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EFEITO ANTINOCICEPTIVO E ANTI-INFLAMATÓRIO DA *Scoparia dulcis Linn* NA OSTEOARTRITE EXPERIMENTAL

MARCUS VINÍCIUS VIÉGAS LIMA

São Luís
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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Maranhão, como pré-requisito para o grau de doutor.

Orientador: Prof. Dr. João Batista Santos Garcia
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O impossível é feito
de várias partes
possíveis

Caio Carneiro

As pessoas felizes
lembram o passado com
gratidão, alegram-se com o
presente e encaram o
futuro sem medo.

Epicuro

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RESUMO

Introdução: A dor na osteoartrite causa incapacidade e comprometimento funcional da articulação afetada, sendo seu manejo considerado um grande desafio. Na busca de novas terapias, surge a *Scoparia dulcis*, conhecida popularmente por vassourinha, cujas propriedades anti-inflamatórias, antinociceptivas e antioxidativas são cientificamente comprovadas e sem estudos até o momento no controle da dor articular na osteoartrite. **Objetivo:** avaliar o efeito antinociceptivo, anti-inflamatório e antioxidante do extrato bruto e da fração clorofórmica de *Scoparia dulcis* Linn em modelo experimental de osteoartrite. **Material e Métodos:** No primeiro momento da pesquisa, foi feita a indução de osteoartrite de joelho, pela injeção intra-articular de monoiodoacetato de sódio. Vinte e cinco ratos Wistar foram divididos em cinco grupos com cinco animais cada: grupo sadio, grupo salina, grupo extrato bruto, grupo fração clorofórmica e grupo meloxicam. Foram avaliados parâmetros clínicos de dor (incapacidade articular, atividade motora, sensibilidade tátil, edema articular e dor espontânea) nos dias zero, cinco, dez, quinze e vinte após a indução. Em um segundo momento, foram avaliados o percentual de inibição da ciclooxigenase, análise química, acoplamento molecular e quantificado as citocinas IFN-γ, IL-6 e IL-10 do líquido sinovial. Os valores de polifenóis e teores totais de flavonóides, atividade antioxidante (métodos DPPH •, FRAP e ABTS • +) foram quantificados. Foram avaliadas as enzimas superóxido dismutase, catalase e glutationa peroxidase. A avaliação histopatológica foi feita na cartilagem articular com a coloração de safranina O e no liquido sinovial com a coloração de hematoxilina e eosina. **Resultados:** O tratamento terapêutico por 15 dias com extrato bruto de *S. dulcis* e fração clorofórmica (500 mg/kg/dia) reduziu o edema, a dor espontânea e a atividade nociceptiva periférica no joelho direito com OA, atenuando as alterações histológicas na membrana sinovial e a cartilagem articular com menores níveis sinoviais de IL-6 e IFN-γ, e níveis mais elevados de IL-10, possivelmente devido à sua ação anti-inflamatória. A concentração testada de 50 µg/mL mostrou a maior inibição para COX-1 e COX-2 no extrato bruto. Todos os compostos mostraram afinidade de alto parâmetro com a estrutura da COX-2, destacando suspensaside e nicotiflorin e realizaram interações com importantes resíduos do sítio ativo da COX, como Arg120 e Glu524 e com resíduos vizinhos. O extrato e a fração clorofórmica apresentaram atividade antioxidante pelos métodos DPPH •, FRAP e ABTS • + e pelas enzimas superóxido dismutase, catalase e glutationa peroxidase. **Conclusão:** o extrato bruto e a fração clorofórmica de *S. dulcis* apresentam potencial terapêutico para o tratamento da osteoartrite devido sua ação anti-inflamatória, antinociceptiva e antioxidante.

Palavras-chave: *Scoparia dulcis*. Osteoartrite. Dor. Inflamação. Tratamento.

ABSTRACT

Introduction: Pain in osteoarthritis causes disability and functional impairment of the affected joint, and its management is considered a major challenge. In search of new therapies, comes *Scoparia dulcis*, popularly known as broom, whose anti-inflammatory, antinociceptive and antioxidant properties are scientifically proven and have not been studied so far in controlling joint pain in osteoarthritis. **Objective:** To evaluate the antinociceptive, antiinflammatory and antioxidant effect of crude extract and chloroform fraction of *Scoparia dulcis* Linn in an experimental model of osteoarthritis. **Material and Methods:** In the first moment of the research, knee osteoarthritis was induced by intraarticular injection of sodium monoiodoacetate. Twenty-five Wistar rats were divided into five groups with five animals each: healthy group, saline group, crude extract group, chloroform fraction group and meloxicam group. Clinical parameters of pain (joint disability, motor activity, tactile sensitivity, joint edema and spontaneous pain) were evaluated on days zero, five, ten, fifteen and twenty days after induction. Secondly, the percentage of cyclooxygenase inhibition, chemical analysis, molecular coupling and quantification of the cytokines IFN-γ, IL-6 and IL-10 of the synovial fluid were evaluated. Polyphenol values and total flavonoid contents, antioxidant activity (DPPH •, FRAP and ABTS • + methods) were quantified. The enzymes superoxide dismutase, catalase and glutathione peroxidase were evaluated. Histopathological evaluation was done on articular cartilage with safranin O staining and on synovial fluid with hematoxylin and eosin staining. **Results:** Therapeutic treatment for 15 days with crude *S. dulcis* extract and chloroform fraction (500 mg / kg / day) reduced edema, spontaneous pain and peripheral nociceptive activity in the right knee with OA, attenuating histological changes in the membrane. synovial and articular cartilage with lower synovial levels of IL-6 and IFN-γ, and higher levels of IL-10, possibly due to their anti-inflammatory action. The tested concentration of 50 µg / mL showed the highest inhibition for COX-1 and COX-2 in the crude extract. All compounds showed high parameter affinity with COX-2 structure, highlighting suspensaside and nicotiflorin and performed interactions with important residues of the active site of COX, such as Arg120 and Glu524 and with neighboring residues. The extract and chloroform fraction showed antioxidant activity by the DPPH •, FRAP and ABTS • + methods and by the enzymes superoxide dismutase, catalase and glutathione peroxidase. **Conclusion:** The crude extract and chloroform fraction of *S. dulcis* have therapeutic potential for the treatment of osteoarthritis due to its anti-inflammatory, antinociceptive and antioxidant action.

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

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1. INTRODUÇÃO

A osteoatrite (OA) é reconhecida com uma das doenças musculoesqueléticas crônicas mais prevalentes, afetando articulações nas mãos, joelhos, quadris e coluna com diferentes graus de deformidade e edema articulares. A dor é reconhecida como um dos principais sintomas na osteoartrite de joelho, sendo a principal razão pela qual pacientes procuram atendimento médico, pois resultam em comprometimento funcional, além da incapacidade (BROWN; BOULAY, 2013) alterando assim a qualidade de vida para as atividades cotidianas (JOSÉ, 2013) e laborais (COIMBRA et al., 2004).

Inúmeros tratamentos clínicos e cirúrgicos já foram tentados para a OA, outros estão em estudo, mas até o momento não há uma terapêutica que resulte na recuperação definitiva ou na cura das lesões articulares, sendo que nos casos mais graves há necessidade de colocação de próteses metálicas nos joelhos. A terapêutica usada tem como objetivo apenas tratar os sintomas, melhorar a mobilidade articular, diminuir a dor e melhorar a qualidade de vida do paciente, sem interferir na fisiopatologia da osteoartrite (TORRES et al., 2009).

Nesse sentido, a fitoterapia e/ou o uso de plantas medicinais tem sido proposto como tratamento para a osteoartrite (ZHANG et al., 2016), pois comparados aos anti-inflamatórios esteriodais e não esteriodais, as plantas medicinais e os fitomedicamentos desempenham ainda um pequeno papel no tratamento desta doença (ROSA; MACHADO, 2007).

O uso de plantas medicinais é regulamentado pela Agência Nacional de Vigilância Sanitária, órgão do Ministério da Saúde, que publica resoluções que regulam quais, quando e como as chamadas 'drogas vegetais' devem ser usadas, a partir do uso de suas partes como: folhas, cascas, raízes ou flores, como opção terapêutica, no Sistema Único de Saúde (BRASIL, 2006) para o tratamento de doenças agudas e crônicas (DUTRA et al., 2016).

Dentre essas espécies, destacamos a *Scoparia dulcis* (S. *dulcis*) também conhecida como vassourinha, erva perene que é encontrada em regiões tropicais e subtropicais (BABINCOVÁ; SCHRONEROVÁ; SOURIVONG, 2008; LANNA et al., 2012; MURTI et al., 2012). Seus constituintes bioativos isolados tem contribuído para demonstrar o efeito medicinal da planta pela presença de

flavonoides, dipertenos, tripertenos e esteroides em processos inflamatórios e nociceptivos (ERUMAL YOGEESWARI, 2005; GIROTTI et al., 2005; FAUJAN et al., 2010; MISHRA et al., 2011; TSAI et al., 2011; PAMUNUWA; KARUNARATNE; WAISUNDARA, 2016). No seu uso popular a *S. dulcis* tem sido utilizada no alivio e desconforto causados pela menstruação, menopausa (MICHEL et al., 2007), dor de parto, inflamação uterina (SOUZA et al., 2014) e lesões gástricas (RATNASOORIYA et al., 2002).

Nessa perspectiva, não se encontrou até o momento dados publicados sobre a ação antinociceptiva, anti-inflamatória e antioxidante da espécie vegetal no tratamento da dor na osteoartrite de joelho em ratos. Sendo assim, o objetivo desse trabalho é avaliar o efeito analgésico, anti-inflamatório e antioxidante do extrato bruto e da fração clorofórmica de *Scoparia dulcis*, em modelo experimental de osteoartrite.

2. REFERENCIAL TEÓRICO

2.1 Osteoartrite

2.1.1 A doença

A OA Caracteriza-se por ser uma afecção dolorosa das articulações, multifatorial e crônica (COIMBRA et al., 2004; VINCENT et al., 2012) com destruição da cartilagem e do osso subcondral, redução do espaço articular, sinovite e formação de osteófitos que leva a incapacidade funcional progressiva (DIEPPE; LOHMANDER, 2005) principalmente nas articulações dos membros inferiores (joelhos) (LIM et al., 2015).

É a forma mais comum de doença articular alterando a qualidade de vida (REZENDE; CAMPOS; PAILO, 2013; STANOS, 2013), sendo a causa mais frequente de dor musculoesquelética (COIMBRA et al., 2004; JOSÉ, 2013; MAN; MOLOGHIANU, 2014) para as atividades de vida diária (JOSÉ, 2013) e laborativas no Brasil e no mundo (COIMBRA et al., 2004).

Não possui cura e tem apresentado um impacto direto na economia em decorrência dos elevados gastos públicos com medicamentos e cuidados hospitalares; perda da produtividade por afastamento do trabalho e elevado índice de cirurgias de artroplastia de joelho (OLALEKAN et al., 2013).

2.1.2 Epidemiologia, fatores de risco e classificação

A OA pode ser classificada de acordo com suas causas ou fatores predisponentes em primária e secundária. A primária não possui etiologia identificável ou causas predisponentes, sendo a mais comum, e, a secundária que decorre de desvios do eixo ou de causa pós traumática (REZENDE et al., 2000).

Fatores de risco como predisposição genética, envelhecimento, idade, obesidade, trauma, uso excessivo da articulação contribuem para iniciar o processo degenerativo dos diferentes componentes da articulação (LEE et al., 2013).

