatment (CLEAN). **** represents significant differences, **** with $p < 0.0001$ comparing the treatments to the saline group.

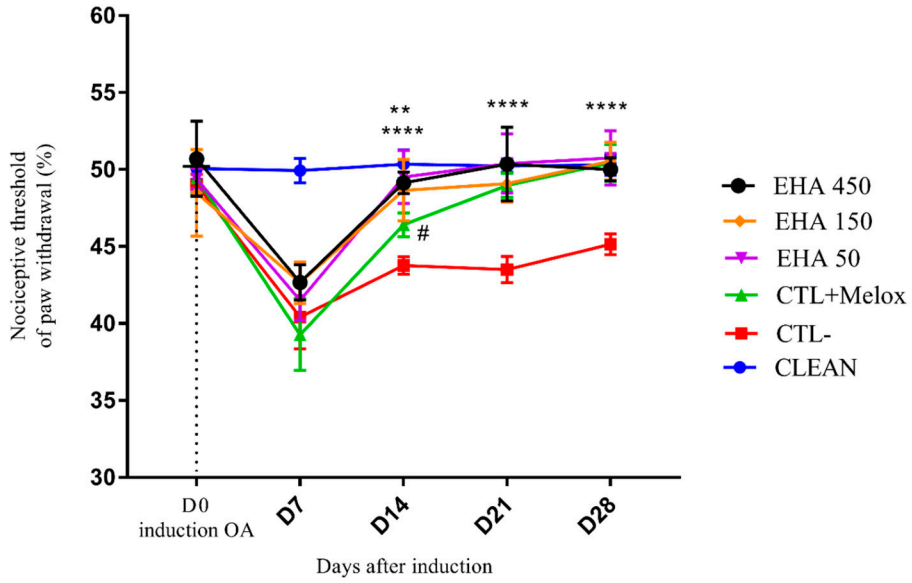

Figure 4. Effects of *A. chica* extract on mechanical hyperalgesia in rats with induced osteoarthritis (OA). The induction of OA was done by the injection of MIA (2 mg) in the knee of Wistar rats. After 3 days, the animals received oral saline (CTL−), Meloxicam (CTL + Melox) and extract of *A. chica* at doses 50, 150, and 450 mg/kg (EHA50, EHA150, and EHA450). After 7, 14, 21, and 28 days of OA induction, the nociceptive threshold of withdrawal of the affected paw was measured by indirect evaluation with Randall Sellito apparatus. The data are represented in mean ± standard deviation of the means. The healthy group is represented by animals without osteoarthritis and without treatment (CLEAN). ** represents significant differences, with $p < 0.005$; **** with $p < 0.0001$ comparing the treatments to the saline group. # represents significant differences, with $p < 0.05$ comparing the EHA450 with CTL + Melox.

2.2.4. Mechanical Allodynia (von Frey Test)

The results of mechanical allodynia evaluation first demonstrated the efficacy of fear of induction by MIA, since the animals immediately after induction (D7) had a significant reduction of the response time to the stimulus compared to the group that was not induced (CLEAN). On day 21 after induction the group treated with extract at the concentration of 450 mg/kg (EHA450) showed a significant difference ($p = 0.0015$) in relation to the saline group, demonstrating that the extract at this concentration is able to promote improvement in allodynia mechanics. On day 28 this result was also observed, but in addition to the EHA450 extracts, the extracts of lower concentration (EHA50) and (EHA150) also produced significant improvement ($p = 0.0108$) in allodynia compared to the saline group (Figure 5).

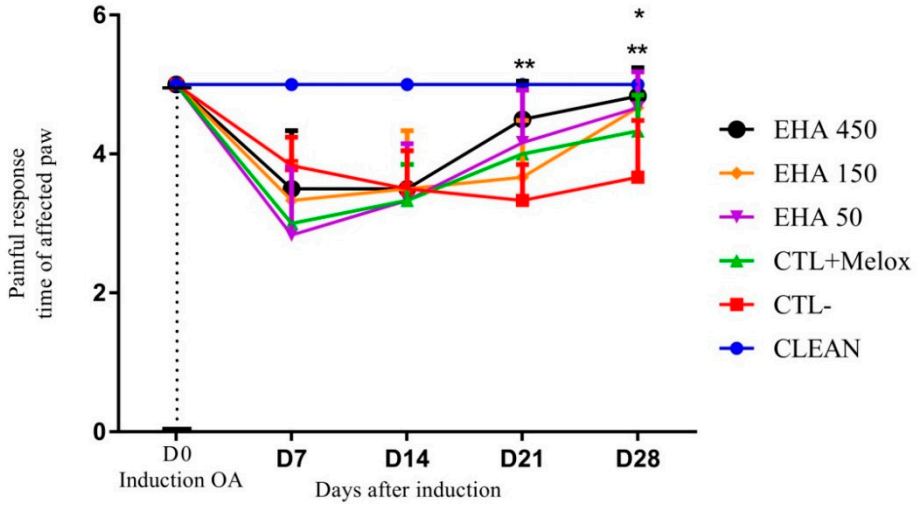

Figure 5. Effects of *A. chica* extract on mechanical allodynia in rats with induced osteoarthritis (OA). The induction of OA was made by the injection of MIA (2 mg) in the right knee of Wistar rats. After 3 days, the animals received oral saline (CTL−), Meloxicam (CTL + Melox) and *A. chica* extract at doses 50, 150, and 450 mg/kg (EHA50, EHA150, and EHA450). After 7, 14, 21, and 28 days of OA induction, mechanical allodynia was measured by indirect evaluation with the von Frey test. The data are represented in mean ± standard deviation of the means. The healthy group is represented by animals without osteoarthritis and without treatment (CLEAN). * represents significant differences, with $p < 0.05$; ** with $p < 0.005$ comparing the treatments to the saline group.

2.3. Radiographic Analysis

In the radiographic analyzes, it was observed that in the group without osteoarthritis (CLEAN), the scores were minimal (grade 0; Figure 6), with preservation of the joint space, without subchondral sclerosis and no formation of osteophytes (Figure 7A) which would be the main features of OA. In the group that had OA induction by MIA and received no treatment (CTL−), a high score was observed, reaching grade 4 (Figure 6), with common radiographic characteristics of the disease, such as reduction of articular space, marked subchondral bone sclerosis, and intense formation of osteophytes as seen in Figure 7(B1,B2). In the groups treated with extract (EHA50, EHA150, and EHA450 (Figure 7C) and Meloxicam, these common radiographic features of OA were much less evident and the score did not exceed grade 2 according to the classification of Kellgren–Lawrence (Figure 6). Thus, the extract in the three concentrations tested was able to significantly reduce ($p < 0.05$) the degree of joint involvement in relation to the saline group (CTL−) (Figure 6).

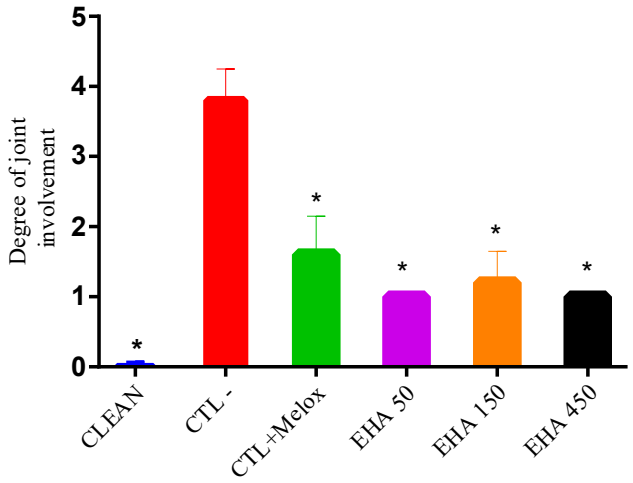

Figure 6. Degree of joint involvement in rats treated with *A. chica* extract (EHA50, 150, and 450 mg/kg, orally), Meloxicam (CTL + Melox) and saline (CTL–) administered from day 3 after OA induction. This analysis was performed with the knees collected on day 29 after OA induction. The data are represented according to classification proposed by Kellgren–Lawrence. * represents significant differences, with $p < 0.05$, between the groups treated with *A. chica* extract and the saline group (CTL–).

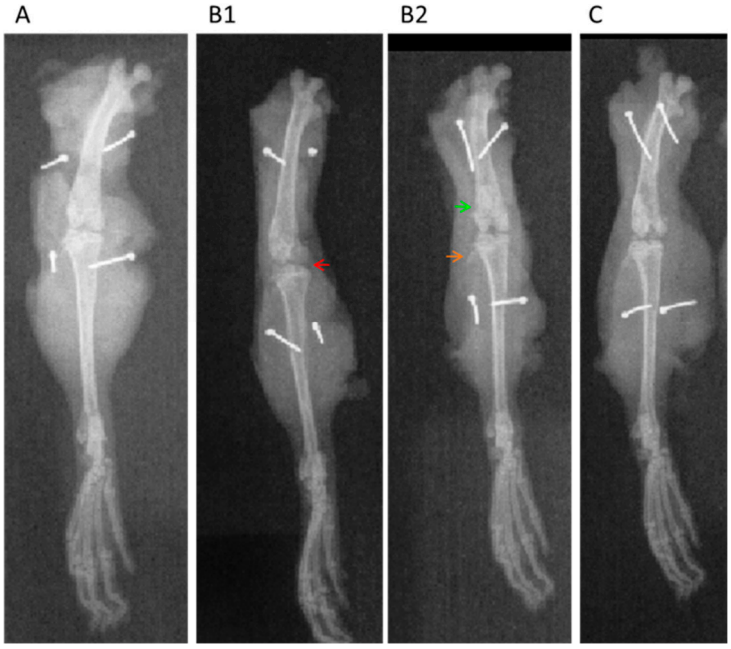

Figure 7. Knee radiographs in the antero-posterior projection. **A**-RX from the healthy rat paw (CLEAN); **B1** and **B2**-RX from the rat paw with experimental model of MIA-induced osteoarthritis–saline group; **C**-RX, *A. chica* extract 450 mg/kg treated group. This analysis was performed with the knees collected on day 29 after OA induction. Arrowhead: red—indicating a remarkable narrowing of the joint space and deformity in the bone contour; green—subchondral sclerosis; orange—large osteophytes.

2.4. Histopathological Analysis

Histopathological evaluation using the OARSI scoring system (see Materials and Methods item 4.5) revealed that the treatment with *A. chica* extract at the highest dose tested (EHA450) was not able to inhibit the OA-induced joint compromise. The OARSI classification system group EHA450 obtained an average grade rating of 5.9 (±0.22), similar to the saline group (CTL−) of 5.4 (±0.47) and the meloxicam-treated group (CTL + Melox) of 5.9 (±0.25) (Figure 8). These grades above 5, according to the OARSI classification, indicate greater articular cartilage injury in these animals, recognized by

denudation, complete erosion of the hyaline cartilage to the level of mineralized cartilage and/or bone, microfractures, as well as fibrocartilaginous bone surface repair (Figure 9).

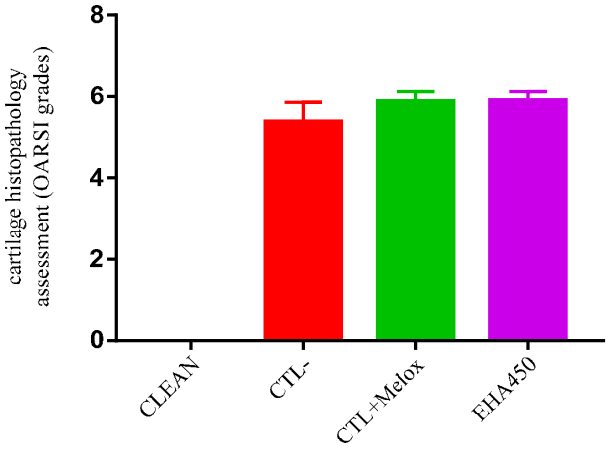

Figure 8. Histopathological evaluation of cartilage classified using the Osteoarthritis Research Society International (OARSI) scoring system. This analysis was performed with the knees collected on day 29 after OA induction. Groups: EHA450 = with sodium monoiodoacetate-induced osteoarthritis (MIA-induced OA) 2 mg and treated with *A. chica* hydroalcoholic extract 450 mg/kg; CTL− = with MIA-induced OA (2 mg) and treated with 0.9% sodium chloride saline; CTL + Melox = with MIA-induced OA (2 mg) and treated with meloxicam 0.5 mg/kg; CLEAN = untreated and uninduced OA, healthy. *Y*-axis: Histopathological evaluation of cartilage (OARSI histological classification: Grade 0—surface intact and cartilage intact; Grade 1—surface intact; Grade 2—surface discontinuity; Grade 3—vertical fissures; Grade 4—erosion; Grade 5—denudation; and Grade 6—deformation).

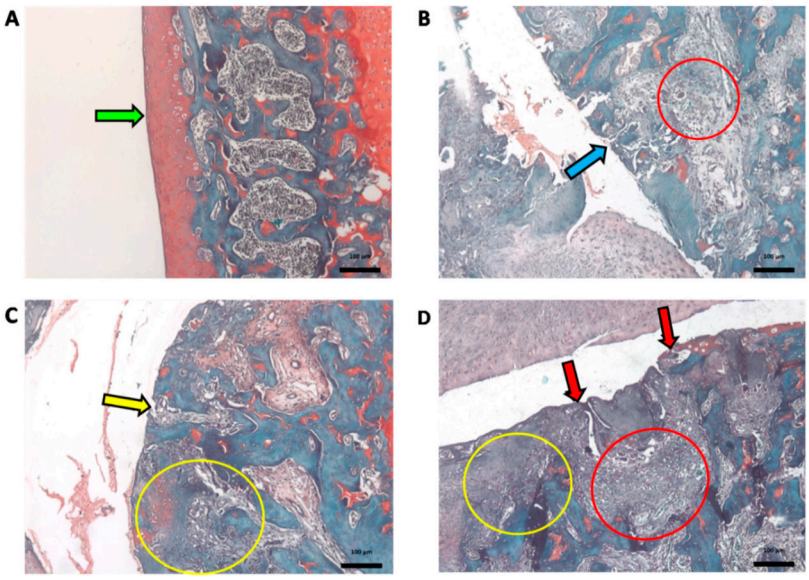

Figure 9. Sections of articular cartilage with different grades of degenerative changes in osteoarthritis (OA) classified using the Osteoarthritis Research Society International (OARSI) scoring system.This analysis was performed with the knees collected on day 29 after OA induction. (**A**) Example of CLEAN group, untreated and uninduced OA, healthy (Grade 0) normal cartilage. (**B**) Example of CTL-group treated with 0.9% saline. (**C**) Example of EHA450 group treated with *A. chica* hydroalcoholic extract 450 mg/kg. (**D**) Example of CTL + Melox group. B, C, and D with degrees on average between 5.4 and 5.9. Green arrow indicates normal cartilage lining (intact surface and cartilage); blue arrow indicates microfractures; red arrow indicates erosion with bone expositon; red circles indicate repair fabric; yellow arrowindicates complete erosion; yellow circles indicate fibrocartilage formation and bone remodeling. (O-safranin stain; Bar = 100 μm; 200×).

Histopathological analyses of the liver, spleen and kidney showed no pathological changes, even at the highest administered dose of *A. chica* extract (450 mg/kg) (Figure 10), thus demonstrating that the extract did not induce any toxicity.

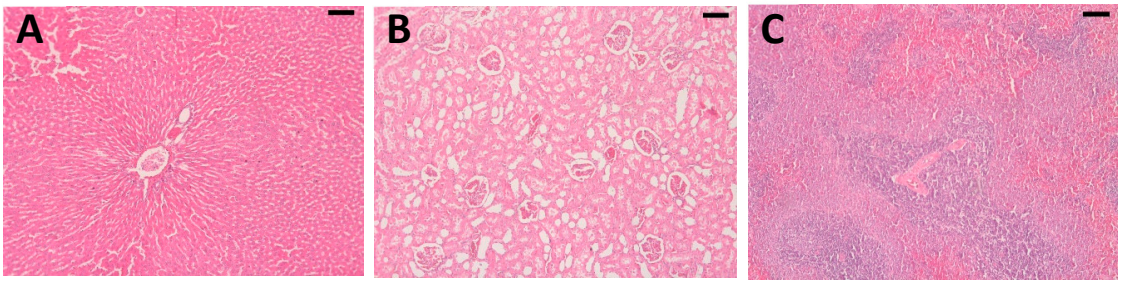

Figure 10. Exemplary histological sections of the liver (**A**), kidneys (**B**), and spleen (**C**) of EHA450 group. The images show no signs of ischemia, necrosis, cell injury and/or inflammation. (Hematoxylin-Eosin—HE stain, Bar = 100 µm, 100×).

2.5. Chemical Analysis

The HPLC-PDA-ESI-IT-MS/MS analysis, in the positive mode, showed the presence of 25 compounds in the hydroethanolic extract of *A. chica* leaves (Figure 11); among them, 22 were identified by dereplication, comparing the masses, retention times, and fragments obtained in mass spectrometry, with the literature (Table 1).

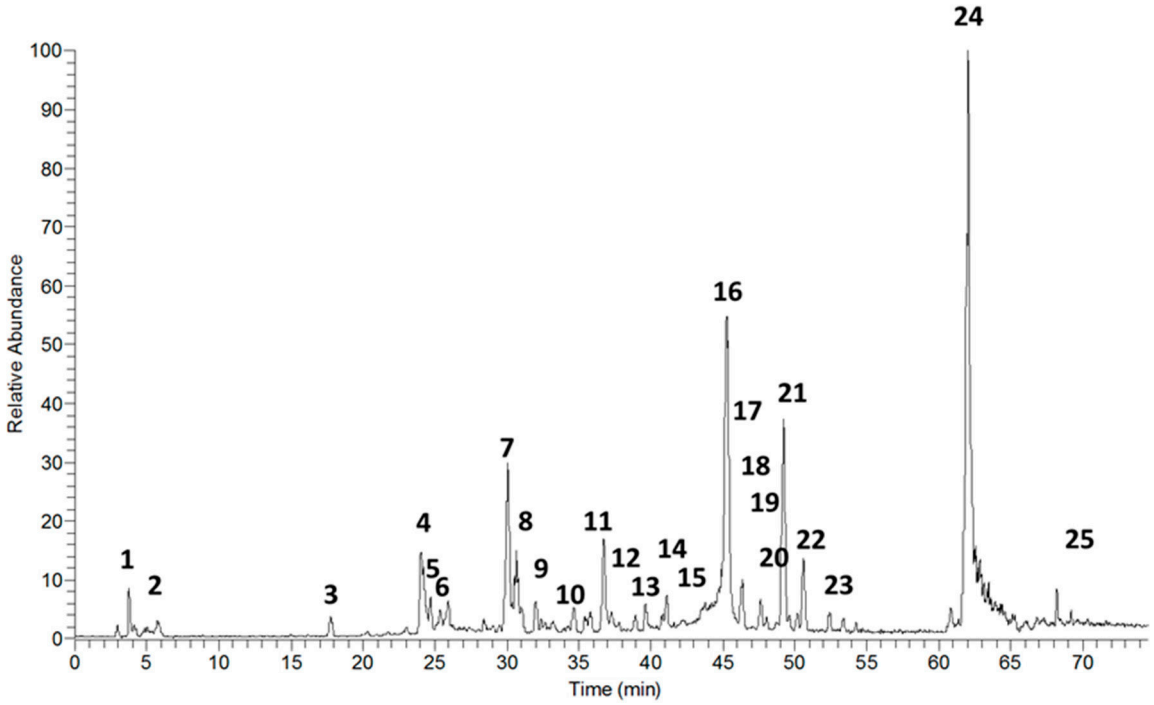

Figure 11. Chromatogram of total ions obtained by HPLC-PDA-ESI-IT-MS/MS, positive mode, from the hydroethanolic extract of *A. chica* leaves.

Table 1. HPLC-PDA-ESI-IT-MS/MS data (detected UV-vis and detected ions) and FIA-ESI-IT-MS/MS^n (product ions) of compounds detected in the *A. chica* leaves hydroethanolic extract.