O risco de seu desenvolvimento aumenta substancialmente após 45 anos de idade (VINCENT et al., 2012), sendo mais prevalente entre os indivíduos com mais de 65 anos de idade (COIMBRA et al., 2004). Atualmente cerca de 40% dos adultos com idade superior a 70 anos sofrem de OA joelho e 80% apresentam limitações funcionais do movimento e 25% tem comprometimento das atividades diárias e laborativas (MELO et al., 2008; JOSÉ, 2013; EDMONDS et al., 2016). É esperado que o número de indivíduos com OA dobre no futuro com o aumento da expectativa de vida, pois é considerada a segunda maior causa de disfunção física e de saúde que mais avança rapidamente (KOTTI et al., 2014).

Cerca de 43 milhões de pessoas são afetadas pela OA nos Estados Unidos (MAN; MOLOGHIANU, 2014) onde, 36% dos indivíduos, com mais de 60 anos possuem OA de joelho (DILLON et al., 2006), apresentando um custo anual de aproximadamente 60 bilhões de dólares (JOSÉ, 2013).

No Brasil, estima-se que a população com mais de 60 anos, irá alcançar em 2050 mais de 64 milhões de pessoas (REZENDE; CAMPOS; PAILO, 2013) com uma prevalência de OA de joelho de 33,7% entre as idades de 70 a 79 anos (PINEDA; SOTOS; GARCES-POENTES, 2014).

2.1.3 Osteoartrite e dor

A dor é uma experiência sensorial (sensitiva) e emocional indesejada e desagradável associada e descrita em termos de lesões teciduais reais ou potenciais, sempre subjetiva em suas experiências, sendo uma das principais queixas dos indivíduos que procuram os serviços de saúde (ROCHA et al., 2007; KLAUMANN; WOUK; SILLAS, 2008).

A OA é uma das doenças que mais causam dor musculoesquelética frequente e persistente, podendo atingir um em cada cinco indivíduos, principalmente idosos, causando dor crônica e incapacidade articular, principalmente nos joelhos (CUNHA-MIRANDA et al., 2015).

A OA no joelho é uma das formas mais incapacitantes da doença pois além de dor e fraqueza muscular, evolui com redução do equilíbrio, déficits de propriocepção, redução da amplitude de movimento e instabilidade articular (GUR et al., 2002; KIERKEGAARD et al., 2015; EDMONDS et al., 2016).

Por não ser bem compreendida, a dor tem sido estudada a partir de uma estrutura biopsicossocial (HUNTER; MCDOUGALL; KEEFE, 2008) que envolve além da perda da função articular, (ZHANG; REN; DUBNER, 2013) uma integração multifatorial de processos biológicos, cognitivos, psicológicos, sociais, emocionais e afetivos a partir de mecanismos periféricos e centrais do sistema nervoso (DIEPPE; LOHMANDER, 2005; HUNTER; MCDOUGALL; KEEFE, 2008; LEE; NASSIKAS; CLAUW, 2011) pois é uma experiência multidimensional (DA SILVA; RIBEIRO-FILHO, 2011).

Do ponto de vista estrutural e biomecânico, a OA é caracterizada pela progressiva degeneração da cartilagem, entretanto, a relação entre a dor nas articulações e a degradação da cartilagem é pouco compreendida pois é avascular e não há uma inervação nervosa sensitiva (OKAMOTO; ATSUTA, 2010).

A inflamação pode causar estímulos mecânicos nocivos devido a presença de fibras nervosas aferentes na cápsula articular, sinóvia, periósteo, osso subcondral, ligamentos, músculo e tendões, pois são ricamente inervados por fibras nociceptivas e podem ser a provável fonte de nocicepção (FELSON, 2005; HUNTER et al., 2013).

Para a inflamação articular, as fibras do tipo II (Aβ) apresentam alterações agudas e transitórias em resposta à manipulação da articulação. Entretanto, as fibras do tipo III (Aδ) e do tipo IV (C) passam a apresentar atividade espontânea dos receptores nociceptivos a partir das terminações nervosas livres, pois seu campo receptivo, localizado dentro das articulações, se expande para áreas adjacentes presentes nessas fibras, que se tornam responsivas a estímulos mecânicos externos aos quais eram insensíveis (TEIXEIRA, 2001).

Além da dor e perda da funcionalidade articular, a inflamação pode também contribuir para a mudança estrutural na articulação pois eleva os níveis de citocinas e proteases (BONNET; WALSH, 2005). Linfocitos T e B e mediadores pró-inflamatórios como o fator de necrose tumoral alfa, a interleucina 1, interleucina 6 (VERRI et al., 2007), interleucina 17 e prostaglandina E_2 (LEE et al., 2013) participam do desencadeamento e agravamento da doença e no desenvolvimento da dor e inflamação. Por outro lado, o líquido sinovial contém vários mediadores inflamatórios, como bradicinina, adenosina trifosfato, acetilcolina e serotonina, e acredita-se que estimulem diretamente os

nociceptores dos terminais nervosos sensoriais e participem do processo de geração de dor (OKAMOTO; ATSUTA, 2010).

A lesão tecidual gera uma sensação subjetiva de dor, pois os mediadores químicos liberados na articulação pela inflamação sensibilizam os nervos aferentes primários através de estimulos nociceptivos intensos e persistentes levando a um quadro álgico espontâneo e a hipersensibilidade mecânica secundária por estimulos neuronais no corno posterior da medula espinhal e/ou nos centros superiores (HUNDER; MCDOUGALL; KEEFE, 2008), através de uma regulação ascendente (O´NEILL et al., 2007) até o tronco, tálamo e córtex cerebral (HUNDER; MCDOUGAL; KEEFE, 2008) produzindo alterações funcionais, chamadas de neuroplasticidade neural através de alterações em canais iônicos ou receptores na distribuição de neurotransmissores e neuromoduladores cerebrais (WOOLF; SALTER, 2000; STAUD et al., 2007).

A neuromodulação leva a um processo de conscientização e interpretação do quadro doloroso na OA a partir das conexões descendentes entre tálamo e córtex pelos sistemas laterais e mediais do trato espinotalâmico. A ativação dos núcleos talâmicos laterais, leva as informações para o córtex somatossensorial, sendo o estimulo nocivo na OA analisado quanto a localização, duração, intensidade e qualidade. Já o medial leva as informações para outras partes do cérebro como a amígdala, fazendo com que a percepção da dor seja através de respostas afetivas-emocionais (raiva, ansiedade ou depressão), de atenção e aprendizado (HUNDER; MCDOUGALL; KEEFE, 2008). Estudos de neuroimagens demostram que a dor na OA pode está associada ao aumento de atividade em áreas relacionadas as emoções e medos ativando áreas dolorosas da região límbica pré-frontal, córtex cíngulo, tálamo e amídala confirmando o processamento da dor aos aspectos emocionais (KULKAMI et al., 2007).

O estado cognitivo (crenças de dor, expectativas, memórias de dor), idade, sexo, condições socioeconômicas, raça, cultura, atividade física, habilidades de comunicação da dor, lesões, traumas e experiências prévias de dor podem contribuir para a percepção da dor na OA (HUNDER; MCDOUGALL; KEEFE, 2008).

2.1.4 Tratamento da osteoartrite

O tratamento da OA deve ser multidisciplinar buscando a melhora funcional, mecânica e clínica da doença (WANDEL et al., 2010), podendo ser farmacológico, cirúrgico e não farmacológico (COIMBRA et al., 2004). Seus objetivos são: educação do paciente sobre a doença e seu controle; controle da dor; melhora da função e alteração do processo de doença e suas consequências (JOSÉ, 2013).

No tratamento farmacológico, as medicações podem ser de uso sistêmico, tópico ou intra-articular sendo divididas em analgésicas e/ou anti-inflamatórias e drogas modificadoras dos sintomas e/ou da evolução da doença (JOSÉ, 2013). A medicação de primeira escolha é o paracetamol até 4g/dia para analgesia (COIMBRA et al., 2004). Os anti-inflamatórios não esteroidais (AINES) são os fármacos mais utilizados na clínica médica para aliviar a dor, em curtos e longos períodos de tempos (VIDOTTO et al., 2013). Administrados oralmente, podem diminuir a inflamação persistente de baixo grau, sendo recomendadas nas diretrizes terapêuticas da *Osteoarthritis Research Society Internacional,* para tratamento da OA em pacientes que não possuem co-morbidades ou OA multiarticular (NAGY et al., 2017).

Os produtos de aplicação tópica são uma alternativa para doentes com contraindicações para o uso de terapêutica sistêmica e um adjuvante dos tratamentos convencionais com resposta inadequada. A eficácia a longo prazo dos AINES tópicos é controversa, mas o uso do diclofenaco e do eltenaco, em tratamento por longo prazo, da dor do joelho, tem se mostrado efetivo. As formas tópicas são úteis e eficazes em problemas superficiais, se aplicadas repetidamente, de modo a assegurar a penetração do fármaco (FERREIRA et al., 2012). A capsaicina tem se mostrado um bom agente terapêutico para a sintomatologia dolorosa e outros anti-inflamatórios não-hormonais tópicos como cetoprofeno, ibutrofeno, felbinaco e piroxicam tem um efeito significativo no tratamento da dor aguda ou crônica (COIMBRA et al., 2004).

As injeções intra-articulares de glucocorticoides (por exemplo, betametasona ou prednisolona) são utilizadas em caso de inflamação local ou derrame articular. Podem ser usadas como adjuvantes da terapêutica oral ou em monoterapia em doentes que não toleram outros tratamentos. As doses

dependem do tamanho da articulação a ser tratada. As injeções de ácido hialurônico aumentam temporária e ligeiramente a viscosidade do líquido sinovial e diminuem a dor (FERREIRA et al., 2012) e estão indicadas para o tratamento de OA grau II e III, nas fases aguda e crônica (COIMBRA et al., 2004).

Analgésicos opioides estão indicados em caso de insucesso de outras opções farmacológicas, como paracetamol, AINES, injeções intra-articulares ou terapia tópica. A terapêutica inicia-se com opioides fracos, como o tramadol, em doses baixas e por vezes associados ao paracetamol. Quando estes se tornam ineficazes, recorre-se ao uso prolongado de opioides (FERREIRA et al., 2012).

Algumas medicações têm sido denominadas drogas modificadoras da doença osteoartrite, pois tem a capacidade de prevenir, retardar, reverter ou estabilizar a alteração da estrutura articular (REZENDE; GOBBI, 2009). São medicamentos feitos a partir do extrato insaponificável de *Persea gratíssima* (abacate) *e Glycene max* (soja), *Aloe vera* (babosa) e do *Harpagophitum procumbens* (planta originária da África). Sem serem derivados de plantas, mas com este mesmo objetivo, são usados o ácido hialurônico, glicosamina (sintética ou retirada da carapaça de crustáceos), a condroitina (TORRES et al., 2009), diacreína, a associação de glicosamina com condroitina, cloroquina e para uso intra-articular, a viscossuplementação com ácido hialurônico (REZENDE; GOBBI, 2009).

Quando a abordagem conservadora não é efetiva no alívio da dor e no restabelecimento da função articular, deverá ser considerada a opção cirúrgica, sendo que os procedimentos cirúrgicos englobam: artroscopia, artroplastia, artrodeses, debridamentos, osteotomia, entre outros procedimentos. A cirurgia é recomendada a doentes com elevada incapacidade funcional da articulação e com dor osteoarticular sem resposta a terapêutica tradicional (JOSÉ, 2013).

Por outro lado, tratamentos não farmacológicos utilizam-se programas educativos sobre esclarecimentos em relação a doença; motivam e envolvem o paciente no seu tratamento, pois ele é considerado um agente ativo no programa de reabilitação. Praticar esportes sob supervisão de um profissional habilitado, orientações ergonômicas, domésticas e laborais podem ser usados. Além disso, exercícios terapêuticos com orientação e prescrição individualizada pela fisioterapia como programas de fortalecimento, exercícios aeróbios, de alongamento, o uso de agentes físicos, órteses, equipamentos de auxílio a

macha, palmilhas e estabilização da articulação podem ser utilizadas (COIMBRA et al., 2004) proporcionando alivio dos sintomas com pouco efeitos colaterais. Tratamentos como acupuntura, moxabustão, massagem e Tai Chi Chuan também tem sido utilizados (ZHANG et al., 2016).

A perda de peso deve ser igualmente considerada em doentes com obesidade ou sobrepeso. O excesso de peso aumenta a carga nas articulações, sendo um fator de agravamento da dor osteoarticular e um fator preditivo de eventual substituição da articulação. A intervenção dietética requer motivação do doente e um programa de perda de peso individualizado e estruturado. A meditação, técnicas de relaxamento, hipnose e psicoterapia ajudam o doente a afastar pensamentos negativos, aumentando a tolerância à dor e melhorando a qualidade de vida (FERREIRA et al., 2012).

2.2 *Scoparia dulcis* Linn

Ao longo do processo evolutivo do homem, as plantas medicinais sempre desempenharam um papel importante na busca pelo alívio da sintomatologia de doenças, sendo consideradas como os primeiros recursos terapêuticos utilizados a partir de experiências populares disseminadas por diferentes etnias em todo o mundo (RODRIGUES, 2014), onde 80% da população mundial faz uso de algum tipo de planta medicinal de acordo com a Organização Mundial de Saúde (CARNEIRO et al., 2014).

A prática do uso de plantas na medicina tradicional está incluída e é reconhecida no sistema primário de saúde nos países em desenvolvimento, já que eles atualmente possuem 67% das espécies vegetais do mundo (CHAVES; BARROS, 2012). Estima-se que cerca de 30% das drogas terapêuticas disponíveis sejam derivadas de fontes naturais, principalmente de plantas e microrganismos. Em algumas áreas terapêuticas, como por exemplo, em oncologia, a quantidade de medicamentos derivados de plantas chega a 60% (MISHRA; TIWARI, 2011; NEWMAN; CRAGG, 2016).

Produtos naturais que possuem propriedades terapêuticas são fontes importantes de novos compostos biologicamente ativos e vêm sendo utilizados em diversas partes do mundo há décadas, atraindo o interesse de muitos pesquisadores (COUTO et al., 2016).

Scoparia dulcis Linn (Scrophulariaceae) também conhecida como vassourinha é uma erva perene que é encontrada em regiões tropicais e subtropicais (BABINCOVÁ; SCHRONEROVÁ; SOURIVONG, 2008; LANNA et al., 2012; MURTI et al., 2012). A presença de metabolitos secundários tem justificado seu uso medicinal e popular (ERUMAL YOGEESWARI, 2005; GIROTTI et al., 2005; BABINCOVÁ; SCHRONEROVÁ; SOURIVONG, 2008; TSAI et al., 2011; PAMUNUWA; KARUNARATNE; WAISUNDARA, 2016). A utilização de extratos e frações através de seus compostos bioativos têm sido utilizados para investigar e pesquisar várias doenças (DE FREITAS et al., 2015; MONIRUZZAMAN; ATIKUR RAHMAN; FERDOUS, 2015).

2.2.1 Nome botânico, cientifico e taxonomia

Possui o seguinte nome botânico, cientifico e taxonomia (MISHRA et al., 2011):

Reino: Plante;
Sub-reino: Trachcobiota;
Divisão: Magnoliophyta;
Classe: Magnoliopsita;
Subclasse: Asteridae
Família: Scrophulariaceae
Gênero: Scoparia
Espécie: dulcis
Nome botânico e cientifico: *Scoparia dulcis* Linn

2.2.2 Sinônimos botânicos

Capraria dulcis (L.) kuntze*, gratiola micrantha* Nutt*, Scoparia dulcis var. tenuifolia Griseb., Scoparia grandiflora Nash, Scoparia nudicaulis Chodat, Scoparia procumbens Jacq., Scoparia purpúrea Ridl., Scoparia ternata Forssk., Ambulia micrantha Raf., Capraria dulcis var. albiflora Kuntze, Capraria dulcis var. coerulea Kuntze, Scoparia nudicaulis Chodat & Hassl* (LIST, 2016; TROPICOS, 2017).

2.2.3 Sinônimos vernaculares

Possui o seguinte nomes vernaculares (MISHRA et al., 2011):

Sânscrito: Asmaghni
Hindu: Mithipatti, Ghodatulsi, Mithi Patti, Ghoda Tulsi, Ban Dhania
Tâmil: Sarakkotthini;
Bengali: Bom-dhonya;
Malaio: Kallurukki;
Inglês: Sweet Broom, Broom weed;
Brasil: Vassourinha

2.2.4 Sinônimos populares

Corrente-roxa, ganha-aqui-ganha-acolá, tapeiçaba, tupeiçava, tupiçaba, tupiçava, tupixaba, tupixava, vassoura, vassourinha, vassourinha-cheirosa, vassourinha-de-botão, vassourinha-doce, vassourinha-miúda, vassourinha-mofina, vassourinha-molfina, vassourinha-tupiçaba (PLANTMED, 2016).

2.2.5 Distribuição geográfica

É uma espécie amplamente distribuída nas regiões tropicais e subtropicais do mundo (LANNA et al., 2012; MURTI et al., 2012), sendo encontrada em abundância na América do Sul e na floresta amazônica, (BABINCOVÁ; SCHRONEROVÁ; SOURIVONG, 2008), principalmente nas seguintes áreas: Argentina, Austrália, Belize, Bolívia, Brasil, Caribe, África, Colômbia, Comoros, Costa Rica, Equador, Guiana Francesa, Gabon, Chana, Guatemala, Guiana, Honduras, Madagascar, Malawi, México, Nicarágua, Panamá, Paraguai, Peru, Filipina, Suriname, Tanzânia, Estados Unidos, Venezuela e Vietnam (TROPICOS, 2017).

2.2.6 Morfologia

A *S. dulcis* é um vegetal popularmente conhecido como vassourinha, possui aspecto perene e pequena que pode crescer até 60cm de altura podendo assumir hábito rastejante, possuindo folhas serrilhadas e flores brancas hermafroditas geralmente axilares com 6-7 mm de diâmetro. Possuem 4-5 sépalas, cálice oblongo medindo 2.5-3.0x0.8-1.0mm com floração quase todo o ano (MISHRA et al., 2011; MURTI et al., 2012; PAMUNUWA; KARUNARATNE; WAISUNDARA, 2016; PAUL; VASUDEVAN; KRISHNAJA, 2017) (figura 1).

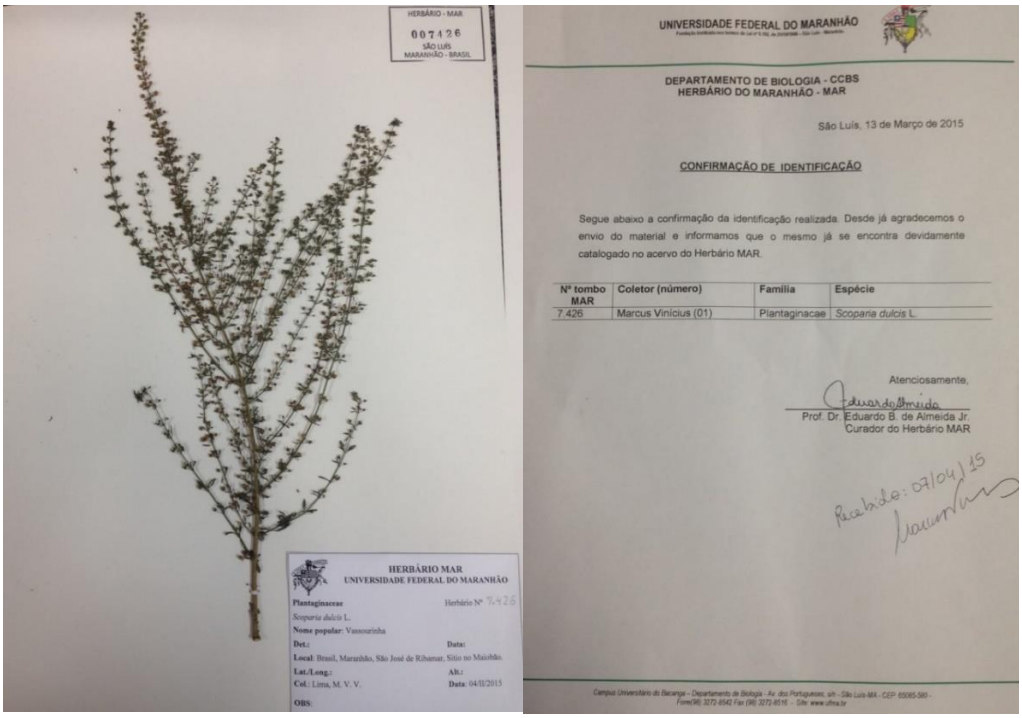

Figura 1: Exicata (nº 7426) de *Scoparia dulcis* identificada no Herbário “Ático Seabra”/UFMA na cidade de São Luís Maranhão, Brasil

2.2.7 Caracterização microscópica

Possui uma folha simples e pinnatificada, de cor verde com odor característico e sabor doce. O caule é lenhoso, a superfície externa é áspera, sabor doce, cor verde e de aspecto irregular e fibroso. Os frutos apresentam superfície externa lisa, cor verde, característica de odor e sabor adstringentes (MISHRA et al., 2012).

A folha da planta, tanto na superfície superior quanto na inferior, contém estômatos anomocíticos, um tricoma glandular séssil único. O caule contém cristais de grãos de amido e oxalato de cálcio na região do córtex. Seu feixe vascular contém vasos espirais, traqueídeos, fibras, etc. A epiderme do caule apresenta tricomas uniseriados, multicelulares e glandulares (MISHRA et al., 2012).

A raiz mostrou a presença de periderme contendo matéria acastanhada. O phellogen mostra duas linhas de células circundando a única camada de phelloderm. O córtex consistia em 4-6 camadas de células do parênquima tangencialmente oblongas e comprimidas radialmente. Algumas células do parênquima continham grãos de amido. O floema consistia em elementos de

peneira e células associadas. O xilema foi lignificado e consiste em elementos do xilema, como parênquima e traqueia. O câmbio separa a região do xilema e do floema. Os raios medulares na região do floema não são lignificados, enquanto os lignificados na região do xilema (MISHRA et al., 2012).

2.2.8 Constituintes químicos

Scoparia dulcis é uma rica fonte de flavonoides, terpenos, esteroides, fenóis, taninos, saponinas, aminoácidos, cumarinas e carboidratos. Os produtos químicos principais incluem os ácidos de scopadulcic A e B, scopadiol, scopadulciol, scopadulin, os ácidos scoparic A – C e o ácido betulínico. Outros produtos químicos incluem: acacetina, amirina, apigenina, benzoxazina, benzoxazolina, benzoxazolinona, cirsimarina, cirsitakaoside, coixol, ácido cumárico, cynaroside, daucosterol, dulcinol, ácido dulcóico, ácido gentísico, glutinol, hymenoxin, linarin, luteolina, manitol, scoparinol, scutellarein, scutellarin, sitosterol stigmasterol, taraxerol, vicenina, e vitexina (MISHRA et al., 2011; PAUL; VASUDEVAN; KRISHNAJA, 2017).

2.2.9 Usos tradicionais

Nas regiões subtropicais e tropicais, seu uso popular tem sido utilizando a planta fresca ou seca tradicionalmente como remédios para problemas de estômago, hipertensão, diabetes, inflamação, dor, bronquite, hemorróidas, problemas hepaticos, (FREIRE et al., 1996; MONIRUZZAMAN; ATIKUR RAHMAN; FERDOUS, 2015) urinários e picada de inseto (RATNASOORIYA et al., 2005).