N	RT	[M+H]	MS^n Fragments	Name Suggestion for Structure	Reference
1	4.48	299	289, 287, 160	carajurin	[17–19]
2	5.38	315	313, 289	6,7,3′-trihydroxy-5,4′-dimethoxy-flavylium	[18]
3	17.61	289	205, 188	2′-hydroxy-a-naphthoflavone	[20] ID BML00331
4	24.13	289		catechin	[21]
5	24.69	301	289	5,7-dimethoxy-4′-hydroxyflavone	
6	25.42	289		epicatechin	[21]
7	29.98	477	301, 289	quercetin-o-gallate	
8	31.12	465	301	Quercetin-o-glucoside	[22]
9	32.04	540	460, 301, 289	amentoflavone	[23]
10	34.72	479	301, 279	isorhamnetin-3-o-glucoside	[24]
11	36.62	301	279	chrysoeriol	[25]
12	37.64	287	279	kaempferol	[26]
13	39.65	463	330, 301	chrysoeriol-o-glucoside	[27]
14	41.24	317	301, 279	isorhamnetin	[24]
15	42.99	287	279	luteolin	[28,29]
16	45.25	303		6-hydroxyluteolin	[29]
17	46.18	477	328, 301, 279	cirsimarin	[30,31]
18	47.42	617	601, 301, 279	hyperin 6″ gallate	
19	47.64	301	279	6,7,3′,4′-tetrahydroxy-5-metoxy-flavilium	[32]
20	47.94	302	301, 288, 285	hispidulin	[29]
21	49.23			n.i.	
22	50.31–50.96	601		n.i.	
23	52.72–54.29	577	301, 289	catechin dimer	[29]
24	62.14	819		n.i.	
25	72.66	593	421, 399	feoforbide A	[33]

n.i.—not identified.

2.6. In silico Analysis

To our molecular docking analysis, we used all metabolites identified by HPLC-MS on *A. chica* extract. On general, all metabolites showed satisfactory parameters affinity with the COX-2 structure. In addition to these molecules, molecular docking of the commercial non-steroidal anti-inflammatory drug meloxicam was performed, with affinity parameters of −8.82 kcal·mol^{-1} and 0.34 μM from free binding energy and the inhibition constant, respectively, while among the *A. chica* metabolites, the best affinity parameters were shown to be amentoflavone and quercetin-o-gallate with −9.21 and −8.86 kcal·mol^{-1} from free binding energy, respectively, and 0.11 and 0.32 μM from the inhibition constant, respectively; these parameters being superior to those presented by meloxicam. The results of the binding energy values of all compounds are shown in Table 2.

Table 2. Free-binding energies and inhibition constant obtained by molecular docking of the compounds identified in the *A. chica* extract with the COX-2 structure.

Ligand	ΔGbind (kcal·mol^{-1}) *	Ki (μM) **
amentoflavone	−9.21	0.11
quercetin-o-gallate	−8.86	0.32
chrysoeriol-o-glucoside	−8.45	0.63
catechin dimer	−8.33	0.78
2′-hydroxy-a-naphthoflavone	−7.98	1.43
6-hydroxyluteolin	−7.95	1.48
hispidulin	−7.81	1.88
cirsimarin	−7.61	2.64
isorhamnetin-3-o-glucoside	−7.56	2.87
epicatechin	−7.49	3.25
catechin	−7.46	3.40
hyperin 6″ gallate	−7.39	3.81
6,7,3′,4′-tetrahydroxy-5-metoxy-flavylium	−7.21	5.19
6,7,3′-trihydroxy-5,4′-dimethoxy-flavylium	−7.11	6.11
carajurin	−7.06	6.71
kaempferol	−7.06	6.73
luteolin	−7.05	6.76
chrysoeriol	−6.90	8.72
quercetin-o-glucoside	−6.85	9.46
isorhamnetin	−6.72	11.86
meloxicam	−8.82	0.34

* ΔGbind: binding energy; ** Ki: inhibition constant.

The map from interactions of the amentoflavone and quercetin-o-gallate with COX-2 amino acids identified in selected configurations obtained through molecular docking calculations are displayed in Figure 12.

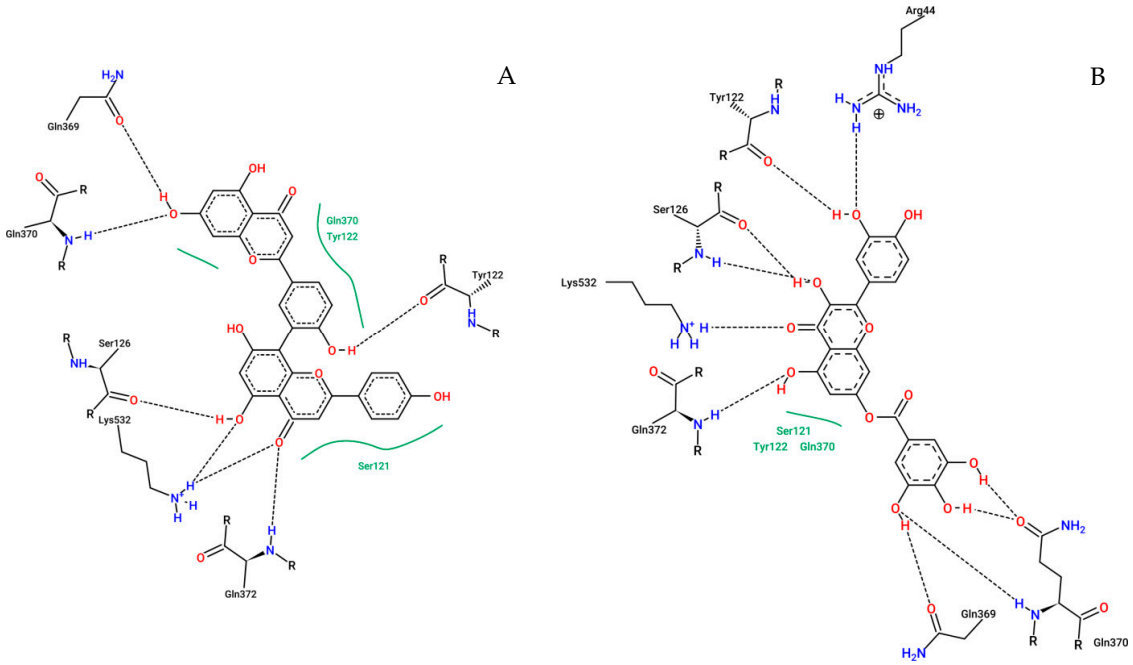

Figure 12. 2D representation of the interactions of COX-2 residues with amentoflavone (**A**) and quercetin-o-gallate (**B**). Dashed lines represent hydrogen bonds; green lines represent van der Waals interactions.

3. Discussion

Herbal medicines have become considerably popular to relieve the symptoms of various diseases, including OA [34]. However, available scientific data are still insufficient to support the use of these products in the clinical management of OA. However, the growth of studies in this sense brings expectations regarding the arrival of reliable, efficient, and safe herbal products that meet the criteria of modern medicine [35].

In order to meet these expectations, the present study evaluated pain behavior in an experimental OA model using *A. chica* extract as an alternative to traditional pharmacological treatments. Since many studies have proven its therapeutic potential, including its anti-inflammatory activity [36–38], and due to the fact that OA is a disease in which the inflammatory process plays a central role in the pathogenesis [8], we evaluated this plant against OA.

The present study shows the anti-inflammatory and analgesic properties of *A. chica* extract through in combo studies: In vitro, by inhibition of cyclooxygenase; in vivo, due to the improvement in clinical and radiological parameters of animals with MIA-induced OA; and also by the demonstration of potential mechanisms of action of *A. chica* secondary metabolites through in silico studies. Chemical analysis of the extract allowed the identification of a large number of molecules with bioactive potential, including metabolitoes were described for the first time in the species.

In this context, *A. chica* extract was evaluated for its ability to inhibit COX enzymes in vitro. COX-2 participates in inflammatory and painful processes, being responsible for the metabolization of arachidonic acid resulting from the action of phospholipase A_2 on membrane lipids, which produce intermediates, prostaglandin G_2, and prostaglandin H_2 which are isomerized to prostanoids such as prostaglandins, prostacyclins, and thromboxanes [39]. Due to the role this enzyme plays in inflammatory processes, it is the target of non-steroidal anti-inflammatory drugs and have represented a potential target for action of new compounds. And in this study, *A. chica* extract was able to inhibit COX-2 and COX-1 by up to 30% (Figure 1A,B), demonstrating its anti-inflammatory potential.

The antinociceptive activity of *A. chica* extract was observed from the improvement of motor activity from day 21 after OA induction, in all doses, similar to meloxicam (Figure 2). Treatment with the dose of 500 mg/kg of *Spinacia oleracea* L. extract also showed improvement in the motor activity of the animals, but only later (day 28) [40].

Treatment with *A. chica* extract also had antinociceptive effects, significantly reducing the weight distribution deficit between the left and right paws, improving OA-induced disability from 14 days after induction, in all doses (Figure 3). The paw weight distribution test is an indicator of OA progression and reveals the efficacy of anti-inflammatory compounds, as observed in the study with an isolated compound from *Zingiber zerumbet* (L.) Smith. which was able to reduce joint discomfort in this same model [41].

The extract also improved the hyperalgesia threshold from day 14 onwards after OA induction, also in all doses (Figure 4). Hyperalgesia is characterized by a painful response accentuated by a previously painful stimulus [42]. Treatment with *Entada pursaetha* DC., hydroethanolic extract 30, 100, and 300 mg/kg also significantly reduced hyperalgesia in the MIA-induced OA model on days 7, 14, and 21. The authors suggest that this effect may be linked to the reduced production of inflammatory mediators responsible for peripheral and central sensitization of pain and hyperalgesia in the model in question [43].

The results of the clinical evaluations of the present study also show that *A. chica* extract 450 mg/kg, from day 21 of treatment, significantly reduced allodynia (pain associated with a stimulus that would normally not cause pain), and for all doses on day 28 [42] (Figure 5). Lima [38] also observed this analgesic effect of *A. chica* extract in an experimental model of neuropathic pain induced by sciatic nerve compression, hence corroborating the results found here. Similar results were also found by our group in Calado et al. [44] when they analyzed the effects of the hydroethanolic extract of *Chenopodium ambrosioides* L. leaves in an experimental OA model using the same methodology.

A possible explanation for improvement of these clinical aspects could be the anti-inflammatory activity of the flavonoids present in the extract, which may be able to inhibit COX, and produce this action, as reported by [45]. *A. chica* extract also demonstrated this effect by inhibiting nuclear transcription factor kappaβ, which consequently prevents the formation of inflammatory mediators such as iNOS, COX-2, 5-LOX, and phospholipase A_2 [18]. Studies by Lima et al. [32] also observed that in lipopolysaccharide-induced peritonitis in mice, oral pretreatment with hydroethanolic extract of the leaves or with the isolated compound 4′,6,7-trihydroxy-5-methoxyflavone (5-o-methyl scutellarein) led to decreased leukocyte migration to the peritoneal cavity, as well as a reduction in proinflammatory cytokine concentrations (TNFα and IL-1β).

Regarding the radiographic findings of the present study, they showed that after OA induction, subchondral sclerosis, osteophytes, and decreased joint space were found in the evaluated knees. Although *A. chica* extract was unable to prevent the most frequent radiological changes that occur in OA, the animals treated with herbal medicine had lower degrees of joint changes according to the Kellgren–Lawrence classification. However, histopathological analyses did not show this improvement for the *A. chica*-treated EHA group.

Importantly, radiological parameters are not always associated with clinical or histological results. This factor generates difficulties in the interpretation of the pain phenomenon in OA; however, people with radiographic alterations compatible with OA have a higher chance of presenting pain than individuals without this type of alteration [46,47].

In the chemical analysis of the extract, 22 compounds were identified, most of which belong to the group of flavonoids (Table 1) that comprise an important class of natural pigments, having a chemical structure consisting of two aromatic rings linked by a chain of three atoms forming an oxygenated heterocycle. The degree of oxidation and the substitution pattern of ring C rank the flavonoids and the substitution pattern on rings A and B specifically defines each compound [48].

Flavonoids in general have been attributed important biological activities, among them an important anti-inflammatory action, due to the ability of these compounds to modulate the action of cellular components involved in the mechanism of inflammation such as pro-inflammatory cytokines TNF-α and IL-1, and the activity of arachidonic acid pathway enzymes such as cyclooxygenase and lipoxygenase [47,48]. Results such as these were observed by studying extract and fractions of *Tabernaemontana catharinensis* DC. leaves, which provide the ability to inhibit leukocyte migration and significantly decreased the levels of various proteins such as MPO, interleukin (IL)-1β, and tumor necrosis factor TNF-α [49]. These actions are attributed at least in part to the flavonoids present in the plant.

Among the 22 compounds identified, it should be noted that 12 are being reported for the first time in species *A. chica*. They are: 2′-hydroxy-a-naphthoflavone; 5,7-dimethoxy-4′-hydroxyflavone; quercetin-o-gallate; quercetin-o-glucoside; amentoflavone; isorhamnetin-3-o-glucoside; chrysoeriol; chrysoeriol-o-glucoside; isorhamnetin; cirsimarin; hyperine 6″ gallate; and catechin dimer.

Considering the potential of these compounds as therapeutic agents and with the encouraging results of the in vitro and in vivo studies, the in silico study was carried out in order to verify the possible pathways of action of the metabolites identified in the extract. Thus, the metabolites had their interactions with the structure of the COX-2 enzyme evaluated through molecular doping. Molecular docking data show that amentoflavone and quercetin-o-gallate were among the metabolites which had more favorable interactions with COX-2.

Negative binding-free energy values between the binder and the macromolecule indicate favorable interactions [50]. The active site of COX-2 involves residues Arg120, Tyr355, Tyr385, Glu524, and Ser530 where arachidonic acid binds, thus forming prostaglandins [51,52]. The result of molecular docking demonstrated that amentoflavone and quercetin-o-gallate performed a large number of interactions (hydrogen bonds and van der Walls interactions) with residues neighboring this region (Figure 12). Silva and colleagues [53] have identified through molecular docking and molecular dynamics simulations that the ursolic acid inside all metabolites identified on the ethylacetate fraction

of *Borreria verticillata* (L.) G. Mey. was responsible for the anti-inflammatory activity of this plant species. The ursolic acid was selected by in silico assay and when evaluated isolated in vivo on mice, showed anti-inflammatory activity higher than indomethacin.

Amentoflavone showed anti-inflammatory activity besides suppressing the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in RAW264.7 cells [54]. Also, amentoflavone promotes the downregulation of COX-2 and iNOS levels in cancer cells. This activity is also well associated with the suppression of PGE_2 biosynthesis [55]. Evaluated against an ulcerative colitis model on mice, the amentoflavone showed decreases in the mucosal injury by lowered colonic wet weight as well as vascular permeability and diminished lactate dehydrogenase (LDH) and myeloperoxidase (MPO) activity reflecting reduced leukocyte infiltration and reducing significantly the tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6 levels as well as the expression of iNOS and COX-2 [56].

4. Materials and Methods

4.1. Collection and Processing of Plant Species

The leaves of *Arrabidaea chica* were collected in the urban area of São Luis city, Maranhão state, Brazil, in May 2016 (2° 33'29" S, 44° 18 '30" W) between 5 to 6 p.m. A sample of the plant was deposited in the "Atico Seabra" Herbarium, Federal University of Maranhão (UFMA), and was identified and cataloged in register 1.067. For preparation of the extract, the collected plant material was dried at 40 °C in an air circulating oven and then pulverized in an electric mill to obtain the powder, which was impregnated in 70% ethanol in a ratio of 1:4 (*m*/*v*) and placed in maceration, under daily manual agitation. The alcoholic extraction of the macerate was carried out by three successive changes every 72 h, with the renovation of the solvent. At the end of this process, the extracts were pooled and gauze filtered. The filtrate was concentrated in a rotary evaporator under reduced pressure and at a temperature of 40 °C. From this process, the hydroethanolic extract (EHA) was obtained.

4.2. In vitro Activity on Cyclooxygenase

Cyclooxygenase (COX) has been associated with the target of non-steroidal anti-inflammatory drugs and has been a potential target for the study of new drugs. Thus, in vitro tests of inhibition of COX by the hydroethanolic extract of *A. chica* were carried out, according to the instructions of the manufacturer of the enzymatic kit (Colorimetric COX Inhibitor Screening Assay Kit, Cayaman Chemical®, Ann Arbor, Michigan, USA.) and percent inhibition of COX-1 and COX-2, calculated from the means of the absorbance values, read at 590 nm. Inhibition assays were performed with extracts at three concentrations 2, 10, and 50 μg/mL in triplicate.

4.3. In vivo Experimental Studies

This study was conducted at the Experimental Laboratory for the Study of Pain (LEED). All procedures were approved in May 22, 2017 by the Ethics Committee on Animal Use of UFMA under No. 23115.000372/2017-09.

4.3.1. Animals

Wistar rats (*Rattus norvegicus*) males, adults approximately 60 days old, which were procured from the Central Vivarium (Biotério Central) of Federal University of Maranhão (UFMA), São Luis, Brazil, were used in this study. These animals, throughout the experiment, were fed standard feed and water ad libitum and kept under a controlled temperature of 23 ± 1 °C and humidity of 40–60% under a 12 h light–dark cycle.

4.3.2. Experimental Design

The animals were divided into 6 groups (n = 6, per group): Group 1—without OA and untreated (CLEAN); Group 2—with osteoarthritis and treated with saline (NaCl 0.9%, 0.1 mL/kg) (CTL−); Group 3—with osteoarthritis and treated with Meloxicam® (0.5 mg/kg) (CTL + Melox) [57]; Groups 4, 5,and 6—with osteoarthritis and treated with *A. chica* hydroethanolic extracts at doses of 50, 150, and 450 mg/kg (EHA50, EHA150, EHA450), respectively.

The CLEAN group did not undergo any type of intervention. The other groups (CTL−, CTL + Melox, EHA50, EHA150, EHA450) received intra-articular injections of MIA (2 mg) for induction of osteoarthritis in the knee. And after three days, these groups received their respective treatments, as described above, orally (once daily) for 25 days (Figure 13).

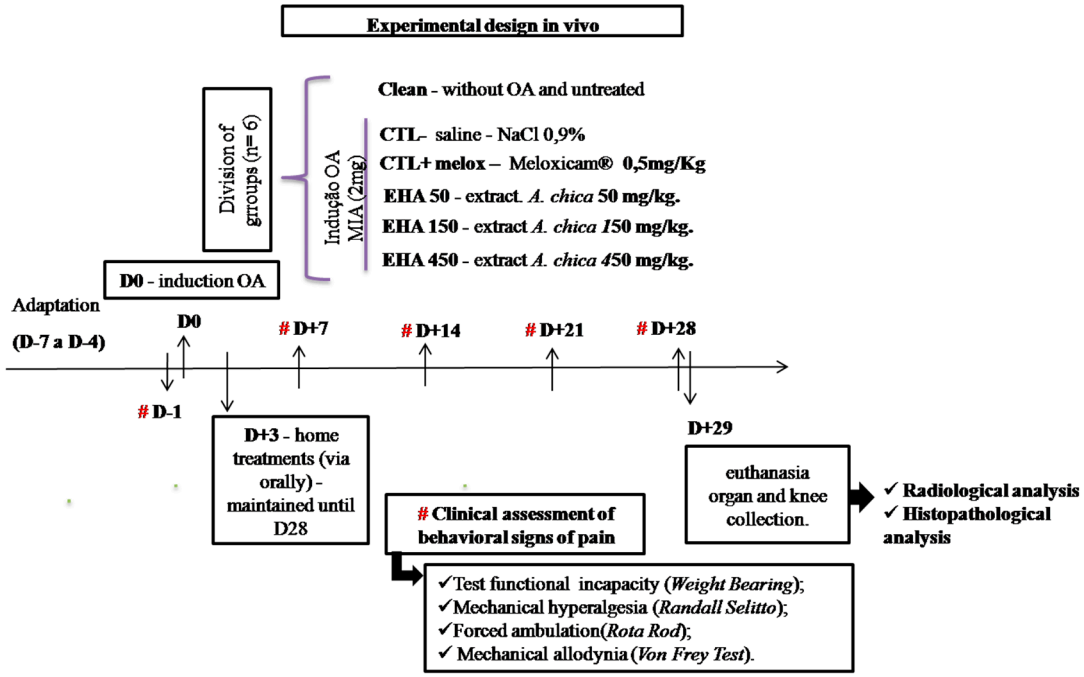

Figure 13. Experimental design.

These groups were evaluated clinically for disability, allodynia, mechanical hyperalgesia, and motor activity every 7 days, and were slaughtered on day 29 after initiation of OA induction. The animals were euthanized with a 2:1 anesthetic solution of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (80 mg/kg), after which the blood of all animals was collected through the abdominal artery. Liver, spleen, and kidney were collected for histopathological analysis, as well as the right paw induced for radiographic and histopathological analyses (Figure 13).