Outras atividades têm sido observadas, como: atividade antidiurética, analgésica, antipirética (RATNASOORIYA et al., 2005), antioxidante (COULIBALY et al., 2011), antibacteriana, anti-HIV, anti-malaria, anticancer (TSAI et al., 2011), antifúngica, anti-herpética, anti-séptico, antiespasmódico, antiviral, emoliente e expectorante (DE FREITAS et al., 2015).

Partes da planta têm sido usadas para uma variedade de comorbidades, como (MISHRA et al., 2011):

Peças aéreas: tosse, diarreia, expectoração, febre e dores no estômago;

Folha: diabetes, diarreia, problemas oculares, febre, dores de cabeça, hemorroidas, infecções, picadas de insetos, vermes intestinais, doença renal, distúrbios do fígado, malária, distúrbios menstruais, enxaquecas, picadas de cobra, distúrbios do estômago, tônico, úlceras, distúrbios do trato urinário, vômitos, feridas, anemia, queimaduras e tosse;
Raiz: bronquite, diarreia, febre, icterícia, distúrbios do fígado, malária, distúrbios menstruais, infecções de pele, dores de estômago;
Planta inteira: anemia, bronquite, queimaduras, tosse, diabetes, diarreia, disenteria, expectorante, febre, distúrbios gástricos, dor de cabeça, hemorroidas, hepatite, hipertensão, infecções, picadas de insetos, vermes intestinais, icterícia, doença hepática, malária, menstrual distúrbios, dor, erupção cutânea, picadas de cobra, inchaço e dor de dente.

2.2.10 Usos terapêuticos

Os efeitos anti-inflamatórios e analgésicos da planta (PAULINO et al., 2011; DE MEDEIROS; LADIO; ALBUQUERQUE, 2013) foram demonstrados, para uso no alívio e desconforto causados pela menstruação, menopausa (MICHEL et al., 2007), dor de parto, inflamação uterina (SOUZA et al., 2014), lesões gástricas, bem como uma alternativa para atenuar efeitos colaterais e adversos de analgésicos em humanos (RATNASOORIYA et al., 2005).

2.2.11 Fitoquímica e atividades biológicas

Sua composição fotoquímica e as atividades biológicas relacionadas estão descritas abaixo nas tabelas de 1 a 6.

Tabela 1: Flavonoides e atividades biológicas

Componente	Atividade biológica	Referência
Apigenina	Antioxidante, eliminador de radicais, anti-inflamatório, promotor do metabolismo de carboidratos, modulador do sistema imunológico	Mishra et al. (2011)
Acacetina	Inibe as correntes de potássio atriais humanas, repolarização, antioxidante, sequestrador de radicais, anti-inflamatório, promotor do metabolismo dos hidratos de carbono	Hashimoto; Yamashila (2006)
Cirsimarina	Estimular a lipólise, anti-inflamatória, antidiabética e hipotensiva	Girotti et al. (2005); Saikat et al. (2009)
Cirsitakaoside	Doença respiratória, distúrbios gástricos, hepáticos, anti-inflamatórios, antidiabéticos e hipotensão	Saikat et al. (2009)
Cynaroside	Antioxidante, antidiabético	Park et al. (2002); Xiao Sun et al. (2011)
Hymenoxin	Estrogênico, antiespasmódico	Mishra et al. (2011)
Linarin	Propriedades sedativas e melhoradoras do sono	Sebastián Fernández et al. (2004)
Luteolina	Antioxidante, anticâncer, imunomodulador, anti-inflamatório	Chowdhury et al. (2002); Kelley; Johnson (2008)
Scutellarein	Induzir a apoptose de células tumorais de ovário e mama in vitro	Mishra et al. (2011)
Vicenina 2	Anticâncer e anti-inflamatório	Nagaprashantha et al. (2011)
Vitexina	Antioxidante e hipotensivo	Kim et al. (2005)
Vitexin, iso	Antioxidante e hipotensivo	Kim et al. (2005)

Tabela 2: Diterpeno e atividade biológica

Componente	Atividade biológica	Referência
Ácido Scopadulcico A	Falciparum malária	Hayashi et al. (1992)
Ácido Scopadulcico B	Antiviral, atividade antitumoral em várias linhas celulares humanas	Mishra et al. (2011)
Ácido Scoparic A	Inibição da B-glucuronidase	Kawasaki et al. (1987); Hayashi et al. (1992)
Ácido Scoparic B	Antiviral	Hayashi et al. (1992)
Ácido Scoparic C	Inibição da B-glucuronidase	Hayashi et al. (1992)
Dulcitol	Atividade antiviral e citotóxica	Mishra et al. (2011)
Scoparinol	Anti-inflamatório e analgésico	Mishra et al. (2011)

Tabela 3: Terpenóides e atividades biológicas

Componentes	Atividade biológica	Referência
Amirina, alfa	Atividade anti-elastase, e modula a fluidez da membrana inibição da liberação de PGE2, forte atividade anti-inflamatória, inibidor de PKA, bem como um inibidor seletivo de protease	Mishra et al. (2011)
Ácido Betulínico	Potencial agente anti-melanoma, eficaz como agente anti-inflamatório não esteróide, anti-malárico, anti-HIV, prostaglandina antagonista	Girotti et al. (2005); Perumal; Dharmarajan, (2005); Fulda (2008); Faujan et al. (2010)
Ácido dulcioico	Efeito inibitório significativo na produção de citocinas, antiespasmódico	Girotti et al. (2005);
Friedelin	Estrogênico, antiinflamatório, analgésico e antipirético	Mishra et al. (2011)
Glutinol	Anti-inflamatório e analgésico	Freire et al. (1991); Freire et al. (1993); Mishra et al. (2011)
Ácido Iffaiónico	Hipotensor	Mishra et al. (2011)

Tabela 4: Esteróides e atividades biológicas

Componentes	Atividade biológica	Referência
Daucosterol	Imunomodulador	Lee et al. (2007)
Sitosterol	Antioxidante, anti-câncer, anti-tumoral, reduz os níveis de colesterol no sangue	Bouic, (2001)
Estigmasterol	Anti-câncer, colesterol sérum inferior, antioxidante, hipoglicêmico	Panda et al. (2009)
Taraxerol	Anti-câncer e anti-tumor	Mishra et al. (2011)

Tabela 5: Outros componentes e atividades biológicas

Componentes	Atividade biológica	Referência
Benzoxazin-3-ona, 1-4: 2 (h): hidroxi	Antimicrobiano, anticâncer e antiinflamatório	Mishra et al. (2011)
Benzoxazolinona	Propriedades adrenérgicas e anti-hipertensivas	Mishra et al. (2011)
Benzoxazolin-2-ona, 6-metoxi	Antimicrobiano e anti-inflamatório	Perumal; Dharmarajan, (2005); Faujan et al. (2010)
Benzoxazolona, 2 (3H) 6-metoxi	Antimicrobiano, analgésico e anti-inflamatório	Mishra et al. (2011)

Tabela 6: Outros compostos e atividades biológicas

Componentes	Atividades biológicas	Referência
Ácido gentísico (benzenoide)	Antiespasmódico, anestésico local, antioxidante e anticonvulsivo	Mishra et al. (2011)
Manitol, d (carboidrato)	Diurético, doença de Alzheimer, quimioterapia para tumores cerebrais	Ikeda et al. (2002)
Ácido Coumarico, para (fenilpropanóide)	Inibe o desenvolvimento de câncer de estômago	Ferguson; Shuo-Tun; Harris, (2005)

2.2.12 Estudos farmacológicos

2.2.12.1 Atividade antioxidante

Babincová;Sourivong (2001) utilizaram o ensaio DPPH para demonstrar a forte atividade antioxidante do extrato bruto de *S. dulcis*. Coulibaly et al. (2011) estudaram a propriedade antioxidante dos extratos hexano, clorofórmio e metanol da planta pelos ensaios DPPH e FRAP.

Os extratos, aquoso, etanólico e clorofórmio, mostraram ter significativa capacidade antioxidante (DPPH, FRAP, B-caroteno branqueamento e TBARS) (LATHA et al., 2004; PARI; LATHA, 2004) . O extrato de clorofórmio exibiu a maior atividade antioxidante pelo possível conteúdo fitoquímico rico em metabolitos secundários, como os flavonoides (COULIBALY et al., 2011) .

2.2.12.2 Atividades analgésicas e anti-inflamatórias

Freire (1993) avaliaram o edema de pata induzida por carragenina em ratos e contorção induzida por ácido acético em camundongos e após a administração de glutinol (30mg/kg), a contorção induzida por ácido acético foi reduzida em camundongos em 40% e o edema de pata induzido por carragenina em ratos em 73%. Não foram detectados efeitos analgésicos ou antiedema significativos em animais pré-tratados com extrato aquoso (1mg/kg). Os resultados indicam que a atividade analgésica de *S. dulcis* pode ser explicada por uma atividade anti-inflamatória provavelmente relacionada ao triterpeno glutinol.

Tsai et al. (2011) estudaram o efeito do extrato etanólico sobre o edema de pata induzida por carragenina em camundongos, sugerindo que a S. *dulcis* e o ácido betulínico têm atividades anti-inflamatórias. Os mecanismos anti-inflamatórios parecem estar relacionados com a redução da COX-2, oxido nítrico, diminuição das citocinas inflamatórias (TNF-α e IL1-β), bem como inibição dos níveis de MDA através de atividades de SOD, GPx e GRd aumentadas.

Zulfiker et al. (2010) avaliou o potencial analgésico da *S. dulcis* pela sua ação central usando a placa quente e as ações farmacológicas periféricas usando o teste de contorção induzida por ácido acético nos ratos. O extrato bruto apresentou atividade analgésica significativa ($p < 0,001$) na dose oral de 100 e 200 mg/kg, nos modelos testados.

Coulibaly et al. (2011) obtiveram diferentes extratos de *Scoparia dulcis* por extração sucessiva com hexano, clorofórmio e metanol. Esses extratos apresentaram capacidade antioxidante e anti-inflamatória significativa, provavelmente relacionado à sua composição fitoquímica em termos de conteúdo de polifenóis e carotenóides. O extrato de clorofórmio foi mais rico em

fitoquímicos e obteve melhores resultados para a atividade antioxidante e anti-inflamatória. Esses efeitos parecem estarem relacionados com a peroxidação lipídica e lipoxigenase, embora, Tsai et al. (2011) relatam que o mecanismo do efeito anti-inflamatório não é totalmente elucidado na espécie vegetal.

Adicionalmente, as propriedades analgésicas e hiperanalgésicas do extrato aquoso de S. *dulcis* foi verificado em modelos de ratos (RATNASOORIYA et al., 2005). A analgesia da S *dulcis* tem sido demostrada pelos constituintes ativos presentes na composição fitoquímica da planta, como, o glutinol (FREIRE et al., 1991; FREIRE, 1993) e scoparinol (AHMED et al., 2001). Isolados do extrato vegetal, esses componentes fitoquímicos parecem atuar através de um mecanismo de ação periférica semelhante a agentes anti-inflamatórios não esteróidais, como a indometacina e o diclofenaco sódico (MISHRA et al., 2011) atuando no início da fase do processo inflamatório agudo através de possíveis mecanismos centrais e periféricos (FREIRE, 1993).

Zulfiker (2010) relatam que são necessários mais estudos para compreender o mecanismo preciso de analgesia da *S. dulcis*. Em experimentos futuros, estudos com frações purificadas do extrato podem ser utilizadas para posterior caracterização farmacológica e toxicológica, como a investigação dos mecanismos envolvidos no efeito analgésico central e periférico.

2.2.12.3 Atividade Nefroprotetora

A suplementação de extrato etanólico de S. *dulcis* reduziu a creatinina sérica elevada, os níveis de peroxidação lipídica e melhorou a depuração da creatinina. Os resultados deste estudo sugerem nefroproteção significativa da *S. dulcis* contra nefrotoxicidade por cisplatina (JEDAGE, 2014).

2.2.12.4 Atividade antiúlcera

Babincová; Schronerová; Sourivong (2008) constatou que o extrato aquoso de S. *dulcis* tem uma atividade gastroprotetora, pois inibiu significativamente a formação de úlceras induzidas por indometacina.

2.2.12.5 Atividade sedativa e hipnótica

No estudo de Moniruzzaman; Atikur Rahman; Ferdous (2015) foi avaliado o efeito sedativo e hipnótico do extrato etanólico de plantas inteiras de S. *dulcis*. O extrato etanólico de S. *dulcis* diminuiu significativamente o tempo de indução para dormir e prolongou a duração do sono, induzida por tiopental sódico. O estudo sugere que o extrato etanólico de plantas inteiras de S. *dulcis* pode possuir princípios sedativos com propriedades hipnóticas potentes.

2.2.12.6 Avaliação toxicológica

Foram avaliados por 30 dias ratos Wistar com doses de 250 e 500mg/Kg e foi observado os sinais de toxicidade por 30 minutos/dia. A administração diária do extrato por 30 dias não produziu sintomas ou mortes toxicológicas brutas. Os efeitos histopatológicos da administração de 250 e 500 mg/kg por dia dos extratos e frações de éter de petróleo, clorofórmico e butanólico de *S. dulcis* aos ratos não mostraram nenhuma evidência da necrose do tecido no coração, no fígado, nos testículos e no pulmão. Não houve alterações adversas marcadas ou degeneração dos tecidos, uma vez que estes órgãos vitais mostraram arquiteturas normais não sugerindo alterações morfológicas em comparação com o grupo controle, sendo portanto, uma indicação da baixa toxicidade do extrato, portanto a *S. dulcis* poderia ser dita relativamente segura para uso terapêutico (ABERE et al., 2015).

3. OBJETIVOS

3.1 Geral

Avaliar o efeito analgésico, anti-inflamatório e antioxidante do extrato bruto e da fração clorofórmica de *Scoparia dulcis*, em modelo experimental de osteoartrite.

3.2 Específicos

a) avaliar os efeitos na alodinia mecânica e hiperalgesia primária;
b) descrever os efeitos na avaliação edematogênica;
c) avaliar os efeitos na dor espontânea*;*
d) verificar a inibição da COX-1 e da COX-2;
e) realizar a análise química do extrato bruto e fração clorofórmica;
f) avaliar o total de flavonoides e polifenóis totais;
g) analisar o efeito antioxidante;
h) analisar o acoplamento molecular dos compostos identificados e sua interação com a COX-2;
i) analisar a resposta inflamatória através da dosagem de citocinas;
j) verificar o efeito da lesão articular através da análise histológica.

4. RESULTADOS

4.1 CAPITULO I – ARTIGO 1

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

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Dados não publicados sobre a atividade antioxidante do extrato bruto: anexo 2 (página 86)

Article

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

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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 of osteophytes [1,2]. OA mainly affects the joints that are given greater weight, such as the knees, [3] It alters an individual's the quality of life [4,5], is the most frequent cause of musculoskeletal disease and pain [6,7], and impacts daily living and work activities [8].

Although OA has long been defined as a degenerative disease characterized by increased pressure on a particular joint, the current understanding of OA has shifted from cartilage "wear and tear" to an inflammatory joint disease. Proinflammatory cytokines and chemokines have been shown to disrupt homeostasis in the cartilage matrix of OA patients, with increased production of interleukin-1 (IL-1)β and tumor necrosis factor (TNF)α by articular chondrocytes characteristic of established OA. In addition, IL-1β has been shown to induce chondrocytes to produce other inflammatory mediators, including IL-6 and nitric oxide, further amplifying detrimental cellular responses [9].

Advances in understanding the pathophysiology of OA, through the influence of biochemical stress, abnormal biomechanics of the joint, and the inflammatory pathways involved, have allowed an increase in therapeutic alternatives [10]. There has been a constant search for substances that can be combined with conventional therapy for OA. Currently, conventional therapy consists of a combination of nonpharmacological measures such as aerobic exercises, weight loss, and joint protection techniques, as well as symptomatic pharmacological treatments including anti- inflammatory nonsteroidal analgesics and corticosteroids or local intra-articular lubricants until, eventually, surgical intervention is required [6].

Medicinal plants and their derivatives represent a frequent alternative for the treatment of diseases [11]. Among these species, we highlight *Scoparia dulcis* (*S. dulcis)*, also known as "*vassourinha*", a perennial herb that is found in tropical and subtropical regions [12,13]. Its isolated bioactive constituents have contributed to demonstrate the plant′s medicinal effect by the presence of flavonoids, terpenes, tannins, saponins and steroids in inflammatory and nociceptive processes [14–16], which are the most widely used in the literature. *S. dulcis* has been used to relieve discomfort caused by menstruation, menopause [16], labor pain, uterine inflammation [17], and gastric lesions [18], although the exact mechanism of action remains unclear (15).

No published data on the anti-nociceptive and anti-inflammatory action of the plant species in treating pain caused by knee OA in rats have been found so far. Therefore, the objective of this research is to evaluate the analgesic and anti-inflammatory effect of the crude extract of *S. dulcis*, in an experimental model of OA.

2. Results

Twenty animals were divided into four experimental groups, five animals each: healthy group (GS): untreated healthy animals not induced for OA; saline group (GSAL): OA animals treated orally at 1 mL/kg/day with 0.9% sodium chloride (NaCl); *Scoparia dulcis* Group (GSD): OA animals treated orally with 500 mg/kg/day of crude *S. dulcis* extract; meloxicam group (GM): OA animals orally treated with 1 mg/kg/day meloxicam reported in the results below

2.1. Evaluation of Mechanical Allodynia—Von Frey

Induction of OA reduced the nociceptive threshold of paw withdrawal in all groups in a similar way. However, after 10 days, nociceptive threshold increase and decrease of mechanical allodynia occurred in the GSD and GM groups, which presented values similar to GS at the end of 20 days (Figure 1).

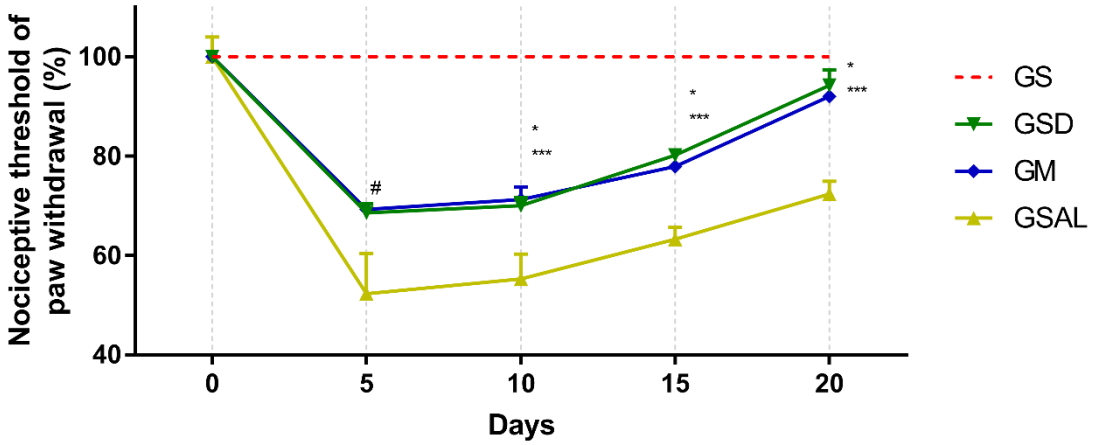

Figure 1. Effect of crude extract of *S. dulcis* on the tactile sensory evaluation using the Von Frey test. Results were expressed as mean ± standard mean error; # difference between healthy group (GS) and saline group (GSAL) in D5 ($p < 0.0001$), GS and *Scoparia dulcis* Group (GSD) in D5 ($p < 0.0001$), GS and meloxicam group (GM) in D5 ($p < 0.0001$); * difference between GSD and GSAL in D10 ($p = 0.0015$), D15 ($p = 0.0002$) and D20 ($p < 0.0001$); *** difference between GM and GSAL in D10 ($p = 0.0004$), D15 ($p = 0.0016$) and D20 ($p < 0.0001$), using 2-way ANOVA with the multiple comparisons test ($p < 0.05$).

2.2. Assessment of Motor Activity/Forced Walking—Rotarod test

The induction of OA reduced gait score in all groups in a similar way. However, only on the 20th day did GSD and GM show an increase in their score (Figure 2).

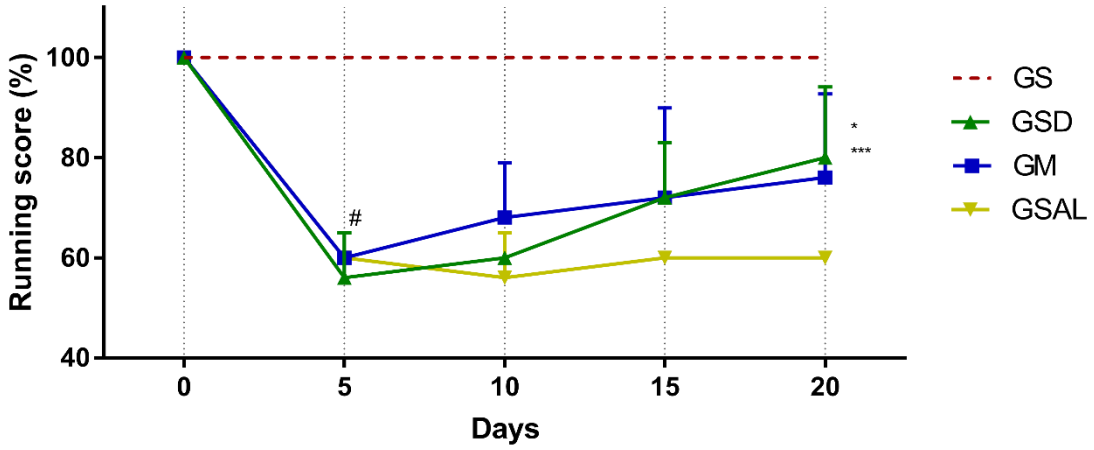

Figure 2. Effect of crude extract of *S. dulcis* on the evaluation of motor activity/forced ambulation using the Rotarod test. Results were expressed as mean ± standard mean error; # difference between GS and GSAL in D5 ($p < 0.0001$), GS and GSD in D5 ($p < 0.0001$), GS and GM in D5 ($p < 0.0001$); * difference between the GSD and GSAL in the D20 ($p = 0.0061$); *** difference between GM and GSAL in the D20 ($p < 0.0472$), using 2-way ANOVA with the multiple comparisons test ($p < 0.05$).

2.3. Functional Incapacity—Weight Bearing Test

Induction of OA reduced the nociceptive threshold of paw withdrawal in all groups in a similar way. However, after 15 days there was an increase in functional capacity (GSD and GM). At the end of 20 days GM is equal to GS (Figure 3).