4.3.3. MIA-Induced OA Model

The animals were anesthetized by inhalation of isoflurane 1%. After certifying the anesthetic plane, a trichotomy was performed in the right knee and, subsequently, a topical solution of 10% iodopovidone was applied for local asepsis. An articular lesion was induced by a single intra-articular injection of 2 mg sodium MIA (diluted in a maximum volume of 25 µL) into the right knee through the patellar ligament [58,59].

4.3.4. Clinical Evaluations

Evaluation of Motor Activity/Forced Deambulation (Rotarod Test)

The animals were placed on a swivel bar (IITC Life Science, Woodland Hills, CA, USA.) at a speed of 16 rpm for a period of 300 s. The use of the affected limb was assessed by forced deambulation. The use of the paw was graded using a numerical scale ranging from 5 to 1, where: 5 = normal use of the paw, 4 = mild claudication, 3 = severe claudication, 2 = intermittent disuse of affected paw, and 1 = complete disuse of affected paw [60].

Incapacitation/Weight Distribution Test on Hind Legs (Weight Bearing)

The animals were placed in a glass bowl angled and positioned so that each hind leg laid on different platforms. The weight exerted on each back paw (measured in grams) was evaluated for 5 s. The final measurement of weight distribution was the mean of three measurements [61]. Changes in the weight distribution on the paws were calculated as follows:

$$\text{Weight distribution } (\%) = \frac{\text{APW}}{\text{APW} + \text{CPW}} \times 100 \quad (1)$$

where APW was affected paw weight and CPW was contralateral paw weight.

Mechanical Hyperalgesia (Randall–SelittoTest)

Mechanical hyperalgesia was assessed by evaluating the nociceptive threshold paw withdrawal following the application of mechanical pressure using an analgesiometer (IITC Life Science, Woodland Hills, CA, USA). A wedge-shaped device (area, 1.75 mm^2) was applied to the dorsal surface of the hind paws with increasing linear pressure until the animal responded by withdrawing the paw. Three measurements were performed in the ipsilateral and contralateral paws. A cut-off threshold pressure of 250 g was programmed to prevent tissue damage. The paw withdrawal reflex was considered to represent the hypernociceptive threshold. The nociceptive paw withdrawal threshold (NPWT) was recorded in grams and defined as the percentage pressure required to provoke a withdrawal of the ipsilateral affected paw, and was calculated as follows:

$$\text{NPTW } (\%) = \frac{\text{NAPWT}}{\text{NAPWTW} + \text{NCPWT}} \times 100 \quad (2)$$

where NPWT was nociceptive paw withdrawal threshold, NAPWT was nociceptive affected paw–withdrawal threshold, and NCPWT was nociceptive contralateral paw withdrawal threshold [62,63].

Mechanical Allodynia (von Frey Test)

The Von Frey test was performed with a filament with an adapted tip, which was pressed with a constant force. For this evaluation the animals were placed in individual transparent acrylic boxes on raised platform to allow access to the lower part of their bodies. The response given at time of paw withdrawal to the filament stimulus was measured in 3 applications lasting up to 5 s each, always performed by the same evaluator [64].

4.4. Radiological Analysis

After euthanasia, the right hind paws of the animals were amputated and submitted to radiographs on anteroposterior and profile incidences, in order to evaluate the decrease in joint space, sclerosis of the subchondral bone and presence of osteophytes in the knees evaluated. The AP incidence was used to classify osteoarthritis by the [65], according to Table 3.

Table 3. Classification of Kellgren–Lawrence.

Grade 0	**No arthrosis**—Normal radiology
Grade I	**Doubtful arthrosis**—Doubtful articular space narrowing and possible
Grade II	**Minimal osteoarthritis**—Possible joint narrowing, defined osteophytes
Grade III	**Moderate arthrosis**—Defined joint narrowing, multiple moderate osteophytes, some subchondral sclerosis and possible deformity in the bone contour
Grade IV	**Severe arthrosis**—Significant articular space narrowing, severe subchondral sclerosis, defined deformity in the bone contour and large osteophytes

4.5. Histopathological Analysis of Articular Cartilage

On day 29, the knee of each animal was removed after euthanasia and fixed in 10% buffered formalin. Then, they were subjected to decalcification in 20% ethylenediaminetetraacetic acid (EDTA) for 28 days. Subsequently they were submitted to the inclusion protocol in paraffin blocks, cut into sections of 5 µm, and the proteoglycans of the organic cartilage matrix were stained specifically with 0.5% O-safranin.

The histopathological evaluation was performed according to the guidelines of the Osteoarthritis Research Society International (OARSI). The slides were analyzed blindly by two pathologists, who graded them on a scale of 0 to 6, according to the severity of the articular cartilage lesion. The classification considered the most severe lesion observed on the slide regardless of the extent of the lesion. Grade 0 indicates morphologically intact cartilage, Grade 1 indicates an intact surface with possible focal lesions or abrasion, Grade 2 shows discontinuity in the articular surface, Grade 3 shows vertical fissures, Grade 4 presents erosions, Grade 5 exhibits denudations with sclerotic bone or fibrocartilaginous tissue repair or both, and Grade 6 shows remodeling and bone deformation with changes in the contour of the articular surface [66], according to Table 4.

Table 4. Osteoarthritis cartilage histopathology assessment classification system (Osteoarthritis Research Society International) [66].

Grade (Key Feature)	Subgrade (Optional)	Associated Criteria (Tissue Reaction)
Grade 0: Surface intact, Cartilage intact	No subgrade	Intact, uninvolved cartilage
Grade 1: Surface intact	1.0 Cells intact 1.5 Cell death	Matrix: superficial zone intact, edema and/or fibrillation; Cells:proliferation (clusters),hypertrophy; Reaction must be more than superficial fibrillation only.
Grade 2: Surfacediscontinuity	2.0 Fibrillation through superficial zone 2.5 Surface abrasion with matrix loss within superficial zone	As above +Discontinuity at superficial zone; ±Cationic stain matrix depletion (Safranin O or Toluidine Blue) upper 1/3 of cartilage (mid zone); ±Disorientation of chondron columns
Grade 3: Verticalfissures	3.0 Simple fissures 3.5 Branched/complex fissures	As above; ±Cationic stain depletion (Safranin O or Toluidine Blue) into lower 2/3 of cartilage (deep zone); ±New collagen formation (polarized light microscopy, Picro Sirius Red stain)
Grade 4: Erosion	4.0 Superficial zone delamination 4.5 Mid zone excavation	Cartilage matrix loss, cyst formation within cartilage matrix

Table 4. *Cont.*

Grade (Key Feature)	Subgrade (Optional)	Associated Criteria (Tissue Reaction)
Grade 5: Denudation	5.0 Bone surface intact 5.5 Reparative tissue surface present	Surface is sclerotic bone or reparative tissue including fibrocartilage.
Grade 6: Deformation	6.0 Joint margin osteophytes 6.5 Joint margin and central osteophytes	Bone remodeling; Deformation of articular surface contour (more than osteophyte formation only); Includes: microfracture and repair

4.6. FIA-ESI-IT-MS/MSn and HPLC-ESI-IT-MS Analysis Instrumentation

For the FIA-ESI-IT-MSn assay, 10 mg of *A. chica* hydroethanolic extract was dissolved in 1 mL MeOH:H_2O (1:1, *v*/*v*). The sample was filtered through a 0.22 µm PTFE filter, and 20 µL aliquots were injected into the LC-MS and directly into the FIA-ESI-IT-MSn system. Chromatographic profile of the crude extract of *A. chica* was performed on LCQ Fleet (Thermo Scientific®, San Jose, CA, USA), Kinetex® C18, 100 Å (4.6 × 100 mm.; 5 µm). The mobile phase was ultra-pure water (eluent A) and acetonitrile (eluent B), both containing 0.1% formic acid in an exploratory gradient starting with 10% to 100% B in 60 min at a flow rate of 1.0 mL/min.

The sample was ionized by electrospray (ESI) and the fragments were obtained in multiple stages (MSn), in ion trap type interface. The experimental conditions were: Capillary voltage35 V, spray voltage 5000 V, capillary temperature at 350 °C, drag gas (N2), and flow 60 (arbitrary units). The acquisition range was m/z 100–2000, with two or more scanning events performed simultaneously on the spectrum. The direct flow infusion of the sample was performed on an LTQ XL Ion trap type analyzer equipped with a positive mode electrospray ionization (ESI) source (Thermo Scientific®, San Jose, CA, USA). A 280 °C stainless steel capillary tube, a spray voltage of 5.00 kV, a capillary voltage of 90 V, a tube lens of −100 V and a flow of 5 µL min^{-1} were used. The complete scan analysis was recorded in the m/z range of 100–1000. Multiple-stage fragmentation (ESI-MSn) was performed using the collision-induced dissociation (CID) method against helium for ion activation.

The first event was a full sweep mass spectrum to acquire data on ions in this m/z range. The second scanning event was an MS/MS experiment performed using a data-dependent scanning on the [M + H] molecules of the compounds of interest with a collision energy of 30% and an activation time of 30 ms. The product ions were then subjected to further fragmentation under the same conditions, until no further fragments were observed. The identification of the different compounds in the chromatographic profile of the hydroethanolic extract was accomplished by the mechanisms of fragmentation and comparing their mass spectral data with the literature.

4.7. In silico Studies

4.7.1. Predictive Models and Theoretical Calculations

The metabolites identified in the *A. chica* hydroethanolic extract had their geometric, electronic, and vibrational properties optimized using the Gaussian program 09 (Gaussian, Inc., Wallingford CT) [67]. The GaussView 5.0.8 (Semichem Inc., Shawnee Mission, KS) [68] was used to obtain 3D structural models. Geometric optimization calculations were performed according to the Functional Density Theory (DFT) method, combining the functional hybrid B3LYP and the set of bases 6-31++G(d, p).

4.7.2. Molecular Docking

All docking procedures utilized the Autodock 4.2 package [69]. The structure of the cyclooxygenase 2 (COX-2) (PDB ID 1DDX) and ligands were prepared for docking simulations with AutoDock Tools version 1.5.6 [70]. Docking methodology described in literature were used [70] with some modifications [44,53]. Gasteiger partial charges were calculated after addition of all hydrogens, both ligand and macromolecule. Non-polar hydrogens from COX-2 and *A. chica* metabolites were

subsequently merged. The dimensions of the cubic box in the X-, Y- and Z-axes were 120 × 120 × 120 Å, respectively, with a spacing of 0.375 Å between grid points. Grid box was centered on oxygen atom from residue Arg120 from COX-2 and the Lamarckian genetic algorithm (LGA) was chosen to search for the best conformations, with 100 runs for each compound. Initial coordinates of COX-2 and *A. chica* compounds interaction complexes were chosen based on the criterion of the better docking conformation of the cluster with the lowest energy in addition to visual inspection.

4.8. Statistics

The comparison of the means of different experimental groups was performed using Student's *t*-test or univariate analysis of variance (One-way ANOVA), followed by the Tukey test. In the evaluation of two sources of variability, bivariate variance analysis (Two-way ANOVA) was used. The p value <0.05 was considered as indicative of significance and the data obtained were analyzed through the software GraphPad Prism 7.0® software for Windows® (CA, USA).

5. Conclusions

The results of the present study provide evidence that *A. chica* extract has the potential to be used in the treatment of OA. It has been shown to inhibit the enzyme COX-2; additionally, oral treatment for 25 days with *A. chica* hydroethanolic extract (50, 150, and 450 mg/kg) showed antinociceptive activity, producing improvements in incapacitation, motor activity, allodynia and hyperalgesia parameters in rats with OA experimentally induced by MIA.

The extract was able to produce radiological improvements in the affected knees; however, histopathological analysis revealed that the extract apparently did not act significantly on cartilage regeneration. Investigations into prophylactic use of the extract before OA induction may show whether the extract would be effective in preventing cartilage deterioration. Thus, more study is needed.

The results of the present study also showed that *A. chica* extract is rich in flavonoids, which have a large biological potential and may help in the treatment of OA. The in silico assays indicate a possible way of action of the compounds present in the extract, suggesting that through an interaction with cyclooxygenase-2 these compounds reduce the inflammatory process and improve OA.

Author Contributions: Conceptualization, C.C.V. and M.d.S.d.S.C.; methodology, C.C.V., A.J.O.L., E.L.F.S., D.S.C., G.E.B.S., C.Q.d.R. and M.d.S.d.S.C.; formal analysis, C.C.V., M.d.S.d.S.C., A.J.O.L., C.Q.d.R, J.B.S.G., G.E.B.S. and F.C.V.M.L.; investigation, C.C.V., A.J.O.L., E.L.F.S., D.S.C., F.C.V.M.L., G.E.B.S., C.Q.d.R. and M.d.S.d.S.C.; writing—original draft preparation, C.C.V.; writing—review and editing, C.C.V., M.d.S.d.S.C., C.Q.d.R., A.J.O.L. and J.B.S.G.; supervision, M.d.S.d.S.C.; project administration, C.C.V. and M.d.S.d.S.C.; funding acquisition, M.d.S.d.S.C.

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Conflicts of Interest: The authors declare no conflict of interest and that the funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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4.2 CAPÍTULO II

Artigo "*Arrabidaea chica* **Verlot fractions reduces MIA-induced osteoarthritis progression in rats' knees",** submetido a revista ***Inflammopharmacology*** (ISSN 0925-4692).

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Arrabidaea chica Verlot fractions reduce MIA-induced osteoarthritis progression in rat knees

Cleydlenne Costa Vasconcelos[1]*, Alberto Jorge Oliveira Lopes[1], Emilly de Jesus Garcia Ataide[1], Kevin Waquim Pessoa Carvalho[1], Maria Fernanda Freitas de Brito[2], Marineide Sodré Rodrigues[1], Sebastião Vieira de Morais[1], Gyl Eanes Barros Silva[1,2], Claudia Quintino da Rocha[3], João Batista Santos Garcia[1] and Maria do Socorro de Sousa Cartágenes[1]*

[1] Biological and Health Sciences Center, Federal University of Maranhão, Av. dos Portugueses 1966, São Luís, MA, Postal Code: 65085–580, Brazil;

[2] Hospital Universitário Presidente Dutra, HUPD, Federal University of Maranhão, São Luís, MA, Brazil;

[3] Chemistry Department, Federal University of Maranhão, São Luís, MA, Brazil;

* Correspondence: cleydlenne@yahoo.com.br; scartagenes@gmail.com

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Abbreviations

OA	osteoarthritis
IL-1	Interleukin-1
IL-6	Interleukin-6
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
NSAIDS	nonsteroidal anti-inflammatory drugs
Hex	n-hexane
EtAc	ethyl acetate
But	butanol
SISGEN	National System of Genetic Heritage Management and Associated Traditional Knowledge
ECAU	Ethics Committee in Animal Use
UFMA	Federal University of Maranhão
MIA	sodium monoiodoacetate
NaCl	Sodium chloride (saline)
APW	affected paw weight
CPW	contralateral paw weight
EDTA	Ethylenediaminetetraacetic acid
OARSI	Osteoarthritis Research Society International
GC/MS	gas chromatograph and mass spectrometry
EI-MS	Electron-impact mass spectra
DFT	Density Functional Theory
ANOVA	Analysis of variance
iNOS	Inducible nitric oxide synthase
PGE2	Prostaglandin E2
TNF-α	Tumour necrosis factor-α
NF-κB	Nuclear factor kappa B

p38MAPK	p38 mitogen-activated protein kinases
DPPH	2,2 Diphenyl 1 picrylhydrazyl
ABTS	2,2'-azinobis-3-ethylbenzothiazoline-6- sulfonic acid
NO	nitric oxide
IL-1β	Interleukin-1β
IFN-γ	*Interferon-gamma*
LPS	*Lipopolysaccharide*
PBMC	peripheral blood mononuclear cell

Abstract: This study aims to investigate the activity of n-hexane, ethyl acetate and butanol fractions obtained from *Arrabidaea chica* Verlot against MIA-induced osteoarthritis (OA). The antinociceptive potentials of each fraction were evaluated through a cyclooxygenase (COX) 1 and 2 inhibition test and an *in vivo* OA-model. In addition, toxicity assessments in the liver, spleen and kidney, as well as radiographic and histopathological knee analyses, were performed. The chemical composition of the n-hexane fraction was elucidated, and a molecular docking protocol was carried out to identify which compounds are associated to the detected bioactivity. The n-hexane *A. chica* fraction preferentially inhibits COX-2, with 90% inhibition observed at 10 µg/mL. The fractions also produced significant improvements in OA incapacity, motor activity and hyperalgesia parameters and in radiological knee conditions. However, concerning the histopathological evaluations, these improvements were only significant in the hexane and ethyl acetate fraction treatments, which resulted in better average scores, suggesting that these fractions slow OA-promoted joint injury progression. Histopathological organ analyses indicate that the fractions are not toxic to animals. Twenty compounds were identified in the n-hexane fraction, comprising fatty acids, terpenes and phytosterols. *In silico* analyses indicate the presence of favourable interactions between some of the identified compounds and the COX–2 enzyme, mainly concerning alpha-tocopherol (Vitamin E), squalene and beta-sitosterol. The findings indicate that *A. chica* fractions display analgesic, anti-inflammatory properties, are non-toxic and are able to slow OA progression, and may, therefore, be prioritized as natural products in OA human clinical trials.

Keywords: new drug candidates; cartilage damage; molecular docking; joint degeneration.

1. Introduction

Osteoarthritis (OA) is a musculoskeletal disorder of significant public health importance worldwide, because, as it is a currently irreversible and debilitating condition and increases mortality and cardiovascular event risks, generating relevant socioeconomic impacts (Hsu et al. 2016; Turkiewicz et al. 2019). Musculoskeletal diseases now represent the second main cause of years of life lived with a disability, with OA responsible for 7.1% of this burden (Kloppenburg and Berenbaum 2020). OA is a multifactorial disease presenting higher incidence risks with old age, overweight, genetic factors, diet, gender, trauma and physical or occupational activities that imply biomechanical joint tension (Smith et al. 2004; McWilliams et al. 2011; Murphy et al. 2016).

OA is a degenerative disease, characterized by progressive damage to the articular cartilage and changes in the subchondral region and perichondral muscle, which hinder free movement and cause pain (Alves-Neto; et al. 2009; Robinson et al. 2016). Inflammation is a common finding in this disease, since the osteoblasts in the subchondral region exhibit a pro-inflammatory phenotype, with increases in several inflammatory mediators, such as prostaglandins, produced mainly by the activity of cyclooxygenase-2 (COX-2), and interleukins, such as IL-1 and IL-6 (Martel-Pelletier et al. 2003; Henrotin et al. 2006; Robinson et al. 2016).

The current pharmacological treatment for OA consists in the use of analgesics, COX-2 enzyme inhibitor drugs and nonsteroidal anti-inflammatory drugs (NSAIDs), although this approach is still

insufficient, since these drugs cannot reverse the progress of this disease and result in important side effects, important, in addition to the persistent nature of pain to current treatments (Bjordal et al. 2007; Tang 2019).

A treatment perspective for OA is Alternative and Complementary Medicine, which comprises, among others, the use of nutritional and botanical products, also known as herbal medicines, that may represent an alternative not only for symptom treatment, but also for the natural progression of disorder's or disease's (Akhtar et al. 2011; Umar et al. 2014; Li et al. 2017).

Arrabidaea chica (Humb. & Bonpl.) Verlot is noteworthy among popular medicinal plants. This species is found predominantly in tropical regions of the Americas and Africa (von Poser et al. 2000) and is traditionally used for several therapeutic purposes, mainly due to its anti-inflammatory and healing activities concerning skin and blood disorders and leukaemia (Lorenzi and Matos 2008; Aro et al. 2013; Michel et al. 2015; de Araújo et al. 2017). Previous studies by our research group have reported the analgesic properties of *A. chica* hydroethanolic extracts (Lima 2017; Vasconcelos et al. 2019), although the search for the active fraction of this plant and the identification and/or elucidation of its active principle justifying the aforementioned biological activities is paramount in obtaining an effective and safe herbal medicine.

In this context, the present study aims to investigate the use of *A. chica* fractions as a therapeutic alternative for OA treatment through *in vitro*, *in silico* and in vivo assays. We aim, therefore, to suggest its possible mechanisms of action and propose new drug alternatives for OA management, so that one can intervene not only in OA symptoms, but also in OA pathogenesis, including improvements to cartilage health and inflammation inhibition.