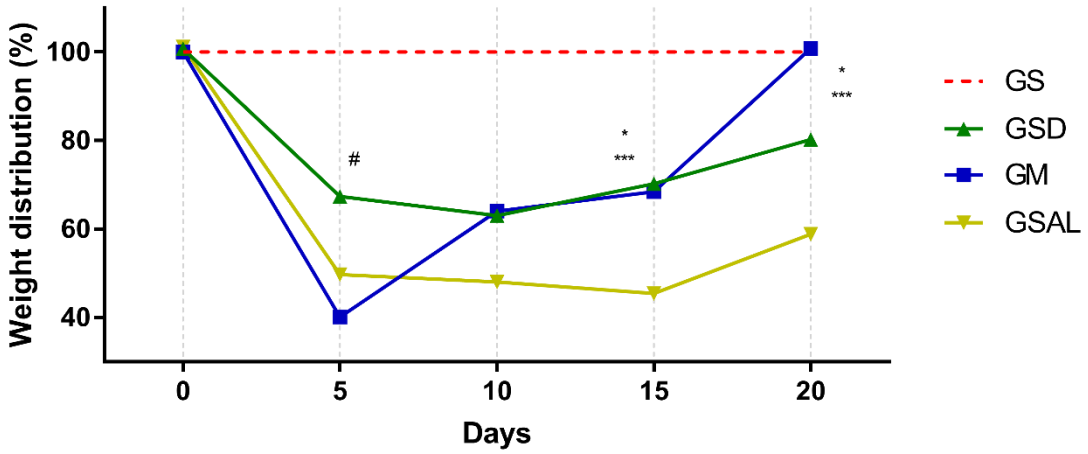

Figure 3. Effect of the crude extract of *S. dulcis* on the assessment of the weight distribution in the hind paws using the weight bearing test. Results were expressed as mean ± standard mean error; # difference between GS and GSAL in D5 ($p < 0.0001$), GS and GSD in D5 ($p < 0.0001$) and GS and GM in D5 ($p < 0.0001$); * difference between GSD and GSAL in D15 ($p = 0.0025$) and D20 ($p = 0.0037$); *** difference between GM and GSAL in D15 ($p = 0.0062$) and D20 ($p < 0.0001$), using 2-way ANOVA with the multiple comparisons test ($p < 0.05$).

2.4. Edematogenic Evaluation

The induction of OA increased the circumference of the right knee characterizing edema in all groups in a similar way. However, after 10 days the edema reduction occurred in the treated groups (GSD and GM), which presented values similar to GS at the end of 20 days (Figure 4).

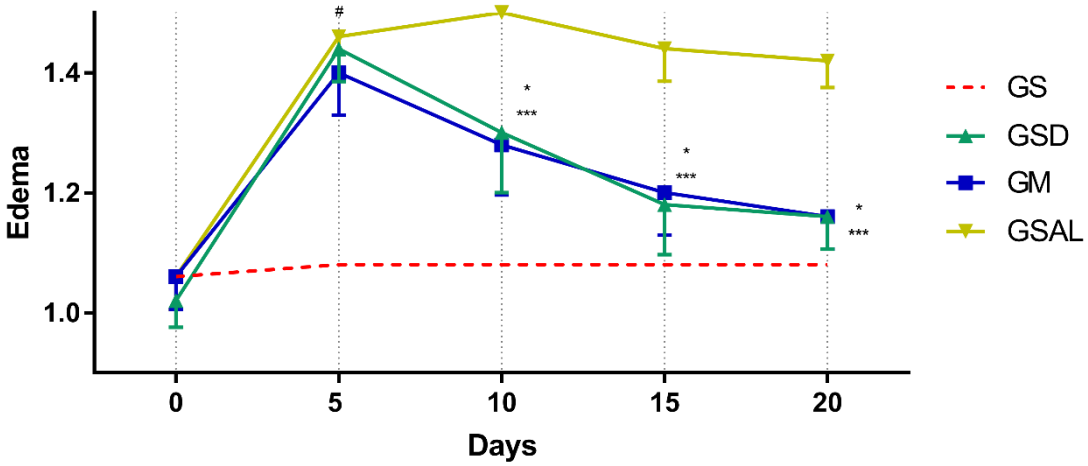

Figure 4. Effect of the crude extract of *S. dulcis* on the edematogenic evaluation of the circumference of the knee by the pachymeter. Results were expressed as mean ± standard mean error; # difference between GS and GSAL in D5 ($p < 0.0001$), GS and GSD in D5 ($p < 0.0001$) and GS and GM in D5 ($p < 0.0001$); * difference between GSD and GSAL in D10 ($p < 0.0001$), D15 ($p < 0.0001$) and D20 ($p < 0.0001$); *** difference between GM and GSAL in D10 ($p < 0.0001$),

D15 ($p < 0.0001$) and D20 ($p < 0.0001$), using 2-way ANOVA with the multiple comparisons test ($p < 0.05$).

2.5. Rating by Mouse Grimace Scale

The induction of OA increased spontaneous pain in all groups of the same magnitude. However, after 10 days the scores decreased in the treated groups (GSD and GM) (Figure 5).

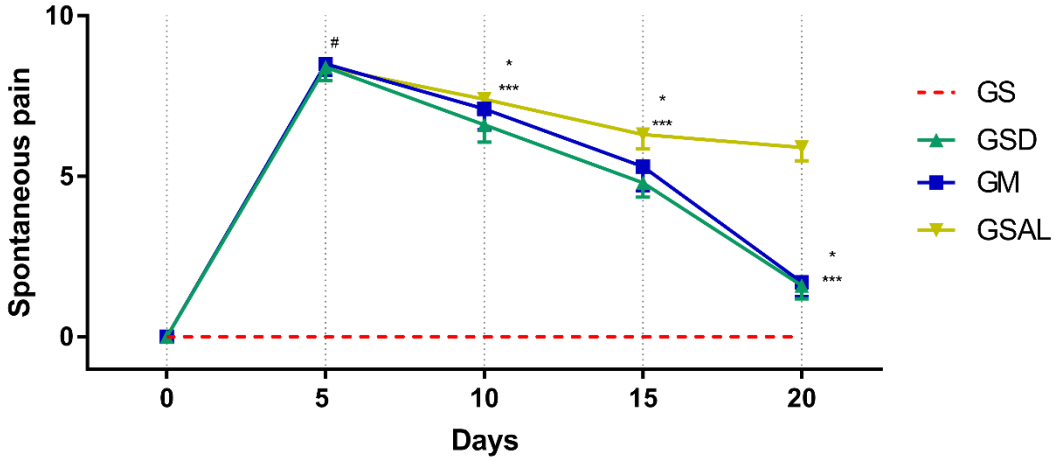

Figure 5. Effect of crude extract of *S. dulcis* on the evaluation of spontaneous pain by Mouse Grimace Scale. Results were expressed as mean ± standard mean error; # difference between GS and GSAL in D5 ($p < 0.0001$), GS and GSD in D5 ($p < 0.0001$), GS and GM in D5 ($p < 0.0001$); * difference between GSD and GSAL in D10 ($p = 0.0062$), D15 ($p < 0.0001$) and D20 ($p < 0.0001$); *** difference between GM and GSAL in D10 ($p = 0.0068$), D15 ($p = 0.0003$) and D20 ($p < 0.0001$), using 2-way ANOVA with the multiple comparisons test ($p < 0.05$).

2.6. Determination of Inhibition of Cyclooxygenase

Cyclooxygenase inhibition assays 1 and 2 showed that the extract at the highest concentration tested (50 µg/mL) can inhibit COX-1 by up to 11.69% and COX-2 by 12.57% suggesting that the extract apparently does not exert greater selectivity for one of these enzymes specifically. The results of the three concentrations (2, 10, and 50 µg/mL) evaluated for COX-1 inhibition also suggest that the extract appears to exert dose dependent effect. However, the results observed at the same concentrations (2, 10, and 50 µg/mL) for COX-2 inhibition do not appear to demonstrate the same dose-dependent effect, as the intermediate concentration 10 µg/mL showed very low inhibition. 0.83% for COX-2, which is lower than that observed for the dose of 2 µg/mL (Figure 6).

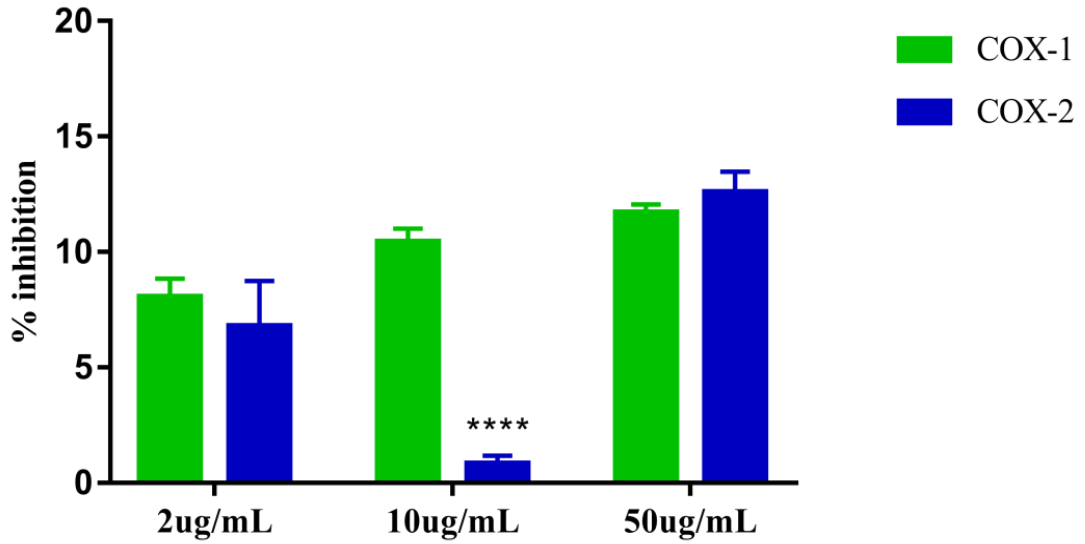

Figure 6. Percent inhibition of in vitro Cyclooxygenase-1 (COX-1) e Cyclooxygenase-2 (COX-2), induced by the crude extract of *S. dulcis*, tested in three concentrations: 2 µg/mL, 10 µg/mL, and 50 µg/mL. The data are represented in mean + standard deviation of the means. **** Represents significant differences, with $p < 0.0001$ comparing inhibition of COX-1 and COX-2.

2.7. Chemical Analysis

The HPLC-ESI-MS analysis exhibit 18 secondary metabolites in the *S. dulcis* crude extract (Figure 7), highlighting flavonoids kaempferol derivates. Sixteen molecules were elucidated compounds (Table 1).

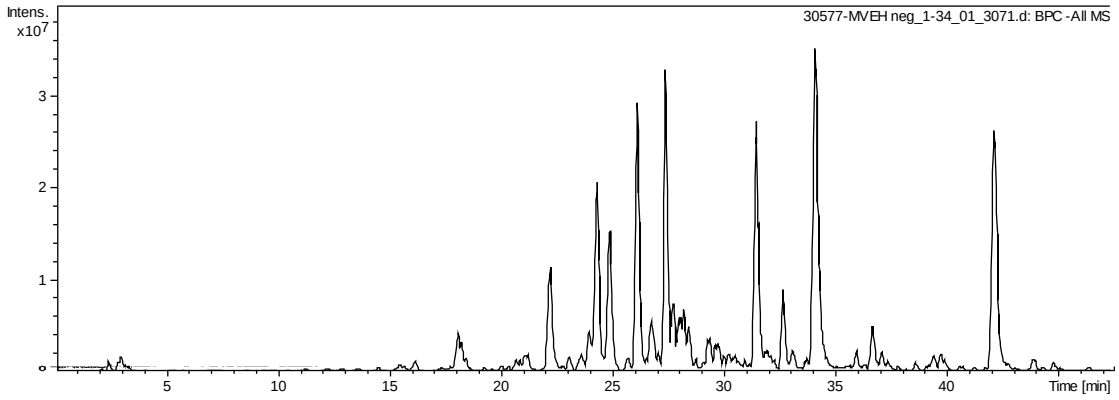

Figure 7. Total Ion Chromatogram of extract from *S. dulcis* acquired by LC-ESI-MS (*m/z*: 100–1500 Da).