2. Material and Methods

2.1. Plant collection and fraction preparation

Arrabidaea chica leaves were collected in São Luis (2 ° 33′29 ″S, 44 ° 18 ′30″ W), in the state of Maranhão, Brazil, from 5 pm to 6 pm. One sample was deposited at the "Atico Seabra" Herbarium, Federal University of Maranhão (UFMA), and was identified and catalogued as no. 1.067. The plant material was dehydrated at 40 °C in an air circulation and renewal oven and then pulverized in an electric mill. An hydroethanolic extract was obtained as described previously (Vasconcelos et al. 2019). The hydroethanolic extract (70% v/v) was then dissolved in methanol/water (80:20 v/v) under stirring, and the extractive solution was then submitted to liquid-liquid partition using n-hexane, ethyl acetate and butanol. The solutions were filtered and concentrated using a rotary evaporator at 40ºC under vacuum, to obtain the n-hexane (Hex), ethyl acetate (EtAc) and butanol (But) fractions, which were freeze-dried before use, thus ensuring total solvent removal. This study is registered at the National System of Genetic Heritage Management and Associated Traditional Knowledge (SISGEN) under code AAF93D3, as determined by Brazilian legislation for studies that use Brazilian genetic heritage, samples.

2.2. In vitro COX inhibition test

In-vitro COX-1 and 2 tests were performed to assess the potential of *A. chica* fractions in inhibiting these important enzymes implicated in the inflammatory process, since they catalyse the synthesis of prostaglandins from arachidonic acid. The tests were performed according to the manufacturer's

instructions (Colorimetric COX Inhibitor Screening Assay Kit, Cayman Chemical ®) and COX-1 and 2 inhibition percentages were calculated from absorbance values determined at 590 nm. The inhibition tests were performed with the n-hexane, ethyl acetate and butanol *A. chica* fractions solubilized in 70% ethanol/water (70:30, *v*/*v*) at three concentrations (2, 10 and 50 µg/mL). This solvent mixture was used in all control reactions, with and without enzymes, as described previously (Lopes et al. 2019).

2.3 In vivo experimental studies

All *in vivo* experimental assessment protocols were approved and authorized on May 22, 2017 by the Federal University of Maranhão (UFMA) Ethics Committee in Animal Use (ECAU), under protocol code 23115.000372/2017- 09 and performed according to the International Association for the Study of Pain (IASP) Guidelines for the Use of Animals in Research.

2.3.1 Animals

The *in vivo* experimental model comprised adults male Wistar rats (*Rattus norvegicus*) approximately 60 days-old obtained from UFMA's Central Vivarium (Biotério Central) . During the entire experimental period, the animals were fed standard chow and water, *ad libitum*, and maintained under controlled temperature (23 ± 1 ºC), humidity (40–60%) and 12-hour light-dark cycle.

2.3.2 Experimental design

The animals were randomly divided into groups (N = 6). The CLEAN group did not undergo any type of intervention, while other groups received an intra-articular sodium monoiodoacetate (MIA) injection (2 mg) to induce osteoarthritis in the right knee, followed by their respective treatments: saline solution - NaCl 0.9% (vehicle) (CTL -), Meloxicam ® - 0.5 mg/kg (CTL + Melox), *A. chica* Hex fraction 5 mg/kg; *A. chica* EtAc fraction 5 mg/kg, *A. chica* But fraction 5 mg/kg (with sample sonication for complete solubilization). The animals received this daily dose orally by the orogastric route, using the gavage method, from D3 to D28 day (Fig. 1). These groups were clinically assessed for antinociceptive activity by incapacitation (Weight Bearing test), mechanical hyperalgesia (Randall-Selitto Test) and motor activity (Rotarod test) every 7 days, and were euthanized on the 29th day after of OA induction, using a 2:1 anesthetic ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (80 mg/kg) solution. The liver, spleen and kidneys were collected for toxicity assessment. The right OA-induced paw was also collected for radiographic and histopathological analyses (Fig. 1).

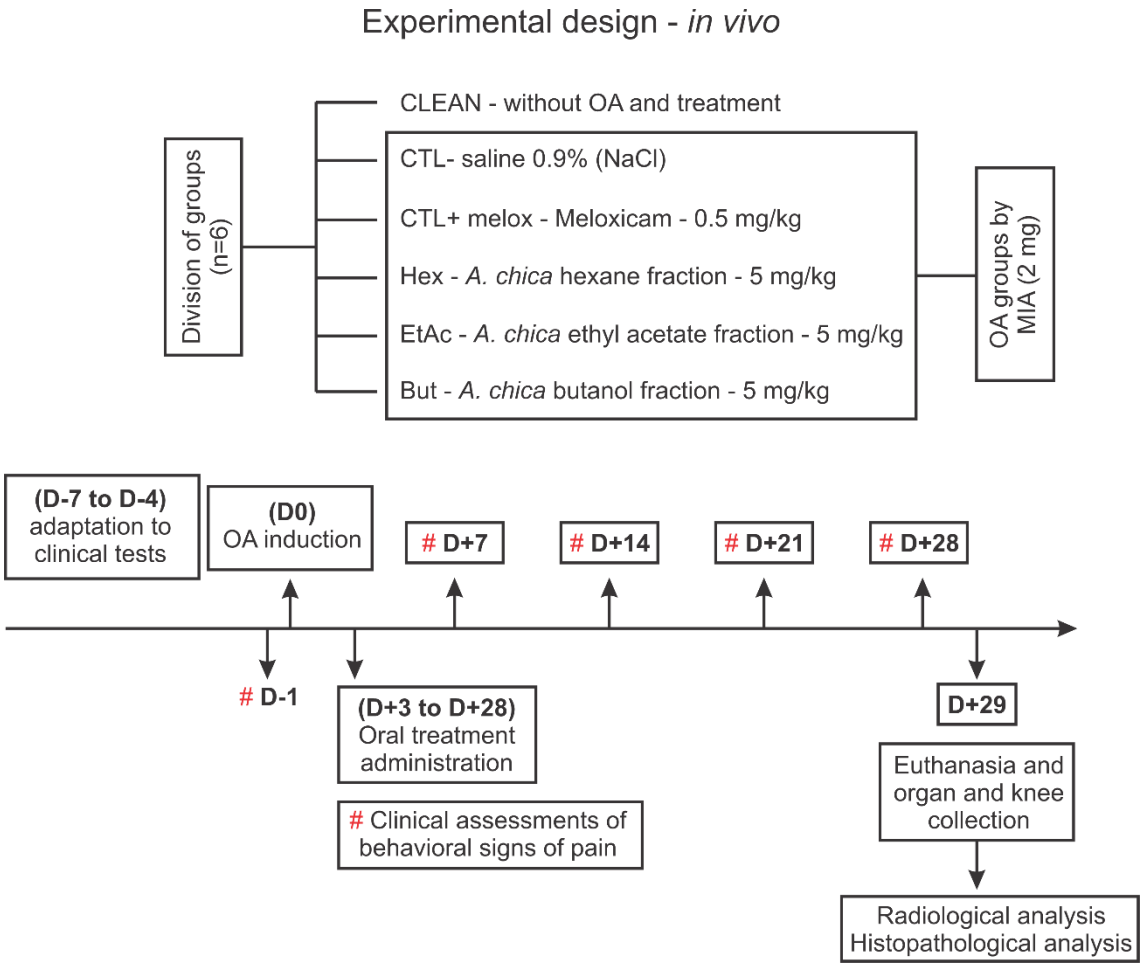

Fig. 1. Experimental design applied in the present study.

2.3.3 Clinical assessments

2.3.3.1 Evaluation of Motor Activity/Forced Deambulation (Rotarod Test)

The animals were placed on a rotating bar (IITC model, Life Science) at 16 rpm for 300 s. The use of the affected limb was assessed by forced walking. Paw use was assessed on a 5 to 1 numerical scale, where: 5 = normal paw use, 4 = mild claudication, 3 = severe claudication, 2 = intermittent disuse of the affected paw and 1 = complete disuse of the affected paw (Kalff et al. 2010).

2.3.3.2 Incapacitation/Weight Distribution Test on Hind Legs (Weight Bearing)

The animals were placed in an angled glass chamber and positioned so that each hind leg rested on different platforms. The weight exercised on each hind leg (measured in grams) was evaluated for five seconds. The final measurement was given by an average of three measurements (Schött et al. 1994).

Changes in weight leg distribution were calculated as follows:

Weight distribution (%) = APW / (APW + CPW) x 100

Where APW represents the affected paw weight and CPW represents the contralateral paw weight.

2.3.3.3 Mechanical Hyperalgesia (Randall-Selitto Test)

Mechanical hyperalgesia was assessed using the nociceptive threshold of paw removal under mechanical pressure using an analgesimeter (model IITC Life Science, California, United States). A wedge-shaped device (1.75mm² area) was applied to the dorsal surface of the hind legs with increasing linear pressure until the animal produced a response characterized by paw removal. Three measurements were performed on the ipsilateral and contralateral paws. A cut-off pressure of 250g was programmed to avoid tissue damage. The paw withdrawal reflex was considered to represent the hypernociceptive threshold. The nociceptive paw withdrawal threshold (LNRP) was recorded in grams and defined as the percentage of pressure necessary to cause the ipsilateral affected paw to be removed, calculated as follows:

NPWT (%) = NAPWT / (NAPWT + NCPWT) x 100

where NPWT is nociceptive paw withdrawal threshold, NAPWT comprises nociceptive affected paw-withdrawal threshold and NCPWT consists in the nociceptive contralateral paw withdrawal threshold (Randall and Selitto 1957; Santos-Nogueira et al. 2012).

2.3.4 Radiological analysis

After euthanasia, the animals right hind legs were amputated and subjected to radiographs in anteroposterior and lateral views, aiming to assess decreased joint space, subchondral bone sclerosis and the presence of osteophytes in the evaluated knees. The profile view was used to classify osteoarthritis by the Kellgren-Lawrence method (Kellgren and Lawrence 1957), where five degrees of classification are assigned: 0 - no arthrosis, normal radiology; grade I - doubtful arthrosis: doubtful joint narrowing, possible marginal osteophyte; grade II - minimal arthrosis: possible narrowing, definitive osteophyte; grade III - moderate arthrosis: definitive narrowing, multiple osteophytes, some subchondral sclerosis, possible bone deformity; grade IV - severe arthrosis: remarkable joint narrowing, severe subchondral sclerosis, large osteophytes, definite deformity.

2.3.5 Histopathological analysis of the articular cartilage

After euthanasia at D29, the knee of each animal was removed and fixed in 10% buffered formaldehyde, followed by decalcification in 20% ethylenediaminetetraacetic acid (EDTA) for 28 days. A paraffin block inclusion protocol was then performed (5 µm sample sectioning), followed by specific staining of the proteoglycans of the organic cartilage matrix with 0.5% O-safranin. Histopathological evaluations were performed according to Osteoarthritis Research Society International (OARSI) Guidelines. The slides were blindly analysed by a pathologist, who classified the samples on a scale from 0 to 6, according to joint cartilage injury severity. The classification considered the most serious injury observed on the slide, regardless of the extent of the injury. Grade 0 indicates a morphologically intact cartilage, grade 1 indicates an intact surface with possible focal lesions or abrasion, grade 2 indicates discontinuity on the joint surface, grade 3 comprises vertical cracks, grade 4 indicates erosions, grade 5 consists in bone denudations sclerotic or fibrocartilaginous tissue repair or both, and grade 6, bone remodelling and deformation with articular surface contour changes (Pritzker et al. 2006).

2.4 Analysis of the A. chica n-hexane fraction by gas chromatography–mass spectrometry

The *A. chica* n-hexane fraction analysis was performed by gas chromatograph mass spectrometry (GC/MS) using a GC-2010gas chromatograph (Shimadzu, Japan) coupled to a CGMSQP2010 mass spectrometer (Ultra Shimadzu, Japan) with a capillary column fused silica SGE BPX5 column (30m × 0.25 mm x 0.25 µm, SIS, USA). The oven temperature was programmed to linearly increase from 60 °C to 240 °C at 3 ºC/min., resulting in a 60 min elution time. Helium was used as the carrier gas at a flow rate of 1.8 mL/min, and an injection volume of 1 µL at a split ratio 1:5 was applied. The injector and detector temperature was set at 280 ºC. Electron-impact mass spectra (EI-MS; 70 eV) were acquired at the mass-to-charge ratios (m/z) range from 35 to 700. The compounds were identified through comparison of the obtained mass spectra with the NIST14s library.

2.5 In silico studies

2.5.1. Predictive models and theoretical calculations

The identified *A. chica* n-hexane fraction metabolites had their geometric, electronic and vibrational properties optimized using the Gaussian09 software (Frisch et al. 2016). The GaussView 5.0.8 (Dennington et al. 2016) was used to obtain 3D structural models. Geometric optimization calculations were performed according to the Density Functional Theory (DFT) method, combining the functional hybrid B3LYP and the set of bases 6-31 ++ G (d, p).

2.*5.2 Molecular docking*

All docking procedures utilized the Autodock Vina software (Trott and Olson 2009). The structure of cyclooxygenase-2 (COX-2) (PDB ID 1DDX) and the ligands were prepared for docking simulations using the AutoDock Tools, version 1.5.7 software (Morris et al. 2009). A docking methodology described in literature were used (Morris et al. 1998), with modifications (Vasconcelos et al. 2019; Lopes et al. 2020). Gasteiger partial charges were calculated after addition of all hydrogens for both the ligands and COX-2 structure. Non-polar hydrogens from COX-2 and the identified *A. chica* metabolites were subsequently merged. The dimensions of cubic box in the X-, Y- and Z-axes were 30 x 30 x 30. The grid box was centred on the oxygen atom from the COX-2 Arg120 residue. Initial COX-2 coordinates and *A. chica* compound interactions complexes were chosen based on the criterion of better docking conformation with the lowest energy score, in addition to visual inspection.

2.6 Statistical analysis

The comparison of the means of different experimental groups was performed using Student's t test or a univariate analysis of variance (One- way ANOVA), followed by the Tukey test. A bivariate variance analysis (Two-way ANOVA) was used to assess two sources of variability. A p value <0.05 was considered as indicative of significance and the obtained data were analysed using the GraphPad Prism 7.0 ® software for Windows® (CA, USA).

3. Results

3.1. Inhibition of cyclooxygenase 1 and 2

The *in vitro* inhibition of COX-1 and COX-2 indicates that *A. chica* n-hexane, ethyl acetate and butanol fractions exhibit potential inhibitory activity against both enzyme isoforms, varying in COX-1 inhibition from approximately 28% (butanol fraction) up to 55% (hexane fraction) at the highest tested concentration, of 50 µg/mL. At this same concentration, the tested fractions also inhibited COX-2 enzyme 55% (butanol fraction) and 97% (n-hexane fraction) (Fig. 2). The results also indicate that the n-hexane fraction exhibits better inhibition potential, able to inhibit COX-2 by about 95% at 10 µg/mL (Fig. 2B), and COX-1, by around 46% (Fig. 2A). Thus, the n-hexane fraction inhibited COX-2 over 2-fold compared to COX-1, demonstrating higher selectively for the former.

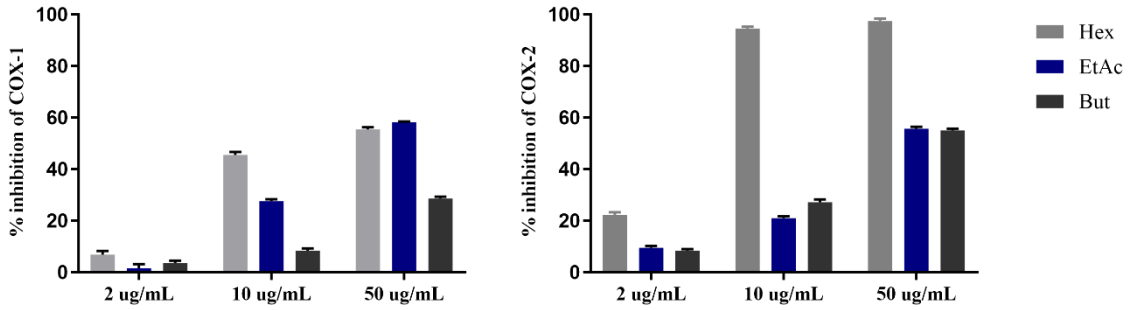

Fig. 2. Percentual *in vitro* inhibition of COX-1 (A) and COX-2 (B), induced by *Arrabidaea chica* n-hexane (Hex), ethyl acetate (EtAc) and butanol (But) fractions, tested at 2, 10 and 50 µg/mL.

3.2. Clinical assessments

3.2.1 Motor Activity/Forced Deambulation (Rotarod Test) evaluation

The deambulation score of all OA-induced groups (CTL-, CTL + Melox, Hex, EtAc and But) was similarly reduced in D7 (Fig. 3) demonstrating effective induction. From D14 after induction, the groups treated with *A. chica* fractions (Hex, EtAc and But) and with meloxicam (CTL + Melox) exhibited significant improvement in motor activity, improving respectively by up to 26.3% and 23.5% when compared to the saline group (CTL-), which received no treatment. On D21, treatment with meloxicam (CTL + Melox) improved motor activity in 27.9%, while the Hex and But fractions both improved motor activity in 35.4% and the EtAc fraction, in 32.6% in relation to the saline group (CTL-). On D28, all treatments also produced significant motor activity improvement in treated animals compared to the saline group (Fig. 3), demonstrating that *A. chica* fractions exhibit analgesic activity and improve the mobility of animals presenting OA.

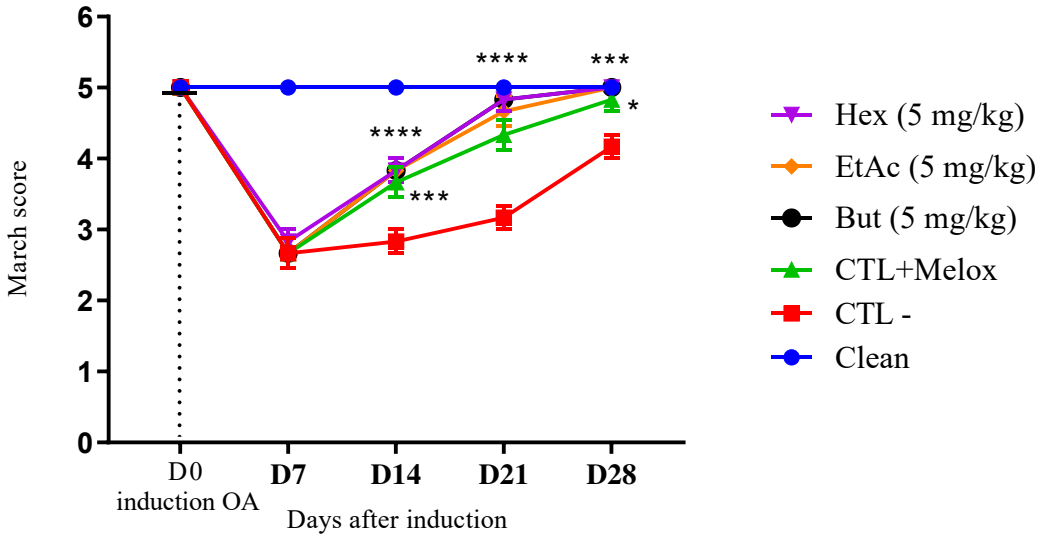

Fig. 3. Effects of *Arrabidaea chica* n-hexane (Hex), ethyl acetate (EtAc) and butanol (But) fractions on the motor activity/forced ambulation of rats presenting induced OA, evaluated using the Rotarod test score. The animals received their respective treatments of oral saline (CTL-), meloxicam (CTL + Melox) and fractions (Hex, EtAc and But - 5 mg/kg) from D3 to D28, and were evaluated on days 7, 14, 21 and 28, after OA induction. Data are represented as means ± standard error of the means (SEM). The healthy group is represented by animals without osteoarthritis and without any treatment (CLEAN). * Represents significant differences, at P <0.05; *** at P <0.0005; **** at P <0.0001 compared to the saline group (CTL-).

3.2.2. Incapacitation/Weight Distribution Test on Hind Legs (Weight Bearing)

The healthy group (CLEAN) exhibited symmetrical support on the two hind limbs after the beginning and at the end of the experiment, with a score of around 50% indicating loss of joint discomfort. The other OA-induced groups (CTL-, CTL + Melo, Hex, EtAc and But) exhibited signs of discomfort on D7, distributing the weight predominantly on the unaffected limb (left side), with marked reduction in weight placed on the affected limb (right side). This pattern demonstrates that OA-induction was efficient. However, on days 14, 21 and 28, the animals in the CTL + Mel, Hex, Acet and But groups displayed significant improvement in weight distribution between the lower limbs and exhibited over 40% of the weight distribution on the affected leg, while the CTL- group exhibited less than 20%. Therefore, the Meloxicam and *A. chica* fraction treatments were able to reduce incapacity compared to the saline group ($p < 0.0001$) (Fig. 4).

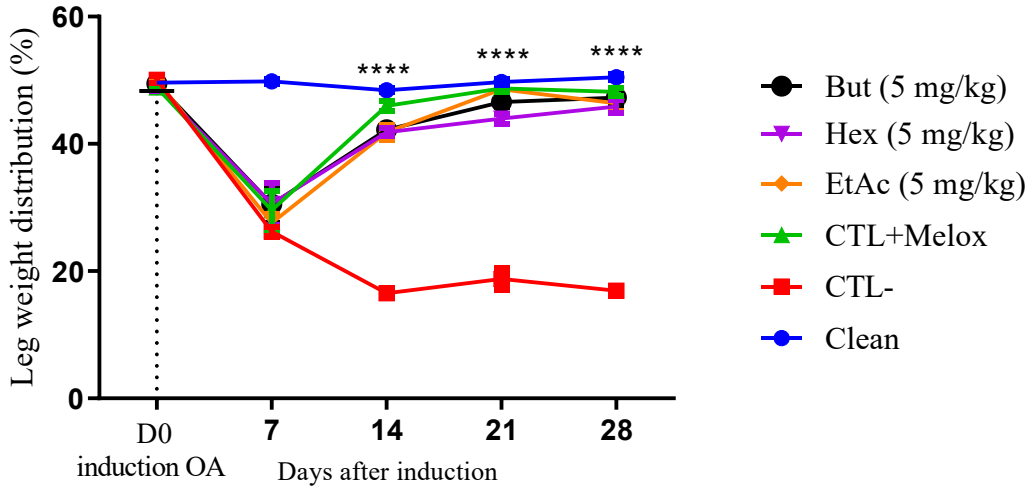

Fig. 4. Effects of *Arrabidaea chica* n-hexane (Hex), ethyl acetate (EtAc) and butanol (But) fractions on degree of incapacitation of rats displaying induced osteoarthritis (OA) evaluated using the Weight Bearing test. The animals received their respective treatments of oral saline (CTL-), meloxicam (CTL + Melox) and fractions (Hex, EtAc and But - 5 mg/kg) from D3 to D28 and were evaluated on days 7, 14, 21 and 28 after OA induction. Data are represented as means ± standard error of the means (SEM). The healthy group is represented by animals without osteoarthritis and without treatment (CLEAN). **** Represents significant differences at $p < 0.0001$ compared to the saline group.

3.2.3 Mechanical hyperalgesia (Randall Selitto test)

On D0, animals were subjected to behavioural testing to determine the basal nociceptive threshold of the hyperalgesia response. At D7, the animals were re-evaluated and a decrease in the nociceptive threshold was observed in all induced groups (CTL-, CTL + Melo, Hex, EtAc and But), characterizing successful OA induction. This decrease was maintained in the CTL-, EtAc and But groups until D14. The Hex group, however, demonstrated an increase in the nociceptive threshold in this period, reaching baseline levels and differing significantly from the saline group ($p < 0.0001$). This group maintained this behaviour both on D21 and D28. The meloxicam-treated group also differed significantly from the saline group on days D14, D21 and D28, but more evident increases in the nociceptive threshold were only observed on D14 and D28, equalling the results of the Hex group (Fig. 5).

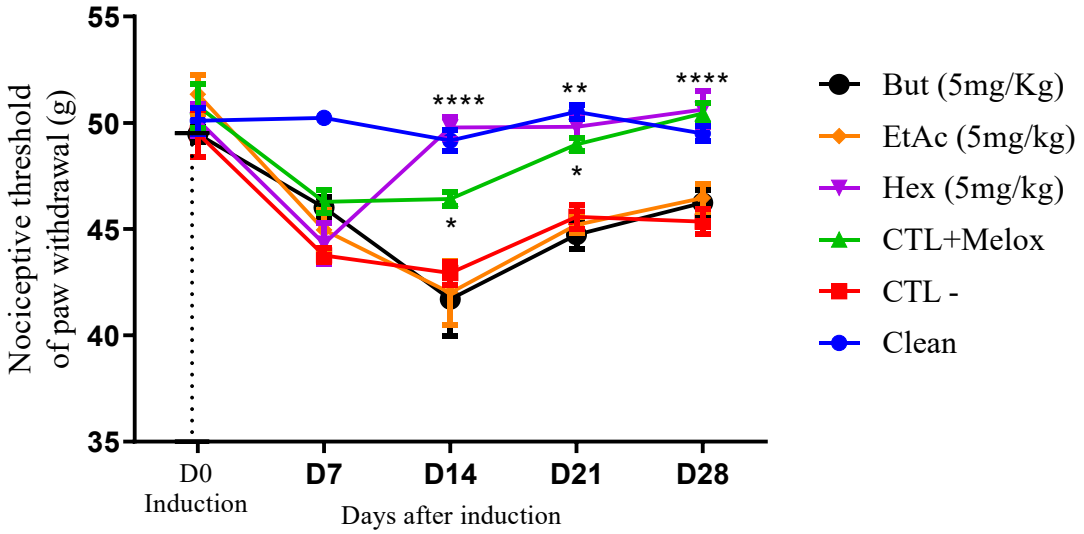

Fig. 5. Effects of *Arrabidaea chica* n-hexane (Hex), ethyl acetate (EtAc) and butanol (But) fractions on mechanical hyperalgesia in rats with induced osteoarthritis (OA) by the nociceptive threshold of affected paw removal, measured by Randall-Sellito test. The animals received their respective treatments of oral saline (CTL-), meloxicam (CTL + Melox) and fractions (Hex, EtAc and But - 5 mg/kg) from D3 to D28 and were evaluated on days 7, 14, 21 and 28 after OA induction. Data are represented as means ± standard error of the means (SEM). The healthy group is represented by animals without osteoarthritis and without treatment (CLEAN). * Represents significant differences, at $p < 0.05$, ** at $p < 0.005$; **** at $P < 0.0001$ compared to the saline group.

3.3 Radiographic analysis

The radiographic analysis indicated that all treatments produced significant improvement ($p < 0.05$) in the degree of articular involvement in the assessed rats (Fig. 6), following the classification proposed by Kellgren-Lawrence. When compared to the saline group (CTL-), which exhibited a high score, the treated rats reached an average score of 3.8 (Fig. 6), with common OA radiographic characteristics, such as reduced joint space, marked subchondral bone sclerosis and osteophyte formation (Fig. 7 CTL-). We also highlight that the groups treated with the *A. chica* Hex and EtAc fractions presented the lowest average scores of joint impairment after OA induction, of 1.2 and 1.4, respectively (Fig. 6). Thus, the common radiographic OA characteristics were much less evident in these groups (Fig. 7, Hex and EtAc).

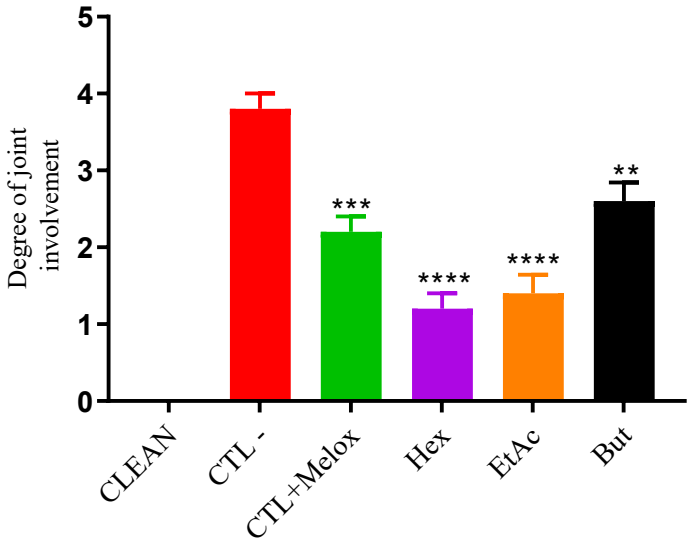

Fig. 6. Degree of joint involvement in rats treated with *Arrabidaea chica* fractions, following the classification proposed by Kellgren-Lawrence. Data are represented as means ± standard error of the means (SEM). CLEAN - group healthy; (CTL-) group presenting induced osteoarthritis (OA); (CTL+Melox) presenting induced OA and treated with Meloxicam; (Hex, EtAc, But) - groups presenting induced OA and treated with *A. chica* hexane, ethyl acetate and butanol fractions, respectively. This analysis was performed with the knees collected on day 29 after OA induction. ** Represents significant differences at $P < 0.005$; *** at $P < 0.0005$, **** at $P < 0.0001$ compared to the saline group (CTL-).

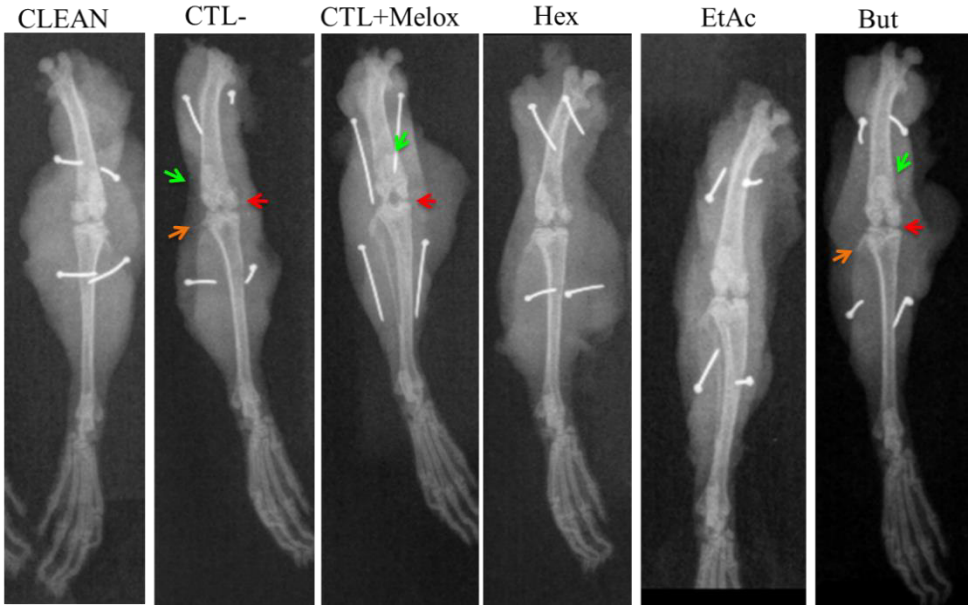

Fig. 7. Effects of *Arrabidaea chica* fractions assessed by radiographic analyses in rats presenting induced osteoarthritis (OA). Representative radiographic images of rat knee joints are shown. CLEAN - healthy; CTL- presenting MIA-induced OA; CTL-+Melox - presenting MIA-induced OA and treated with Meloxicam; Hex, EtAc, But- rats presenting MIA-induced OA and treated with *A. chica* hexane, ethyl acetate and butanol fractions, respectively. This analysis was performed with knees collected on day 29 after OA induction. Arrowhead: red - indicating a remarkable narrowing of the joint space and deformity

in the bone contour; green- subchondral sclerosis; orange - osteophytes. Knee radiographs in antero-posterior projection.

3.4 Histopathological analysis

Considering the classification of the severity or biological progression of the osteoarthritic process into scores as defined by the Osteoarthritis Research Society International (OARSI), treatments with *A. chica* Hex and EtAc fractions exhibited statistical significance in OA progression, with an average rating of 2.9 (± 1.8) for the Hex group and 3.7 (± 1.6) for the EtAc group, both differing significantly from the saline control group (CTL-), which presented a means of 6.0 (± 0.5) (Fig. 8). Thus, the Hex and EtAc fractions were able to decrease OA severity. The But fraction and Meloxicam (CTL + Melox), on the other hand, did not produce significant improvements in OA progression.

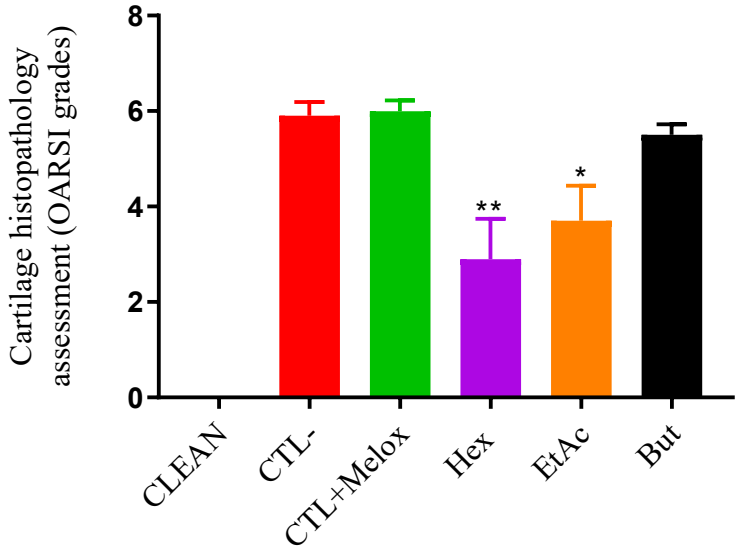

Fig. 8. Histopathological cartilage evaluations, classified by the Osteoarthritis Research Society International (OARSI) scoring system. Data are represented as means ± standard error of the means (SEM). This analysis was performed with the knees collected on 29th day after OA induction. Groups: (CLEAN) - untreated and OA uninduced; (CTL-) presenting induced OA; (CTL+Melox) - presenting induced OA and treated with Meloxicam 0.5 mg/kg; (Hex, EtAc, But) - rats presenting induced OA and treated with *Arrabidaea chica* hexane, ethyl acetate and butanol fractions, respectively. Y-axis: Histopathological evaluation of cartilage (OARSI histological classification: Grade 0 – surface intact and cartilage intact; Grade 1 – surface intact; Grade 2 – surface discontinuity; Grade 3 – vertical fissures; Grade 4 – erosion; Grade 5 – denudation; and Grade 6 – deformation). *Represents significant differences, at $p < 0.05$, ** at $p < 0.005$ compared to the saline group (CTL-).

The OARSI classification scores attributed to animals in the groups treated with Hex (Fig. 9C) and EtAc (Fig. 9D) indicated only changes at the articular cartilage level, without changes to the subchondral bone, observed in the CTL- (Fig. 9B), But (Fig. 9E) and CTL + Melox (Fig. 9G) groups, where the cartilage

was completely corroded and microfracture, repair and bone remodelling processes altered articular surface contour.

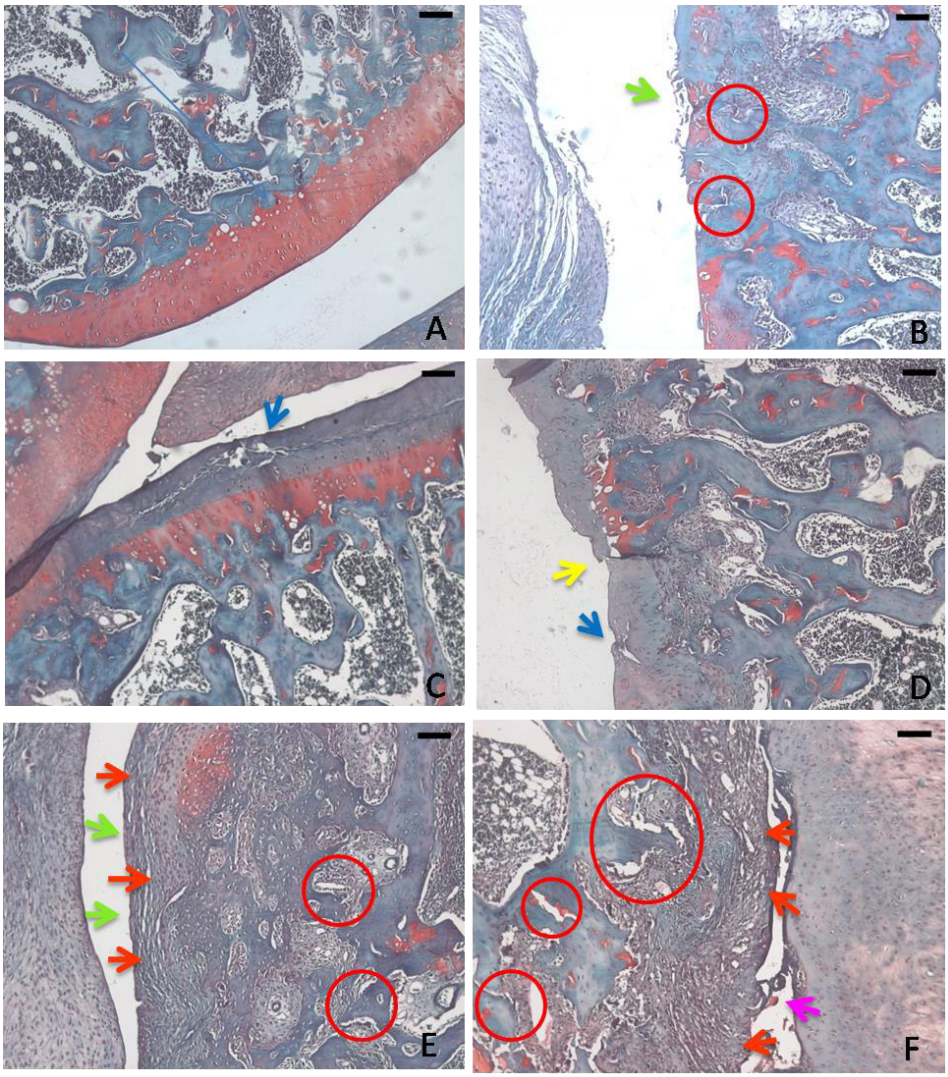

Fig. 9. Articular cartilage sections exhibiting different degenerative change grades in osteoarthritis (OA) classified using the Osteoarthritis Research Society International (OARSI) scoring system. This analysis was performed with knees collected on day 29 after OA induction. (A) example of a CLEAN group representative, untreated and OA uninduced, exhibiting normal healthy cartilage, (Grade 0- surface and cartilage intact); (B) example of a (CTL-) group OA-induced representative treated with 0.9% saline (Grade 6 - deformation); (C) example of a Hex group OA-induced representative and treated with the *Arrabidaea chica* n-hexane fraction (5 mg/kg), (Grade 2.9 on average - vertical fissures); (D) example of an EtAc group OA-induced representative and treated with the *Arrabidaea chica* ethyl acetate fraction (5 mg/kg) - (Grade 3.7 on average - erosion); (E) example of a But group group OA-induced representative and treated with the *Arrabidaea chica* butanol fraction (5 mg/kg), (Grade 5.5 on average – denudation, with reparative tissue surface present); (F) example of a CTL + Melox group group OA-induced representative and treated with meloxicam 0, 5mg/kg (Grade 6 - deformation). Green arrows indicate hyaline cartilage absence; the red circle indicates a microfracture; the yellow arrow indicates erosion; the blue arrow indicates cracks; red

arrows indicate bone plates with fibrocartilage repair resulting in microfractures and the pink arrow indicates joint contour deformation. (O-safranin stain; Bar = 100 µm; 200×).

Histopathological liver, spleen and kidney analyses indicate that the *A. chica* Hex, EtAc and But fractions orally administered to the rats at 5 mg/kg for 26 consecutive days did not produce any pathological changes (Fig. 10), thus demonstrating that the administered doses do not induce toxicity.