Table 1. Identification of compounds in *S. dulcis* crude extract by LC-ESI/MS.

Peak	Tr (min)	[M − H] $^-$	MS^n fragments	Compound	Reference
1	18.0	417	285; 241; 152	kaempferol-3-*O*-pentoside	[19]
2	20.0	327	164; 136	gonzalitosin I	[20]
3	22.3	593	575; 473; 353	nicotiflorin	[21]
4	22.3	639	621; 529; 459	suspensaside	[22]
5	24.0	563	545; 473; 353	apigenin 6-C-pentosyl-8-C-hexoside	[23]
6	24.1	631	563, 479	myricetin-3-*O*-(2″-*O*-galloyl) glucoside	[24]
7	24.4	1127	563	acteoside dimer	[25]
8	26.2	623	563	acteoside (verbascoside)	[25]
9	26.2	623	461; 315	scoparin 7-*O*-glucoside	[26]
10	26.7	563	473; 443; 353	apigenin 6-C-pentosyl-8-C-hexoside isomer	[23]
11	27.4	769	623; 607; 461	deoxyrossicaside A	[27]
12	27.4	791	445; 283	biochanin A *O*-hexoside-*O*-hexoside	[23]
13	31.5	683	637; 313	Ginsenoside F1	[28]
14	31.5	751	705; 381	N.I*	
15	32.6	797	621; 475	N.I*	
16	36.7	299	284	5,7,4′-trihydroxy-3′-methoxyisoflavone (Rhamnocitrin)	[23]
17	42.1	313	297; 283	Diosmetin	[29]
18	44.1	327	291; 229; 211; 171	trihydroxyoctadecadienoic acid	[30]

* Not identified.

2.8. In Silico Assay

To our molecular docking analysis, we used all compounds identified by HPLC-MS on *S. dulcis* crude extract. In general, all compounds showed high parameter affinity with COX-2 structure, highlighting Suspensaside (−9.15 kcal.mol^{-1} and 0.19 μM) and Nicotiflorin (−8.26 kcal.mol^{-1} and 0.88 μM from binding energy and inhibition constant, respectively). In addition to these molecules, the molecular docking of the commercial non-steroidal anti-inflammatory drug meloxicam was performed, with affinity parameters of −8.89 kcal.mol^{-1} and 0.30 μM. The results of the binding energy values of all compounds are presented on Table 2.

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

Ligand	ΔGbind (kcal.mol^{-1})*	Ki (μM)**
Suspensaside	−9.15	0.19
Nicotiflorin	−8.26	0.88
Diosmetin	−7.97	1.44
Rhamnocitrin	−7.72	1.81
Gonzalitosin I	−7.54	1.98
Kaempferol-3-*O*-pentoside	−7.45	3.44
Acteoside	−7.33	4.22
Meloxicam	−8.89	0.30

* ΔGbind, binding energy. ** Ki, inhibition constant.

According to our results, both suspensaside and nicotiflorin performed interactions with important residues from COX active sites like Arg120 and Glu524 and with neighboring residues. Suspensaside performed more interactions of hydrogens bonds, and also performed pi-pi interactions. These interactions are energetically important. Suspensaside also performs seven van der Walls interactions while nicotiflorin, just 4 interactions (Figure 8). The spatial conformation of suspensaside and nicotiflorin with the COX-2 structure was shown on Figure 9.

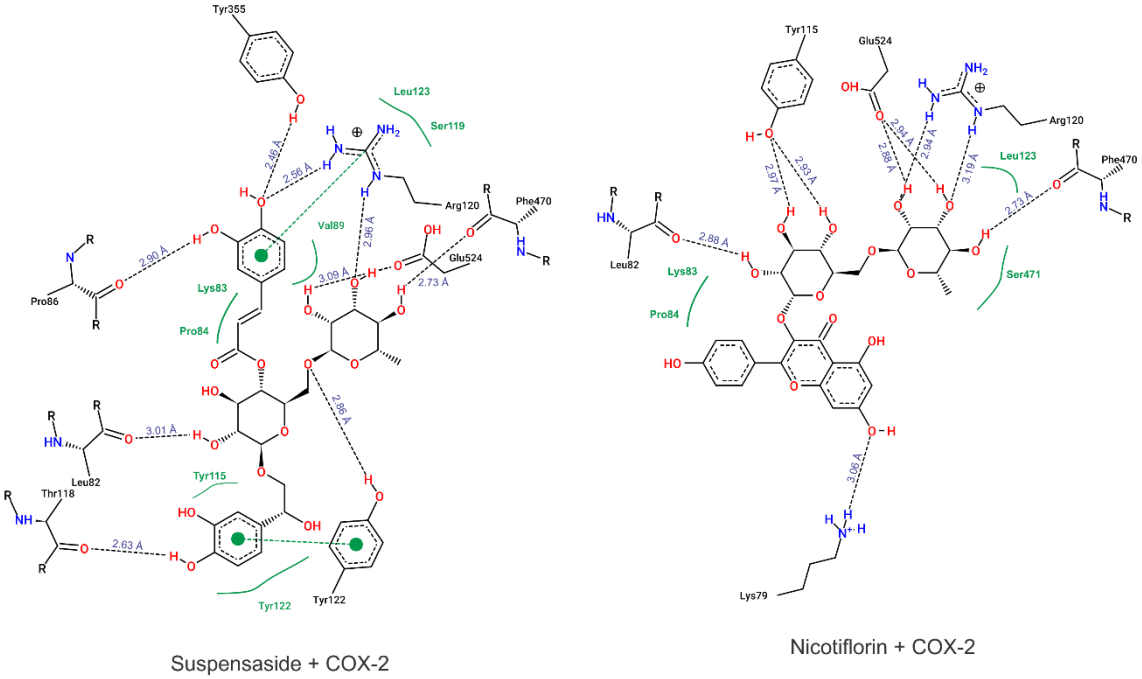

Figure 8. Diagram from interactions performed by Suspensaside and Nicotiflorin with COX-2 structure. Black dashed lines represent hydrogens bonds; green dashed lines represent pi-pi interactions and green full lines represent van der Walls interactions. The Figure was generated with PoseView.

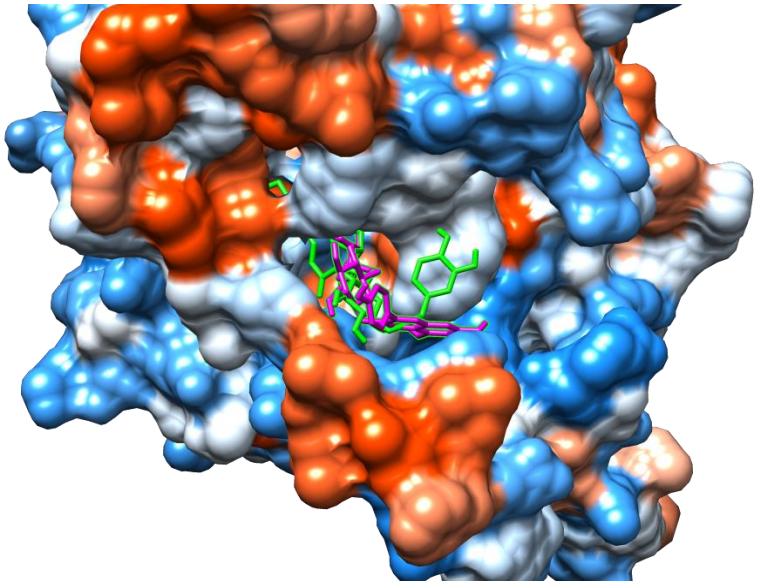

Figure 9. Spatial conformation of suspensaside (green) and nicotiflorin (magenta) on the COX-2 active site (PDB 1DDX). Image generated with USCF Chimera.

2.9. Determination of Cytokines (in Synovial Fluid)

The animals treated with the crude extract and meloxicam showed a reduction of the cytokines IFN-γ and IL-6 when compared to the saline group that was statistically significant. As for interleukin 10, the crude extract and meloxicam groups presented a statistically significant increase when compared to the saline group (Table 3).

Table 3. Effect of crude extract of *S. dulcis* on cytokines.

Cytokines	GSD	GM	GSAL	GS
IFN-γ	1387 ± 71.06 (p = 0.0090)*	1559 ± 117.9 (p = 0.3103)	1748 ± 195.7	1302 ± 157.8
IL-6	35.46 ± 6.78 (p = 0.0300)*	32.19 ± 11.54 (p = 0.0106)***	55.91 ± 8.08	24.73 ± 9.92
IL-10	384 ± 28.09 (p = 0.0035)*	390.5 ± 22.62 (p = 0.0019)***	276.8 ± 46.76	373.2 ± 31.17

* GSD and GSAL for IFN-γ (p = 0.0090), IL6 (p = 0.0300) and IL10 (p = 0.0035); *** GM and GSAL for IL6 (p = 0.0106) and IL10 (p = 0.0019), using One-way ANOVA with the multiple comparisons test (p < 0.05).

2.10. Microscopic Classification Articular Cartilage Histology in Osteoarthritis

The histopathological evaluation using the OARSI scoring system for safranin *O*, the saline group showed a higher classification than the other groups (3.2 ± 0.95), indicating a greater involvement of the articular cartilage in these animals. The group treated with crude extract of *S. dulcis* and meloxicam presented mean scores of 2 ± 1.25 and 2.6 ± 0.81, respectively (Table 4 and Figure 10).

Table 4. Effect of crude extract of S*. dulcis* on articular cartilage.

Variables	GSD	GM	GSAL	GS
Degree	2 ± 1.25	2.6 ± 0.81	3,2 ± 0.95	0 ± 0
***p*-value (GS)**	(p = 0.0006)*	(p < 0.0001)***		
***p*-value (GSAL)**	(p = 0.0492)+	(p = 0.5742)		

* Difference between GSD and GS for histology of articular cartilage (p = 0.0006); *** Difference between GM and GS for histology of articular cartilage (p < 0.0001). + Difference between GSD and GSAL for histology of articular cartilage (p = 0.0492), using One-way ANOVA with the multiple comparisons test (p < 0.05).