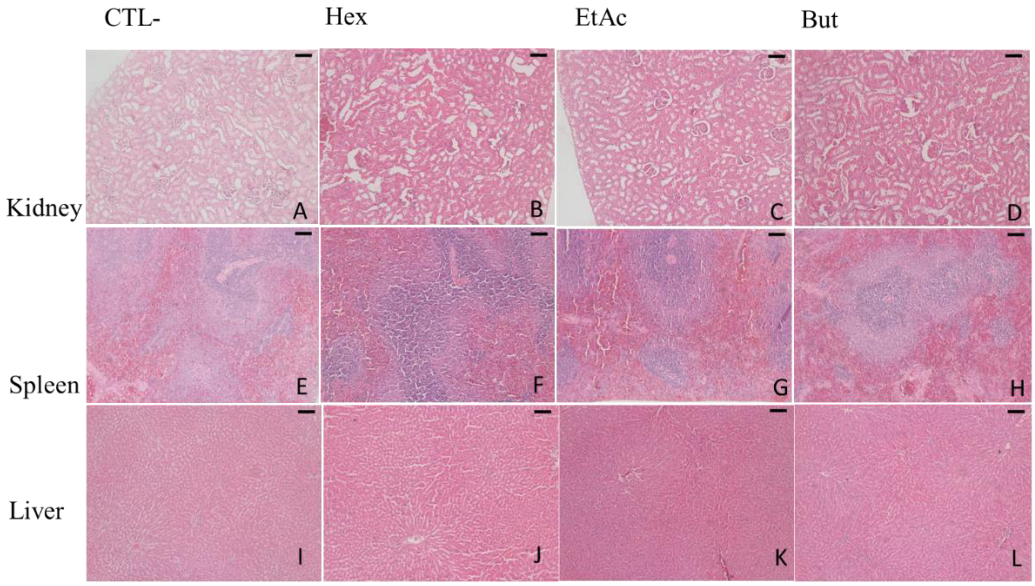

Fig. 10. Representative histological kidney (A, B, C), spleen (D, E, F) and liver (G, H, I) sections of groups treated with *Arrabidaea chica* n-hexane (Hex), ethyl acetate (EtAc) and butanol (But) fractions. The images show no signs of ischemia, necrosis, cell injury and/or inflammation (Hematoxylin-Eosin—HE stains, Bar = 100 µm, 100×).

3.6 Chemical analysis

GC-MS analysis allowed for the identification of 20 compounds in the n-hexane *A. chica* fraction (Figs. 11 and 12/Table 1), classified as fatty acids, terpenes and phytosterols.

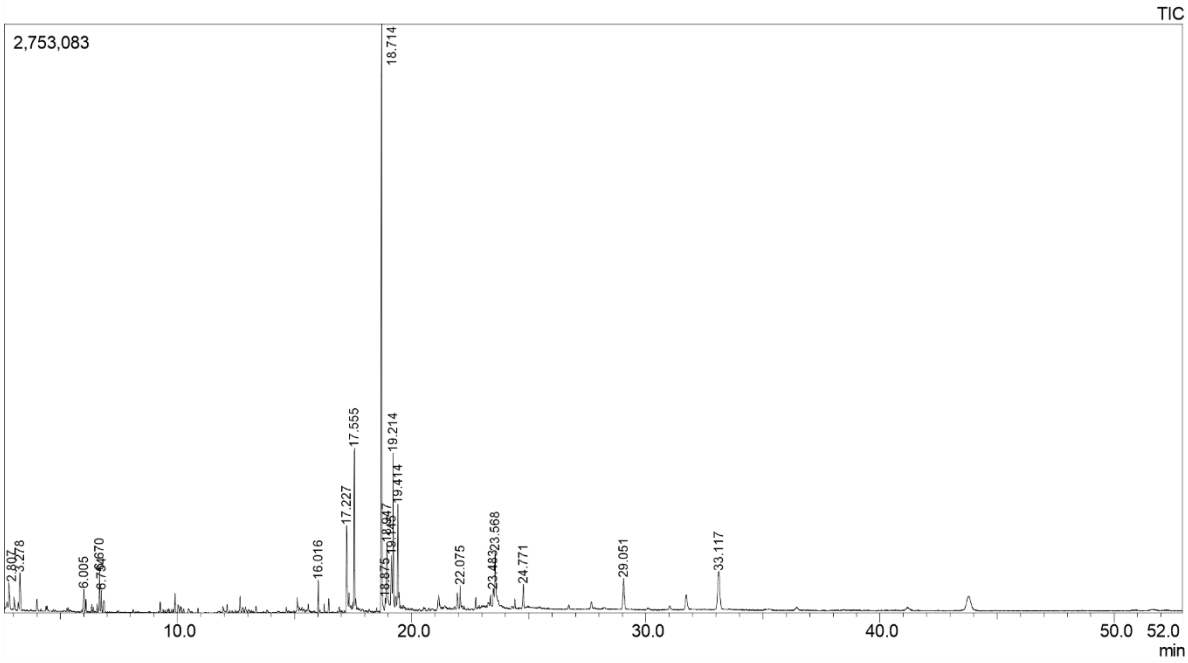

Fig. 11. Total ion chromatogram of the n-hexane *A. chica* fraction.

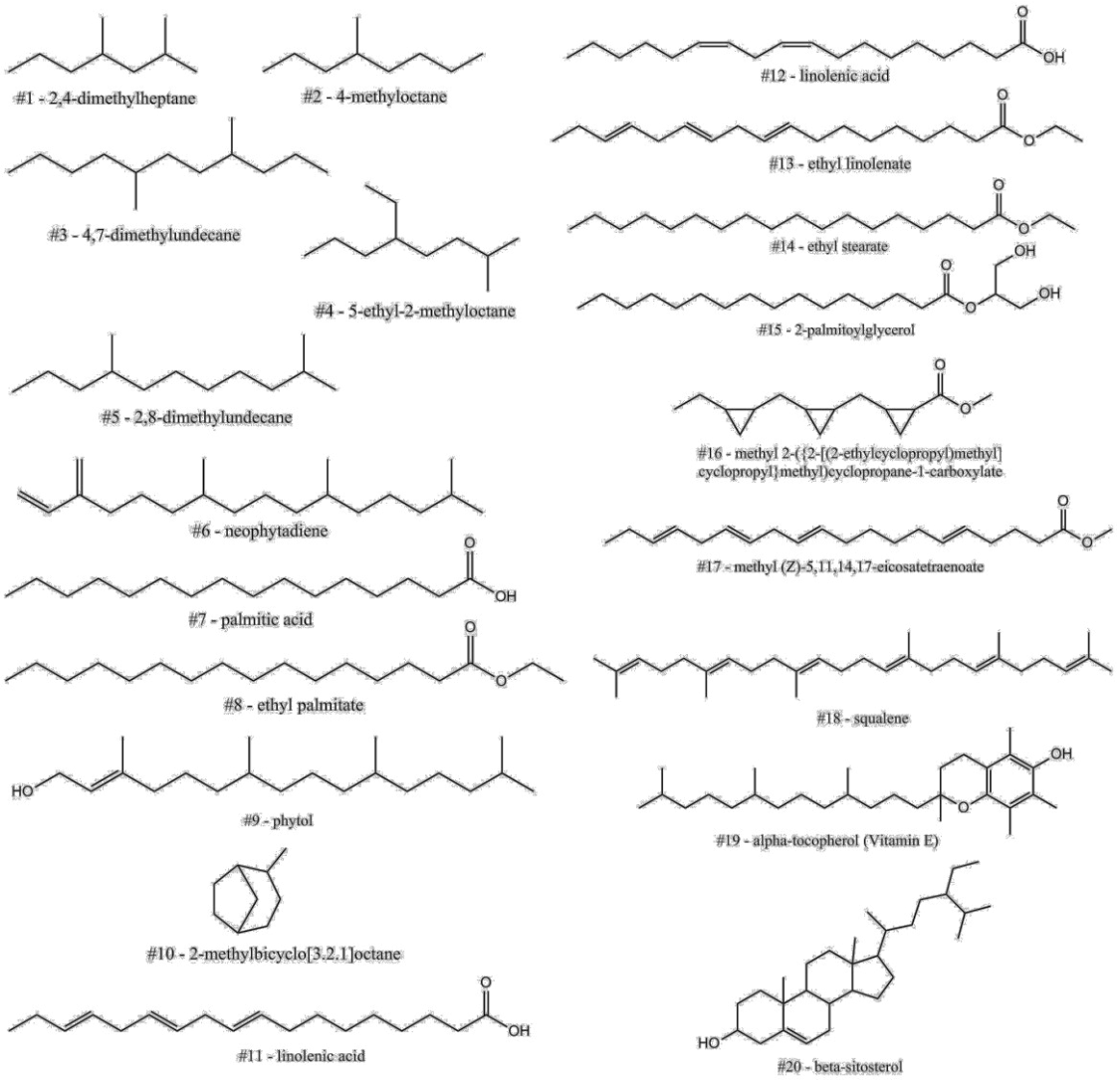

Fig. 12. Chemical structures of the compounds identified by GC-MS in the A. chica n-hexane fraction.

Table 1. Gas chromatography–mass spectrometry (GC/MS) analysis of the *A. chica* n-hexane fraction.

Peak#	Retention Time	m/z	Area (%)	Identified compounds
1	2.807	128.25	1.78	2,4-Dimethylheptane
2	3.278	128.25	2.56	4-Methyloctane
3	6.005	184.36	1.01	4,7-Dimethylundecane
4	6.670	156.31	2.40	5-Ethyl-2-Methyloctane
5	6.754	184.36	1.21	2,8-Dimethylundecane
6	16.016	278.50	1.62	Neophytadiene
7	17.227	256.42	5.28	Palmitic Acid
8	17.555	284.50	8.65	Ethyl palmitate
9	18.814	296.5	31.82	Phytol
10	18.875	124.22	1.12	2-Methylbicyclo[3.2.1]octane
11	18.947	278.40	5.46	Linolenic Acid
12	19.145	280.40	5.73	Linoleic Acid
13	19.214	306.50	9.70	Ethyl linolenate
14	19.414	312.50	7.12	Ethyl stearate
15	22.075	330.50	1.26	2-Palmitoylglycerol
16	23.483	263.35	1.16	Methyl 2-({2-[(2-ethylcyclopropyl)methyl]cyclopropyl}methyl)cyclopropane-1-carboxylate
17	23.568	318.50	3.22	Methyl (Z)-5,11,14,17-eicosatetraenoate
18	24.771	410.70	1.52	Squalene
19	29.051	430.70	2.93	alpha-tocopherol (Vitamin E)
20	33.117	414.70	4.45	beta-Sitosterol

3.7 In silico analysis

All metabolites identified by GC-MS in the *A. chica* n-hexane fraction were used for the molecular docking analysis. In general, all metabolites exhibited satisfactory parameter affinity to the COX-2 structure. Molecular docking of the NSAID meloxicam was also performed, with an affinity parameter of -9.1 kcal/mol for free binding energy, while the best affinity parameters among the n-hexane *A. chica* fraction metabolites were found for alpha-tocopherol (Vitamin E), squalene and beta-sitosterol, with free binding energies of -10.4, -10.4 and -9.8 kcal/mol, respectively, superior to the parameters obtained for meloxicam. The results of the binding energy values of all compounds are presented in Table 2.

Table 2. Free-binding energies by molecular docking of the compounds identified in *A. chica* n-hexane fraction displaying affinity to the COX-2 structure.

Ligand	**ΔG_{bind} (kcal/mol)**
Alpha-tocopherol (Vitamin E)	-10.4
Squalene	-10.4
beta-Sitosterol	-9.8
Methyl 2-({2-[(2-ethylcyclopropyl)methyl]cyclopropyl}methyl)cyclopropane-1-carboxylate	-8.1
Linoleic Acid	-8.1
Methyl (Z)-5,11,14,17-eicosatetraenoate	-8.0
Ethyl linolenate	-7.9
Phytol	-7.6
Linolenic Acid	-7.5
Neophytadiene	-7.5
Ethyl palmitate	-7.0
Ethyl stearate	-6.9
2,8-Dimethylundecane	-6.8
Palmitic Acid	-6.7
4,7-Dimethylundecane	-6.7
5-Ethyl-2-Methyloctane	-6.0
2-Methylbicyclo[3.2.1]octane	-6.0
2,4-Dimethylheptane	-5.9
4-Methyloctane	-5.4
2-Palmitoylglycerol	-5.1
Meloxicam	-9.1

Both alpha-tocopherol, squalene and beta-sitosterol performed only van der Walls interactions with amino acids residues from the COX active site, such as Pro86, Val89, Leu93, Tyr115, Val116, SEr119, Arg120, Thr206, Tyr348, Val349, Leu352, Ser353, Tyr355, Phe357, Leu359, Phe381, Leu384, Tyr385, Trp387, Met522, Val523, Gy526, Ala527, Ser530 and Leu531. Our docking results successfully predicted the ligand position, as alpha-tocopherol, squalene and beta-sitosterol remained in the same region where traditional COX-2 inhibitor drugs, such as Naproxen, Indomethacin and Meloxicam, perform interactions with COX-2 residues (Fig. 12).

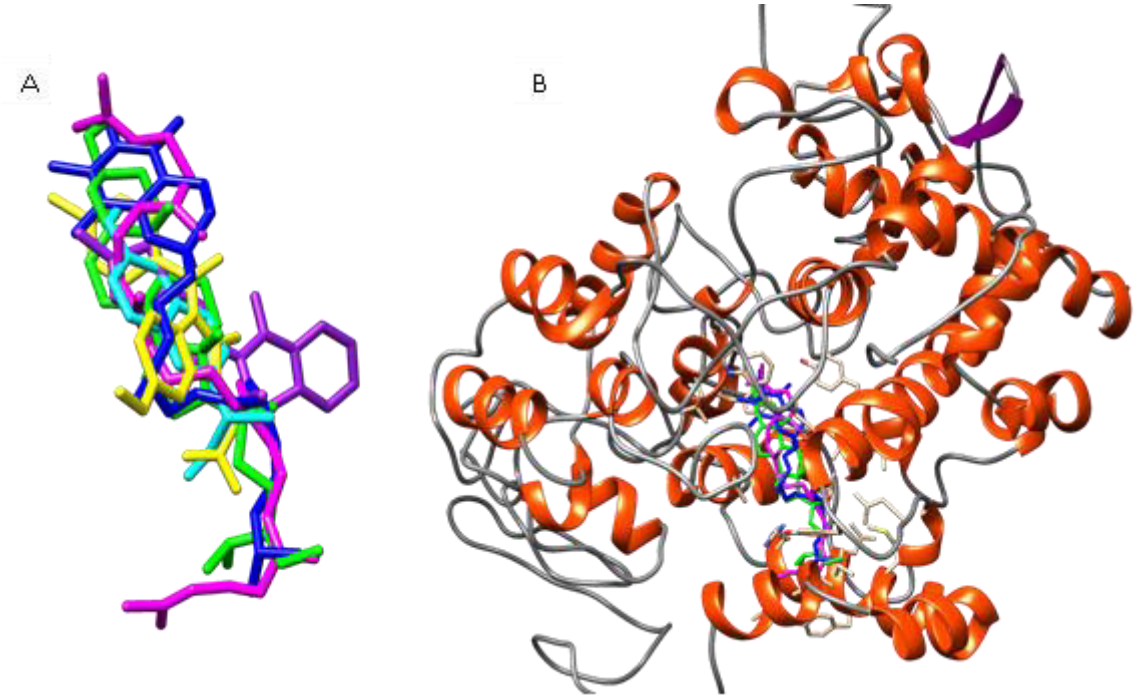

Fig. 12. (A) Spatial docking conformations from alpha-tocopherol (blue), squalene (magenta) and beta-sitoesterol (green), compared to indomethacin (yellow; PDB ID 4COX), naproxen (cyan; PDB ID 3NT1) and meloxicam (purple; PDB ID 4M11) and (B), COX-2 active site ligands.

4. Discussion

The assays carried out in the present study indicate that *A. chica* extract fractions, especially the n-hexane fraction, exhibit significant analgesic and anti-inflammatory properties, thus representing a potent ally in OA-treatment.

OA is a strongly associated with joint pain, resulting mainly from damage induced by an intense inflammatory process. In this regard, it is important to highlight the role of COX-2 in the inflammatory pathway, which is an essential enzyme in this pathway, as it leads to the generation of potent proinflammatory mediators, i.e. prostaglandins. COX-2 is usually expressed at low levels in normal tissues, and highly expressed under inflammatory conditions (Chikanza and Fernandes 2000; Queiroga et al. 2007).

Considering the role of this enzyme and its significance in OA, we evaluated the potential of the fractions obtained from the *A. chica* extracts in *in vitro* COX-2 inhibition. The results indicate that the Hex fraction exhibits significant COX-2 inhibitory activity, inhibiting COX-2 in approximately 95% at a concentration of 10 µg/mL (Fig. 2B), and COX-1 in around 46% (Fig. 2A), demonstrating greater selectivity for COX-2. The EtAc and But fractions also displayed the ability to inhibit both COX-1 and COX-2, although they exhibited similar affinity to both enzymes, and not only COX-2, which is more effectively involved in inflammatory processes.

Previous studies carried out by our research group also evaluated the potential of a hydroethanolic *A. chica* extract in inhibiting these enzymes, which exhibited lower inhibitory potential, of around only 30% for the highest tested concentration of 50 µg/mL, and showed similar affinity to both enzymes (Vasconcelos et al. 2019). One hypothesis that could justify the greater success of the Hex fraction in inhibiting COX-2 in present study is its chemical composition, since fractionation allows for the separation

of compounds found in the total extract, according to their properties (Alves Ferreira 2018). The Hex fraction can potentially contain a higher amount of non-polar compounds, such as lipids, terpenes and sterols (Zapata et al. 2011; Pinheiro et al. 2013). Most non-polar compounds exhibit important anti-inflammatory activities (Jeong et al. 2014; Morales-Del-Rio et al. 2015; Othman et al. 2015).

The analgesic and anti-inflammatory potential of the Hex, EtAc and But fractions was also evaluated *in vivo* using a MIA-induced knee joint OA animal model. MIA is a glyceraldehyde–3–phosphate dehydrogenase inhibitor, which interrupts glycolysis, resulting in chondrocyte death, neovascularization, subchondral bone necrosis and collapse, in addition to inflammation and several types of joint damage. Thus, MIA injections induce mechanical sensitivity in injected limbs, resulting in measurable and quantifiable weight support deficits (Bendele 2001; Marker and Pomonis 2012; Pitcher et al. 2016). Because of this, MIA injection is considered a very useful model to test the effectiveness of pharmacological agents in pain treatment, as it generates reproducible and robust data resembling OA symptoms in humans, validating MIA injections as a useful and significant preclinical model for OA assessments (Im et al. 2010; Kelly et al. 2012; Takahashi et al. 2018).

After OA induction, rats were then subjected to behavioural tests to determine the antinociceptive potential of *A. chica* fractions against OA. Animal mobility was evaluated through the rotarod test, considering affected paw use by forced walking.

As joint movement is significantly affected in individuals presenting OA, due to pain, their ability to perform physical activities is greatly reduced (Bhatia et al. 2013). The mobility test indicated that animals treated with Hex, EtAc and But *A. chica* fractions exhibited significant motor activity improvement from the 14th day of evaluation, maintained until the final evaluation period (D28) (Fig. 3), demonstrating that *A. chica* fractions exhibit potential analgesic activity, resulting in mobility improvement in animals presenting OA.

We also demonstrated the effect of *A. chica* fractions against pain relief through the functional disability test - Weight Bearing. The antinociceptive results were determined by the animal's weight distribution between the unaffected (left paw) and affected (right paw, presenting OA induction) hind limbs. On days 14, 21 and 28, the animals in the groups treated with Hex, EtAc and But *A. chica* fractions, exhibited significant improvement in hind limb weight distribution, with over 40 % of weight distribution put on the affected leg, almost the same as the values observed for the healthy group (CLEAN) (Fig. 4). This demonstrated that *A. chica* fractions significantly reduced affected limb impairment. Mechanical hyperalgesia was also evaluated *in vivo* using the Randall-Selitto test, where only the Hex fraction produced significant analgesic effects compared to the saline group (CTL-), as the Hex group demonstrated a significant nociceptive threshold increase the on the 14th, 21st and 28th assessment days (Fig. 5).

The analgesic potential of *A. chica* fractions demonstrated herein may be attributed to their ability to inhibit COX-2, decreasing the inflammatory process and, consequently, improving OA prognosis, avoiding or reducing pain. This is due to the fact that, by intervening in the COX-2 pathway, the compounds present in this fraction may modulate the entire inflammatory process, thus producing analgesic effects. Hwang et al. (Hwang et al. 2018) reported that decreases in COX-2 and iNOS mRNA and protein levels caused by a hydroethanolic *Zanthoxylum piperitum* (L.) DC (Rutaceae) extract (90%) (100 mg/kg), significantly reduced inflammation and produced remarkable pain relief in animals presenting MIA-

induced OA. The decreases in COX-2 and PGE2 expressions may also decrease aggrecan and collagen II degradation in human chondrocytes presenting OA, thus justifying OA improvement (Jiang et al. 2019).

The radiographic data of OA-affected paws also demonstrated that the meloxicam and hexane, ethyl acetate and butanol *A. chica* fraction treatments produced significant improvements in joint involvement degrees, with the Hex and EtAc *A. chica* fractions exhibiting the lowest average joint involvement scores. Concerning the histopathological evaluation, only the Hex and EtAc fractions resulted in significant improvement in the severity or biological progression of the osteoarthritic process.

These findings suggest that the Hex and EtAc fractions exhibit a significant degree of joint protection, decreasing joint degradation, probably due to cartilage inflammation effects through COX-2 inhibition. The COX-2-dependent prostaglandin PGE2 plays an essential role in OA pathogenesis, as it may interfere with chondrocyte proliferation and prevent extracellular matrix production (Goggs et al. 2003; Li et al. 2010). In this context, COX-2 inhibition can decrease the structural changes that occur during OA progression (Hardy et al. 2002; Li et al. 2010; Rasheed et al. 2016). Lee et al. (Lee et al. 2019) corroborated this hypothesis by demonstrating that COX-2 and metalloproteinase (MMP-2 and MMP-9), suppression by an ethanolic *Mollugo pentaphylla* L. (Molluginaceae) extract in knee joint tissues of rats presenting MIA-induced OA contributed to decreased inflammation and cartilage damage severity observed through histological analyses.

In analgesic terms, the fractions tested herein, especially the Hex fraction, resulted in similar effects as the hydroethanolic *A. chica* extract tested previously (Vasconcelos et al. 2019). However, histological analyses indicate that this fraction was able to prevent OA progression, which was not observed for the hydroethanolic extract (Vasconcelos et al. 2019). Thus, we focused on this fraction.

Considering the results of the *in vitro* and *in vivo* tests, the *A. chica* n-hexane fraction exhibited the most promising results. Therefore, a chemical analysis of this fraction was carried out, in order to investigate its composition. A total of 20 compounds were identified, mostly classified as fatty acids, with the presence of some terpenes and phytosterols (Table 1). Phytol was the major compound of the Hex fraction, although its presence in the n-hexane fraction of *A. chica* was also reported by Rodrigues et al. (Rodrigues et al. 2014), who also identified the presence of linolenic and linoleic acids and vitamin E. Phytol is a acyclic diterpene exhibiting several pharmacological properties, including anti-inflammatory, antioxidant and antinociceptive activities (Santos et al. 2013; Silva et al. 2014; Islam et al. 2018; Carvalho et al. 2020).

Carvalho et al. (Carvalho et al. 2020) demonstrated that phytol was able to decrease the formation of joint oedema and hyperalgesia, reduce myeloperoxidase activity and the release of pro-inflammatory cytokines (TNF-α and IL-6) in synovial fluid, IL-6 and COX-2 in the spinal cord and alter the p38MAPK and NF-κB signalling pathways, indicating that phytol can be a potent antiarthritic agent. Santos et al. (Santos et al. 2013) also demonstrated the antinociceptive and anti-inflammatory effects of phytol in the acetic acid-induced abdominal contortion test, where this compound was able to significantly reduce the number of animal contortions, as well as decrease the time spent on paw licking in the formalin test, both in the neurogenic and in the inflammatory phase; while latency time increased in the hot plate test, also suggesting central action. In addition, the authors also reported a strong antioxidant *in vitro* phytol effect. These findings demonstrate the pharmacological importance of phytol and allow us to suggest that the

analgesic and anti-inflammatory action of the hexane fraction demonstrated in the present study may be associated with this compound.

The chemical analysis of the n-hexane *A. chica* fraction evidenced a high amount of fatty acids and triterpenes. These classes are traditionally recognized as antinociceptive and anti-inflammatory agents. According to the positive *in vitro* and *in vivo* results, an *in-silico* study was carried out to assess potential mechanisms of action of this fraction. Thus, molecular docking analyses for all compounds identified by CG-MS in the n-hexane *A. chica* fraction were performed against the 3D structure of COX-2. The results indicate that alpha-tocopherol, squalene and beta-sitosterol are the metabolites demonstrating the best affinity parameters with COX-2.

The active COX-2 site comprises the Arg120, Tyr355, Tyr385, Glu524 and Ser530 residues, where arachidonic acid binds, thus forming prostaglandins (Rowlinson et al. 2003; Xu et al. 2014). Arachidonic acid binds and interacts in this region, chaining prostaglandins production (Rowlinson et al. 2003; Xu et al. 2014; Lopes et al. 2019). The molecular docking assay indicated that all selected ligands interacted with this group of residues, as well as with neighbouring residues, where the spatial conformations of plant metabolites at the active site of COX-2 were similar to the conformations obtained in X-ray crystallography studies from traditional NSAIDs such as naproxen, meloxicam and indomethacin. This suggests that molecular docking was successful in predicting the spatial conformation of the interaction of these metabolites with COX-2.

Squalene is a polyunsaturated triterpene described as a biochemical precursor of cholesterol and other steroids that has exhibited antioxidant activities detected by the DPPH and ABTS methods (Warleta et al. 2010), thus considered an efficient free radical scavenger. This molecule has been commonly used in various pharmacological applications, such as in the delivery of vaccines, drugs, and other medicinal substances (Reddy and Couvreur 2009; Feng et al. 2019). At low concentrations (25 and 50 μM), squalene significantly reduced COX-2, NO and iNOS expressions in LPS-induced murine macrophages. It also reduced NF-κB gene mRNA and pro-inflammatory cytokine precursor gene expressions, such as TNF-α, IL-1β in human monocytes and TNF-α, IL-1β, IL-6 and IFN- γ in human neutrophils. Reduced iNOS and COX-2 gene expressions have also been reported in LPS-induced human monocytes treated with squalene (Cárdeno et al. 2015).

Alpha-tocopherol has been reported as decreasing the release of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α and the chemokine IL-8, promoting decreased adhesion of monocytes to endothelium and reducing cytokine (IL-1β and TNF-α) production from LPS-activated PBMCs (Singh and Jialal 2004). It has also been evidenced that this molecule improved the clinical symptoms of patients with severe OA due to the reduction of oxidative stress caused by OA progression, indicating significant antioxidant potential (Machtey and Ouaknine 1978; Tantavisut et al. 2017). In addition, alpha-tocopherol is also efficient in slowing the speed of natural OA progression, protecting the subchondral vascular system, reducing necrosis and bone remodelling (Li et al. 2016). The recognized antioxidant activity of this molecule has been associated with inhibition of the NF-kB pathway and the release of other pro-inflammatory mediators (Chin and Ima-Nirwana 2018). Alpha-tocopherol has also been shown to improve the histological score of joints with a significant increase in the amount of proteoglycans in the articular cartilage (Ozkan et al. 2015). Likewise, the present study indicates articular cartilage improvements in

animals treated with the *A. chica* n-hexane fraction. In addition, scanning electron microscopy also evidenced that the articular cartilage of OA rats displayed less damage to the matrix and collagen contents after treatment with alpha-tocopherol (Heidar et al. 2014).

β-sitosterol is a phytosterol, a class of compounds exhibiting extensive reported biological activities(Liu et al. 2019). β-sitosterol has been reported to significantly reduce the production of nitric oxide (NO), and the release of TNF-α, in addition to COX-2 and iNOS expression and activity (Yuan et al. 2019; Sun et al. 2020), the NF-κB pathway and IL-6 expression (Sun et al. 2020).

5. Conclusions

This study demonstrated that *A. chica* fractions display analgesic, anti-inflammatory properties and are able to intervene in OA progression. The assessed fractions inhibited the enzymes COX-1 and COX-2, while the n-hexane fraction was also able to inhibit over 90% of COX-2, an enzyme significantly involved in the OA inflammatory process. Concerning nociceptive activity, the *A. chica* fractions produced significant incapacity, motor activity and hyperalgesia improvements in rats presenting induced OA. The radiological and histopathological analyses indicated fraction ability to intervene in OA progression, with the n-hexane and ethyl acetate fractions noteworthy for significantly reducing joint impairment degree and OA severity. Finally, the chemical analysis of the hexane fraction demonstrated that this fraction contains compounds with significant analgesic and anti-inflammatory potential. An *in silico* evaluation indicated very favourable interactions between some of these compounds and COX–2. Our results explain the traditionally recognized use of *A. chica* by Brazilian populations, indicating potentially safe use and suggesting that it may be the subject of clinical research studies.

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5. CONSIDERAÇÕES FINAIS

Os resultados apresentados neste estudo fornecem evidências que a *A. chica* pode ser um importante plano terapêutico para o tratamento da OA. Visto que o extrato e frações mostraram–se capaz de inibir a enzima COX–2, alvo importante da ação de fármacos analgésicos e anti-inflamatórios. Bem como, o tratamento oral, por 26 dias consecutivos, com extrato hidroetanólico de *A. chica* (50, 150 e 450 mg/Kg) e frações (hexânica, acetato de etila e butanólica – 5mg/Kg) apresentaram atividade antinociceptiva em modelo experimental de OA, induzido por MIA.

Contudo, os dados obtidos nas análises radiológicas e histopatológicas, não são conclusivos sobre a ação do extrato hidroetanólico de *A. chica* na progressão da OA, pois alguns conflitos entre os dados destas duas análises foram evidenciados, confirmando a necessidade de mais estudos. Entretanto, as mesmas análises nos ensaios com as frações indicam que a fração hexânica e acetato de etila, reduziram significativamente a gravidade ou progressão biológica do processo osteoartrítico.

Os resultados do presente estudo evidenciaram ainda, através das análises químicas, que o extrato de *A. chica* é rico em flavonoides, os quais possuem um vasto potencial biológico, comprovado cientificamente, dentre os quais se destaca a capacidade de reduzir a produção de citocinas pró–inflamatórias, podendo auxiliar no tratamento da OA. E que a fração hexânica, que se destacou com melhor potencial para tratar OA, em relação às demais frações testadas, é composta por uma série de ácidos graxos, terpenos e fitoesteróis, os quais também possuem importantes atividades biológicas já registradas na literatura.

O presente estudo também aponta através dos ensaios *in silico* uma possível via de ação dos compostos presentes no extrato hidroetanólico e na fração hexânica de *A. chica*. Sugerindo que através da interação com a ciclo-oxigenase –2 estes compostos reduzem o processo inflamatório e melhoram o quadro da OA.

E por fim, este estudo fornece dados importantes para formulação de um bioproduto, que seja útil para o tratamento da OA. Além de fomentar novos estudos, que investiguem, por exemplo, outros possíveis mecanismos de ação para as moléculas aqui identificadas, e até mesmo seu uso em outras condições patológicas, em que o processo inflamatório seja relevante.

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Apêndice

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ORIGINAL RESEARCH
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Antinociceptive Activity of *Borreria verticillata*: *In vivo* and *In silico* Studies

Rosa H. M. Silva[1], Nathália de Fátima M. Lima[1], Alberto J. O. Lopes[1], Cleydlenne C. Vasconcelos[1], José W. C. de Mesquita[2], Ludmilla S. S. de Mesquita[2], Fernando C. V. M. Lima[1], Maria N. de S. Ribeiro[2], Ricardo M. Ramos[3], Maria do Socorro de S. Cartágenes[1]* and João B. S. Garcia[4]**

[1] Experimental Study of Pain Laboratory, Department of Physiological Sciences, Federal University of Maranhão, São Luís, Brazil, [2] Laboratory of Pharmacognosy, Department of Pharmacy, Federal University of Maranhão, São Luís, Brazil, [3] Research Laboratory Information Systems, Department of Information, Environment, Health and Food Production, Federal Institute of Piauí, Teresina, Brazil, [4] Experimental Study of Pain Laboratory, Department of Pain and Palliative Care, Federal University of Maranhão, São Luís, Brazil

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Edited by:
Thomas J. Anastasio,
University of Illinois at Urbana–Champaign, United States

Reviewed by:
Haroon Khan,
Abdul Wali Khan University Mardan, Pakistan
Birendra N. Mallick,
Jawaharlal Nehru University, India
Paola Patrignani,
University of Chieti-Pescara, Italy
Heinrich Korner,
University of Tasmania, Australia

***Correspondence:**
Rosa H. M. Silva
Lenna1911@hotmail.com
Maria do Socorro de S. Cartágenes
scartagenes@gmail.com
João Batista Santos Garcia
jbgarcia@uol.com.br

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Borreria verticillata (L.) G. Mey. known vassourinha has antibacterial, antimalarial, hepatoprotective, antioxidative, analgesic, and anti-inflammatory, however, its antinociceptive action requires further studies. Aim of the study evaluated the antinociceptive activity of *B. verticillata* hydroalcoholic extract (EHBv) and ethyl acetate fraction (FAc) by *in vivo* and *in silico* studies. *In vivo* assessment included the paw edema test, writhing test, formalin test and tail flick test. Wistar rats and Swiss mice were divided into 6 groups and given the following treatments oral: 0.9% NaCl control group (CTRL), 10 mg/kg memantine (MEM), 10 mg/kg indomethacin (INDO), 500 mg/kg EHBv (EHBv 500), 25 mg/kg FAc (FAc 25) and 50 mg/kg FAc (FAc 50). EHBv, FAc 25 and 50 treatments exhibited anti-edematous and peripheral antinociceptive effects. For *in silico* assessment, compounds identified in FAc were subjected to molecular docking with COX-2, GluN1a and GluN2B. Ursolic acid (UA) was the compound with best affinity parameters (binding energy and inhibition constant) for COX-2, GluN1a, GluN2B, and was selected for further analysis with molecular dynamics (MD) simulations. In MD simulations, UA exhibited highly frequent interactions with residues Arg120 and Glu524 in the COX-2 active site and NMDA, whereby it might prevent COX-2 and NMDA receptor activation. Treatment with UA 10 mg/Kg showed peripheral and central antinociceptive effect. The antinociceptive effect of *B. verticillata* might be predominantly attributed to peripheral actions, including the participation of anti-inflammatory components. Ursolic acid is the main active component and seems to be a promising source of COX-2 inhibitors and NMDA receptor antagonists.

Keywords: ***Borreria verticillata,*** **COX-2, NMDA receptor, molecular docking, molecular dynamics simulations**

INTRODUCTION

Pain is a warning system that informs the body about the occurrence of tissue damage (Nickel et al., 2012). In the pathophysiology of pain several biological actions are involved, including activation of cyclooxygenase 2 enzyme (COX-2) and N-methyl-D-aspartate (NMDA) receptor.

COX-2 is upregulated in the central nervous system in response to inflammatory factors. It is a rate-limiting enzyme for prostanoid production during inflammation

COMPUTATIONAL AND EXPERIMENTAL APPROACHES IN MULTI-TARGET PHARMACOLOGY

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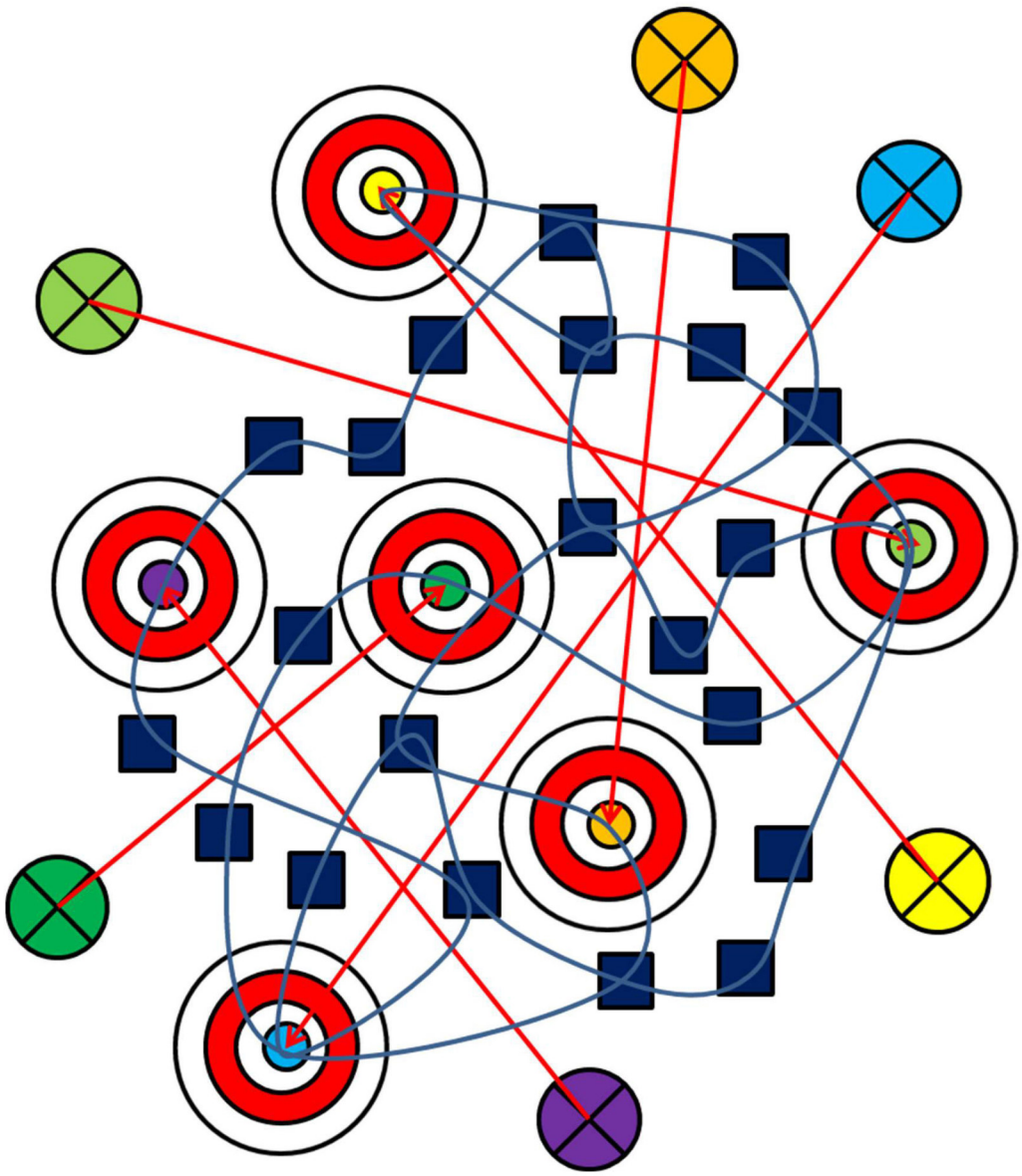

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frontiers in Microbiology

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REVIEW
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Candida Infections and Therapeutic Strategies: Mechanisms of Action for Traditional and Alternative Agents

*Giselle C. de Oliveira Santos[1†], Cleydlenne C. Vasconcelos[1†], Alberto J. O. Lopes[2], Maria do S. de Sousa Cartágenes[2], Allan K. D. B. Filho[3], Flávia R. F. do Nascimento[4], Ricardo M. Ramos[5], Emygdia R. R. B. Pires[6], Marcelo S. de Andrade[2], Flaviane M. G. Rocha[7] and Cristina de Andrade Monteiro[7,8]**

[1] Programa de Doutorado em Biotecnologia da Rede Nordeste de Biotecnologia (RENORBIO), Universidade Federal do Maranhão, São Luís, Brazil, [2] Postgraduate Program in Health Sciences, Universidade Federal do Maranhão, São Luís, Brazil, [3] Departamento de Engenharia Elétrica, Programa de Doutorado em Biotecnologia da Rede Nordeste de Biotecnologia (RENORBIO), Universidade Federal do Maranhão, São Luís, Brazil, [4] Departamento de Patologia, Universidade Federal do Maranhão, São Luís, Brazil, [5] Department of Information, Environment, Health and Food Production, Laboratory of Information Systems, Federal Institute of Piauí, Teresina, Brazil, [6] Department of Biology, Federal University of Maranhão, São Luís, Brazil, [7] Laboratório de Micologia Médica, Programa de Mestrado em Biologia Parasitária, Universidade Ceuma, São Luís, Brazil, [8] Departmento de Biologia, Instituto Federal do Maranhão, São Luís, Brazil

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Octavio Luiz Franco, Universidade Católica de Brasília, Brazil

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Adriano Mollica, Università degli Studi "G. d'Annunzio" Chieti - Pescara, Italy
Camila Guimarães Freitas, Federal Institute of Brasilia, Brazil

***Correspondence:**
Cristina de Andrade Monteiro
cristina.monteiro@ceuma.br

[†]*These authors have contributed equally to this work.*

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The *Candida* genus comprises opportunistic fungi that can become pathogenic when the immune system of the host fails. *Candida albicans* is the most important and prevalent species. Polyenes, fluoropyrimidines, echinocandins, and azoles are used as commercial antifungal agents to treat candidiasis. However, the presence of intrinsic and developed resistance against azole antifungals has been extensively documented among several *Candida* species. The advent of original and re-emergence of classical fungal diseases have occurred as a consequence of the development of the antifungal resistance phenomenon. In this way, the development of new satisfactory therapy for fungal diseases persists as a major challenge of present-day medicine. The design of original drugs from traditional medicines provides new promises in the modern clinic. The urgent need includes the development of alternative drugs that are more efficient and tolerant than those traditional already in use. The identification of new substances with potential antifungal effect at low concentrations or in combination is also a possibility. The present review briefly examines the infections caused by *Candida* species and focuses on the mechanisms of action associated with the traditional agents used to treat those infections, as well as the current understanding of the molecular basis of resistance development in these fungal species. In addition, this review describes some of the promising alternative molecules and/or substances that could be used as anticandidal agents, their mechanisms of action, and their use in combination with traditional drugs.

Keywords: *Candida* infections, *Candida*, antifungals, resistance, alternative antifungal drugs

INTRODUCTION

Candida species, opportunistic pathogens, are a major cause of morbidity and mortality worldwide and thus represents a serious threat to public health (Pfaller et al., 2014; Matthaiou et al., 2015; Pappas et al., 2016). Further, *Candida* species can cause vaginitis, oral candidiasis, cutaneous candidiasis, candidemia, and systemic infections (Wächtler et al., 2012). Candidemia is the most

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Article

Anti-Inflammatory and Antinociceptive Activity of Pollen Extract Collected by Stingless Bee *Melipona fasciculata*

Alberto Jorge Oliveira Lopes [1,2,*], Cleydlenne Costa Vasconcelos [1], Francisco Assis Nascimento Pereira [2], Rosa Helena Moraes Silva [1], Pedro Felipe dos Santos Queiroz [1], Caio Viana Fernandes [1], João Batista Santos Garcia [1], Ricardo Martins Ramos [3], Cláudia Quintino da Rocha [4], Silvia Tereza de Jesus Rodrigues Moreira Lima [1], Maria do Socorro de Sousa Cartágenes [1,*] and Maria Nilce de Sousa Ribeiro [2,*]

1 Experimental Study of Pain Laboratory, Department of Physiological Sciences, Federal University of Maranhão, São Luís 65080-805, Brazil; cleydlenne@yahoo.com.br (C.C.V.); lenna1911@hotmail.com (R.H.M.S.); pedrofelipe.queiroz@gmail.com (P.F.d.S.Q.); caiovianafernandes@outlook.com (C.V.F.); jbgarcia@uol.com.br (J.B.S.G.); silviaterezam@gmail.com (S.T.d.J.R.M.L.)
2 Laboratory of Pharmacognosy, Department of Pharmacy, Federal University of Maranhão, São Luís 65080-805, Brazil; franciscopho2015@gmail.com
3 Research Laboratory Information Systems, Department of Information, Environment, Health and Food Production, Federal Institute of Piauí, Teresina 64000-040, Brazil; ricardo@ifpi.edu.br
4 Chemistry Department, Federal University of Maranhão, São Luís 65080-805, Brazil; claudiarocha3@yahoo.com.br
* Correspondence: lopesajo@gmail.com (A.J.O.L.); scartagenes@gmail.com (M.d.S.d.S.C.); mnribeiro@ufma.br (M.N.d.S.R.)

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Abstract: The stingless bee, *Melipona fasciculata* Smith (Apidae, Meliponini), is a native species from Brazil. Their products have high biotechnological potential, however there are no studies about the biological activities of pollen collected by *M. fasciculata*. In this context, the present study investigated the chemical composition, anti-oxidant, anti-inflammatory, and analgesic activities of hydroethanolic pollen extracts collected by *M. fasciculata* in three cities in Maranhão State, Brazil. We verified the antioxidant activity of the extracts and inhibitory activity against the cyclooxygenase enzyme using in vitro assays and in allowed to select the extract with higher efficiency to be used on in vivo assays. In these trials, the selected extract showed high anti-inflammatory activity as well as nociceptive effects at central and peripheral level, suggesting that this extract acts on inhibition of histamine release and decreased synthesis of prostaglandins and the in-silico study suggested that polyphenols and acids fatty acids in the extract may be associated with these activities. The results of the present study report the high biological potential of pollen extract and we conclude that the pollen collected by *M. fasciculata* can be considered as the object of research for new pharmacological alternatives.

Keywords: pollen; extract; pain; anti-inflammatory; natural products; molecular docking

1. Introduction

Natural products are sources of discovery and development of drugs to treat different diseases for centuries. The search for increasingly powerful and less toxic molecules is constant, where secondary metabolites, especially from medicinal plants, are a promising source for the selection of compounds

molecules

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Article

Therapeutic Use of *Scoparia dulcis* Reduces the Progression of Experimental Osteoarthritis

Marcus Vinícius Viégas Lima [1,2,*], Abner de Oliveira Freire [1], Emerson Lucas Frazão Sousa [1], André Alvares Marques Vale [1], Alberto Jorge Oliveira Lopes [1,3], Cleydlenne Costa Vasconcelos [1], Mônica Virginia Viégas Lima-Aragão [1], Humberto Oliveira Serra [4], Rosane Nassar Meireles Guerra Liberio [1], Ana Paula Silva de Azevedo dos Santos [1], Gyl Eanes Barros Silva [1,4], Claúdia Quintino da Rocha [5], Fernando César Vilhena Moreira Lima [6], Maria do Socorro de Sousa Cartágenes [1,*] and João Batista Santos Garcia [1,*]

1 Centro de Ciências Biológicas e da Saúde, Universidade Federal do Maranhão, São Luís 65080-805, Brazil; abnerfreire@live.com (A.d.O.F.); e.lucasfrazao@gmail.com (E.L.F.S.); andre_amvale@hotmail.com (A.A.M.V.); lopesajo@gmail.com (A.J.O.L.); cleydlenne@yahoo.com.br (C.C.V.); monicavvl@hotmail.com (M.V.V.L-A.); roguerra@globo.com (R.N.M.G.L.); apsazevedo@yahoo.com.br (A.P.S.d.A.d.S.); gyleanes@ig.com.br (G.E.B.S.)
2 Universidade Ceuma, São Luís 65075-120, Brazil
3 Universidade Federal do Maranhão, Coordenação de Ciências Naturais, Campus Bacabal, São Luís 65080-80, Brazil
4 Hospital Universitário Presidente Dutra, São Luís 65020-070, Brazil; hoserra@gmail.com
5 Departamento de Química, Universidade Federal do Maranhão, São Luís 65080-805, Brazil; claudiarocha3@yahoo.com.br
6 Faculdade Santa Terezinha, São Luís 65045-180, Brazil; fernandovilhena15@gmail.com
* Correspondence: m.viegas.lima@gmail.com (M.V.V.L.); scartagenes@gmail.com (M.d.S.d.S.C.); jbgarcia@uol.com.br (J.B.S.G.); Tel.: +55-(98)-3272-9527 (M.V.V.L.)

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Abstract: Pain is recognized as one of the main symptoms in knee osteoarthritis and is the main reason why patients seek medical attention. *Scoparia dulcis* has been popularly used to relieve discomfort caused by various painful conditions. The objective of the study is to evaluate the analgesic and anti-inflammatory effect of the crude extract of *S. dulcis*, in an experimental model of osteoarthritis. The experiment was performed with Wistar rats divided into 4 groups with 5 animals each: healthy, saline, crude extract, and meloxicam groups. Knee osteoarthritis was induced by intra-articular injection of sodium mono-iodoacetate. First, clinical parameters of pain were assessed at days 0, 5, 10, 15, and 20 after induction. Second, the potential cyclooxygenase inhibition was evaluated, and the cytokines of the synovial fluid were quantified. An in silico test and Molecular Docking tests were performed. A histopathological evaluation was made on articular cartilage with safranin O staining. The results showed that a 15-day treatment with crude extract reduced edema, spontaneous pain, peripheral nociceptive activity, and proinflammatory cytokines in the synovial fluid. The highest inhibition of cyclooxygenase 2 in the crude extract occurred at 50 µg/mL. The crude extract of *S. dulcis* presents therapeutic potential for the treatment of osteoarthritis due to its anti-inflammatory and anti-nociceptive action.

Keywords: *Scoparia dulcis*; Osteoarthritis; Pain; Inflammation; Treatment

1. Introduction

Osteoarthritis (OA) is a chronic, complex disease characterized by loss, alteration and progressive degeneration of cartilage and subchondral bone; reduction of joint space; synovitis; pain, and formation

antioxidants

MDPI

Article

Anti-Inflammatory and Antioxidant Activity of Pollen Extract Collected by *Scaptotrigona affinis postica*: in silico, in vitro, and in vivo Studies

Alberto Jorge Oliveira Lopes [1,2,*], Cleydlenne Costa Vasconcelos [1], João Batista Santos Garcia [1], Myssa Sued Dória Pinheiro [2], Francisco Assis Nascimento Pereira [2], Darleno de Sousa Camelo [1], Sebastião Vieira de Morais [1], José Roberto Brito Freitas [3], Cláudia Quintino da Rocha [4], Maria Nilce de Sousa Ribeiro [2] and Maria do Socorro de Sousa Cartágenes [1,*]

[1] Experimental Study of Pain Laboratory, Department of Physiological Sciences, Federal University of Maranhão, 65080-805, São Luís, Brazil; cleydlenne@yahoo.com.br (C.C.V.); jbgarcia@uol.com.br (J.B.S.G.); leno.camelo@gmail.com (D.S.C.); svmcoluna@gmail.com (S.V.M.)

[2] Laboratory of Pharmacognosy, Department of Pharmacy, Federal University of Maranhão, São Luís 65080-805, Brazil; myssa.sued@hotmail.com (M.S.D.P.); franciscopho2015@gmail.com (F.A.N.P.); mnsousaribeiro@gmail.com (M.N.S.R.)

[3] Center for Agricultural and Environmental Sciences, Campus IV, Federal University of Maranhão 65500-000, Chapadinha, MA, Brazil; joserobertobritofreitas@yahoo.com.br

[4] Chemistry Department, Federal University of Maranhão, São Luís 65080-805, Brazil; claudiarocha3@yahoo.com.br

* Correspondence: lopesajo@gmail.com (A.J.O.L); cartagenes.maria@ufma.br (M.S.S.C).

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Abstract: Bees are of great importance for plant diversity for being an important pollinating agents. Stingless bees such as *Scaptotrigona affinis postica*, is cultivated largely due to the products offered by it. Pollen is one of these products, which has been highlighted for exhibit various therapeutic properties. Considering the bioactivity of this natural product, this study investigated the antioxidant, anti-inflammatory, antinociceptive activities, and elucidated the chemical composition of pollen collected extract by *Scaptotrigona affinis postica*. Using in vitro assays, the antioxidant potential and inhibitory activity against the COX enzyme from pollen extract was evaluated. Additionally, tests were performed to measure the anti-inflammatory and antinociceptive activities in animal models. In our results, we found that pollen extract showed antioxidant effects and inhibitory activity against the COX enzyme. The in vivo assays showed that the extract acts on the nervous system in local and systemic levels and that the anti-inflammatory activity is due the prostanoids reducing. Chemical analyses recognize 10 molecules in the extract belonging to the polyphenol and flavonoids classes and the computational study suggests that is responsible for the observed results. Thus, it is reported for the first time the biological potential of *S. aff. postica* pollen extract and we conclude that this bee product can be considered as one source of potential new drugs.

Keywords: stingless bee pollen; biological activity; pain; molecular docking; tubi

1. Introduction

Bees represent a species with wide biodiversity in the world and have an important ecological role, as they are fundamental in maintaining plant diversity, maintaining an intrinsic relationship with these, being responsible for the pollination of several plant species [1]. Bee products have been widely used for generations in human health due to their recognized therapeutic and nutritional

ANEXOS

IPHM-D-20-00401 - Submission Notification to co-author

De: Inflammopharmacology (IPHM) (em@editorialmanager.com)

Para: cleydlenne@yahoo.com.br

Data: segunda-feira, 3 de agosto de 2020 11:19 BRT

Body:
Re: "Arrabidaea chica Verlot fractions reduces MIA-induced osteoarthritis progression in rats' knees"
Full author list: Cleydlenne Costa Vasconcelos; Alberto Jorge Oliveira Lopes; Emilly de Jesus Garcia Ataide; Kevin Waquim Pessoa Carvalho; Maria Fernanda Freitas de Brito; Marineide Sodré Rodrigues; Sebastião Vieira de Morais; Gyl Eanes Barros Silva; Claudia Quintino da Rocha; João Batista Santos Garcia; Maria do Socorro Sousa Cartágenes, Ph.D.

Dear Dr Vasconcelos,

We have received the submission entitled: "Arrabidaea chica Verlot fractions reduces MIA-induced osteoarthritis progression in rats' knees" for possible publication in Inflammopharmacology, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Professor Maria do Socorro Sousa Cartágenes who will be able to track the status of the paper through his/her login.

If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

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Editorial procedure

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Manuscript Submission

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Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title pa[137] The names of funding organizations should be written in full.

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Units of measurement, abbreviations and symbols should follow the International System of Units (SI). For chemical nomenclature, the conventions of the International Union of Pure and Applied Chemistry and the official recommendations of the IUPAC -- IUB Combined Commission on Biochemical Nomenclature should be followed.

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References

Citation

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

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).

- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 19[138]; Medvec et al. 1999, 2000).

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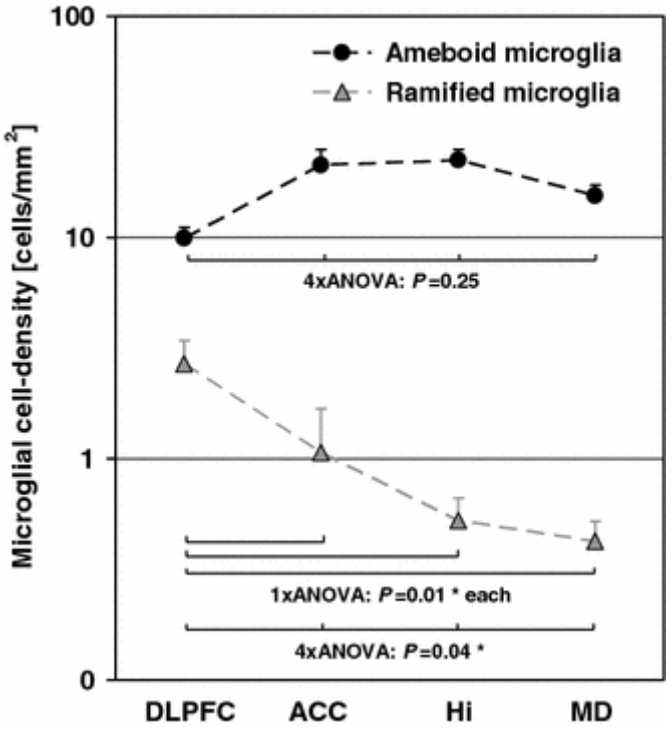

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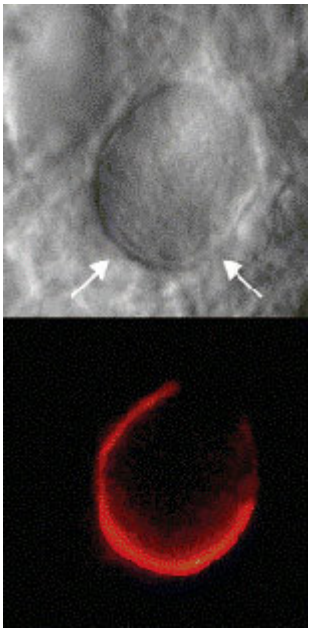

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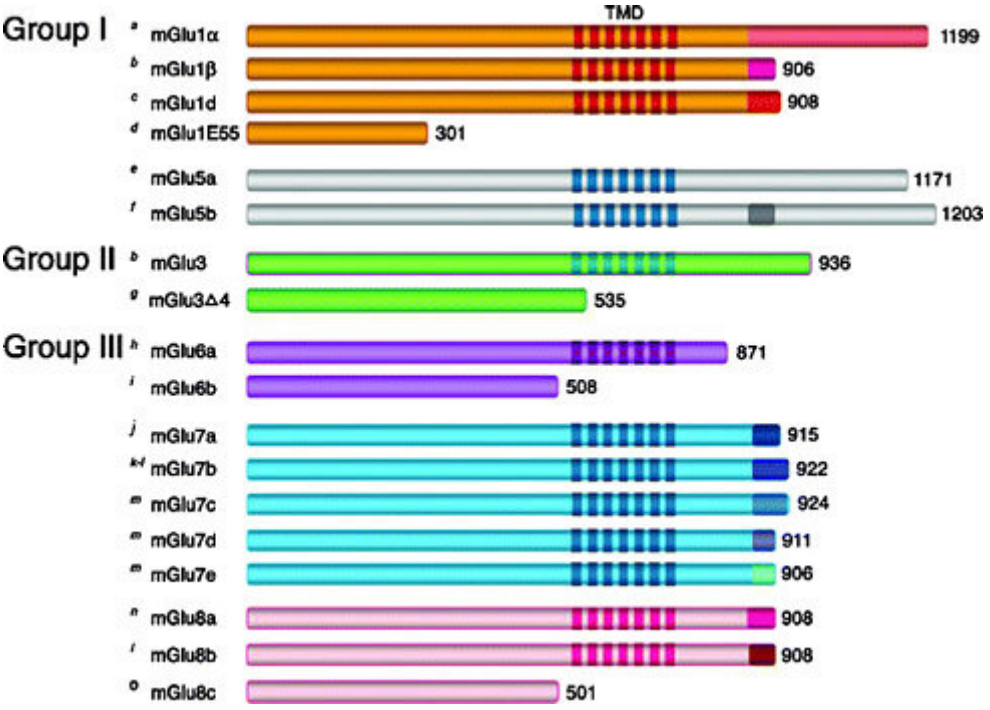

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It should also be stated clearly in the text that all persons gave their informed consent prior to their i[145]usion in the study. Details that might disclose the identity of the subjects under study should be omitted.

These statements should be added in a separate section before the reference list. If these statements are not applicable, authors should state: The manuscript does not contain clinical studies or patient data.

The editors reserve the right to reject manuscripts that do not comply with the above-mentioned requirements. The author will be held responsible for false statements or failure to fulfill the above-mentioned requirements

- Human and Animal Studies

Studies in humans or those involving the use of human tissues, or laboratory animals should comply with internationally acceptable standards detailed requirements of which are specified in Inflammopharmacology 15 (2007): 179-180.

Studies involving investigation of pain responses or those which are inherently painful in laboratory animals should comply with the International Association for the Study of Pain (IASP) Guidelines for the Use of Animals in Research.

IASP-guidelines

Important notes:

- The date of the approval, file, reference or certificate number, and name of the person who authorized the study should be stated.
- Please follow the advice by the Editor-in-Chief in the download below:

Ethics of Animal Investigations (Download pdf, 9 kB)

- Studies with Natural Products

There are specific requirements for studies involving investigations of natural products or nutraceuticals, details of which can be found in Inflammopharmacology (2007): 15:179-180. In general, studies will only be acceptable providing the products have been fully characterised and details of chemical composition provided. The investigations must be directed to understanding the pharmacological properties (e.g. anti-inflammatory activities) of the test substance with comprehensive supporting data including full dose-(in vivo) or concentration (in vitro) responses to the test agents. Studies should also include investigations of the major isolated or chemically-known components in the test materials or reference to those in the Discussion.

Under no circumstances will papers be acceptable if they have involved simple screening of plant or other natural product extracts for anti-oxidant, **acute** anti-inflammatory or non-specific biological activities. The submitted paper must clearly show how the study will result in advances for medical treatments.

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- Asking a colleague who is a native English speaker to review your manuscript for clarity.

UNIVERSIDADE FEDERAL DO MARANHÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-CEUA
CIAEP:01.0341.2014

CERTIFICADO

Certificamos que a proposta intitulada "**Efeitos do extrato, frações e metabólitos secundários de** *Arrabidae de chica* **no controle de dor nociceptiva, induzida em modelo experimental de osteoartrite**" registrada com o nº **23115.000372/2017-09**, sob a responsabilidade de **Maria do Socorro de Sousa Cartagenes**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi considerado **APROVADO** pela Comissão de Ética no Uso de Animais (CEUA - UFMA) da Universidade Federal do Maranhão em reunião de 22/05/2017.

FINALIDADE	( )Ensino (x) Pesquisa ( )Extensão
Vigência da autorização	19/06/2017 à 19/06/2019
Espécie/linhagem/raça	Ratos/Winstar/
Nº de animais	96 animais
Peso/Idade	250 a 300g – 60 dias
Sexo	Macho
Origem	Biotério Central UFMA

Profa. Dra. Lucilene Amorim Silva
Presidente da Comissão de Ética no uso de animais-CEUA
UFMA