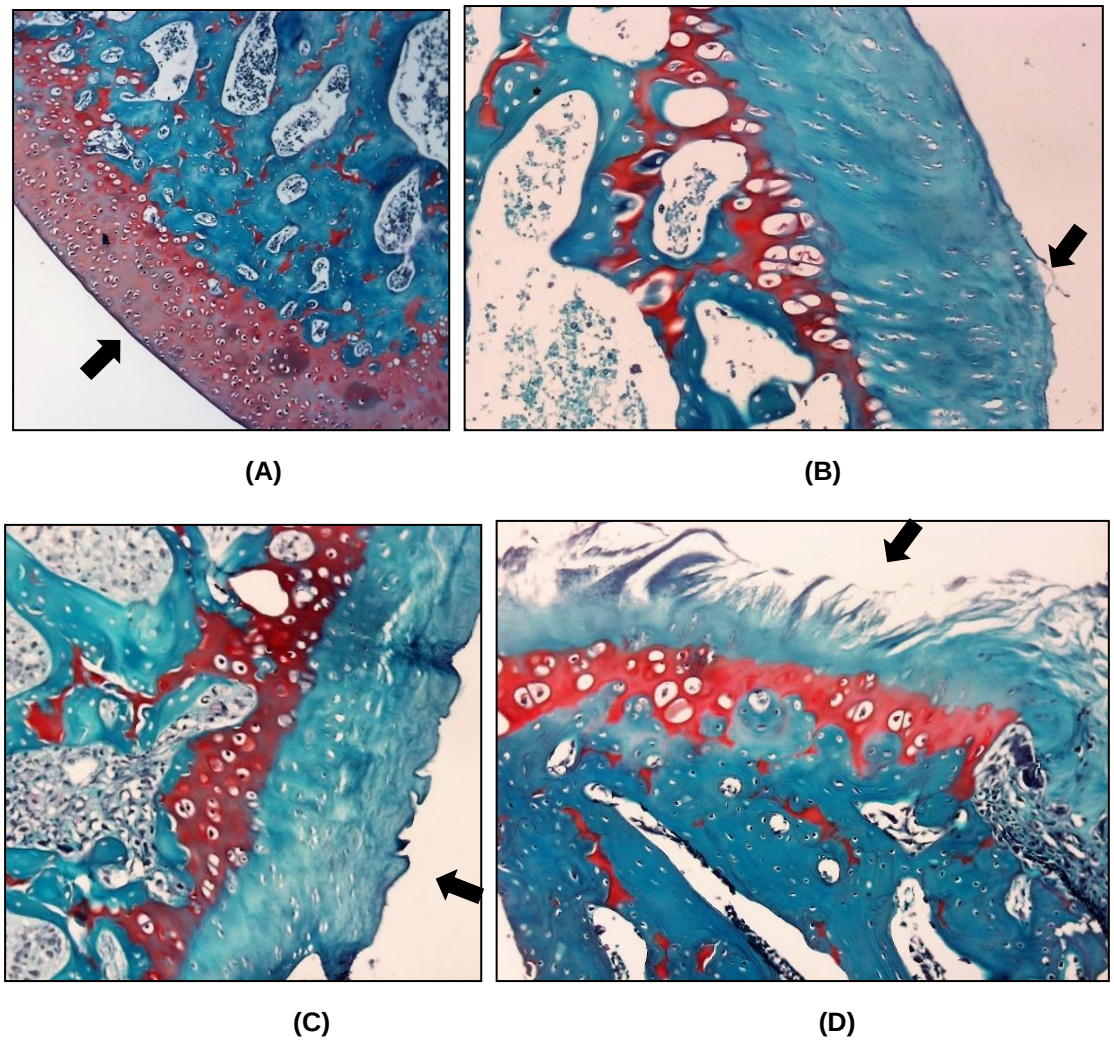

Figure 10. Effect of crude extract of S*. dulcis* on classification articular cartilage histology. (**A**) For articular cartilage, the score 0 represents the surface and morphology of intact cartilage (GS) (Figure 10A). (**B**) Score 1, an intact surface with superficial fibrillation (GSD) (Figure 10B). (**C**) For score 2 there is already a discontinuity of the articular cartilage (GM) surface (Figure 10C). (**D**) For score 4 (GSAL), the surface has vertical crack-like cracks with erosion areas (Figure 10D).

3. Discussion

This study aimed to evaluate the anti-nociceptive and anti-inflammatory effect of the crude extract of *S. dulcis* in an experimental model of knee OA in rats, with meloxicam as the positive control. It is the first study to evaluates the effect of crude extract of *S. dulcis* on pain and inflammation in knee OA in rats.

Throughout the study, the following markers were observed: an improvement in the gait score; the reduction of mechanical allodynia; the distribution of the weight discharge in the hind paws; signs of spontaneous pain and edema; reduction in inflammation; an increase in the peripheral nociceptive threshold; decrease in proinflammatory cytokines, inflammation and edema by synovial membrane histology; as well as a discontinuity of articular cartilage degradation.

The results of the present study showed similarity in the effects of the crude extract of *S. dulcis* to those of meloxicam, perhaps because they act more effectively in inhibiting the COX-2 and arachidonic acid cycle, which could result in decreased pain, inflammation, and degeneration of the articular cartilage by reducing primary nociception, edema, and the production of pro-inflammatory molecules [31].

According to Zulfiker et al. [32] all of the previously mentioned secondary metabolites isolated from the plant extract have a mechanism of peripheral action similar to non-steroidal anti-inflammatory agents such as indomethacin and diclofenac sodium. However, some compounds such as scoparic acid A, scoparic acid D, scutellarein, luteolin, coixol [33], scoparinol [34], glutinol [35], and betulinic acid [36] common in the plant species *S. dulcis* were not present in the crude extract.

Computational methods have been used as a quick and inexpensive alternative to experimentally screen large compound libraries to allow the identification of new target proteins from natural therapeutic products and thus reduce the number of experiments needed to determine their molecular mechanisms of action [37,38].

The anti-inflammatory effect of crude extract of *S. dulcis* through the suspensaside constituent appears to be closely related to inhibition of proinflammatory mediators (TNF-α, IL-1β, and IL-6), nitric oxide, and PGE_2 in stimulated in lipopolysaccharide stimulated BV2 microglia cells through the activation of the Nrf2/HO-1 signaling pathway and down-regulation of NF-κB, JAK-STAT and p38 MAPK signaling pathways [39]. Nicotiflorine has antioxidant, anti-inflammatory, and neuroprotective effects by reducing proinflammatory cytokines, including TNF-α, IFN-γ, IL-1, and IL-6, which may explain the anti-inflammatory effect of the extract. [40].

Structurally, the active site of COX-2 consists of a lipophilic channel whose entry is formed by Arg120, Tyr355, and Glu524 residues [41] and its activation causes arachidonic acid metabolism from interacting with COX Arg120, Tyr355, Tyr385, and Ser530 residues, leading to prostaglandin production [42]. The molecular docking results shows that the secondary metabolites identified on *S. dulcis* crude extract interact favorably and spontaneously with these residues and as neighboring residues that are also important for interaction with drugs such as ibuprofen [43], meloxicam, and isoxicam [42]. This finding suggests that this plant species may be considered as a potential source in the search for new therapeutic alternatives.

In patients with OA, chondrocytes, as well as synovial cells, produce increased levels of inflammatory cytokines, such as interleukin 1β and TNF-α, which in turn decrease collagen synthesis and increase catabolic mediators, such as metalloproteinases and other inflammatory substances such as interleukin 8, interleukin 6, prostaglandin E_2, and nitric oxide. Moreover, mechanical stress, both by static and dynamic compression, increases the production of nitric oxide by the chondrocytes [44].

The cytokine IFN-γ is associated with the activation of microglial cells and nerve sensitization. IL-6 is a cytokine that promotes maturation and activation of neutrophils and macrophages, as well as differentiation/maintenance of cytotoxic T lymphocytes and natural killer cells. It also activates astrocytes and microglia in the dorsal region of the spinal cord [45].

The decrease in IFN-γ and IL-6 cytokines in the present study suggests that there was a reduction in spinal cord neuroinflammation, which can lead to the attenuation of pain signals and improvement of hypersensitivity and hyperalgesia.

IL-10 is an anti-inflammatory cytokine that inhibits proinflammatory cytokines, mainly TNF, IL- 1, and IL-6, enhances the proliferation of mast cells, and prevents the production of IFN-γ by natural killer cells [46]. The increase in IL10 in synovial fluid may be related to decreased IL-6 and IFN-γ levels in the study and may be associated with improved clinical signs, with a possible reduction in inflammation and increase in peripheral circulation and nociceptive threshold.

Prostaglandins E_2 and leukotrienes when released play a key role in the genesis of signs and symptoms of the inflammatory process, such as spontaneous edema and pain, which are evaluated in the crude extract group in the study, in addition to hypersensitizing the polymodal nociceptors of the C fibers to mechanical stimuli, chemical [47] and cytokines [48]. A possible inhibition of COX-2 by the *S. dulcis* extract seems to suggest a decrease in the production of prostaglandins E_2 in the joint, showing its possible similarity to the positive control of the study.

Decreasing IL-6 and IFN-γ cytokines may have led to a progressive improvement in mechanical allodynia over the 15 days of the study, but other unstudied mechanisms may be associated with pain.

Functional incapacity and forced motor activity/ambulation improved only between the 15th and 20th day of treatment. The proliferation of fibrous tissue in the knee, as demonstrated by the synovial membrane histology of the present study, can cause biomechanical and functional alterations that can result in a postural adjustment to compensate and protect the injured knee by, reducing the weight to maintain the joint in flexion and, thus minimizing pain [49].

Another possibility for this result includes the decrease in joint space caused by sodium mono-iodoacetate induction (MIA), which could lead to joint misalignment and concentration of intra-articular stress, and thus, increase the impact of weight discharges on ambulation, increased friction in the joint articulation, which increases or maintains joint pain, and possibly a compensatory postural adjustment [50], as seen in the functional incapacity test.

During the inflammatory process, there is an increase in vascular permeability, capillary extravasation, and cell migration [51] as demonstrated by the histology of the study. Thus, a classic sign of the inflammatory process is the formation of edema that can continue for days and reduce joint mobility [52].

A histopathological analysis of the synovial membrane and articular cartilage showed encouraging results, suggesting the possible efficacy of *S. dulcis* in the treatment of OA at the dose tested. Hyaline cartilage is the most relevant articular tissue in the pathogenesis of this disease [6]. Although OA is characterized by subchondral bone sclerosis, it is uncertain that bone changes are the cause or consequence of the lesions, given that the cartilage properties depend on the bone bed, which can be affected by the mechanical function of the cartilage [53].

Velosa et al [54] reported that up to now, OA treatment is based on drugs that control the pain and inflammation associated with synovitis, but do not reduce the destruction of the articular cartilage. Therefore, a herbal remedy with the potential to protect this tissue has excellent clinical relevance. Thus, the possible protective effect of the crude extract of *S. dulcis* on the cartilage demonstrated in the present study may justify the translation of its use for this purpose.

The reduction of the proinflammatory cytokines in synovial fluid may be associated with a reduction in the inflammatory infiltrate in the synovial membrane and, consequently, reduction of edema, as our results show, since synovial inflammation in OA is usually found next to pathologically damaged areas with bone and cartilage, releasing proteinases and cytokines capable of accelerating joint destruction [55].

Corroborating our results, Nagy et al. [56] in an MIA-induced mouse OA model reported that low doses (0.2 mg/kg) and high doses of meloxicam (1 mg/kg) had a chondroprotective effect and that high doses, also protected against subchondral bone lesions, suggesting the interruption of the low grade inflammatory pathway accompanied by chronic deterioration of cartilage, as shown by the possible effects of the crude extract in the study.

Changes in the subchondral bone and bone marrow support and perpetuate the deterioration of cartilage [56], thus the possible chondroprotective effect of *S. dulcis*, suggests that oral therapy with a dose of 500 mg/kg of crude extract may attenuate the progression of the disease in the chronic phase.

The effects of the crude extract of *S. dulcis* on histological progression, pain behavior, and inflammatory process in OA presented in the study are impressive, however, this work had some limitations that need to be highlighted, such as not evaluating the antioxidant activity of the crude extract by enzymatic and non-enzymatic methods. Also, in future studies, a centrally acting medication could be used as a positive control, in addition to evaluating different doses and their possible effects on the inhibition of COX-1 and COX-2 and possible toxic effects.

4. Materials and Methods

The study was conducted at the Experimental Laboratory for Study of Pain (LEED), after approval by the Ethics Committee on Animal Use of the Federal University of Maranhão—Brazil (CEUA-UFMA number 23115.018030/2014-94).

4.1. Animals

Twenty male approximately 60-day-old Wistar rats, *Rattus norvegicus* species (albinus variety) were used in the study. The animals were obtained from the Central Animal Facility of the Universidade Federal do Maranhão. They remained in the vivarium of the LEED and were fed a standard ration and water ad libitum and maintained under controlled conditions of light and temperature.

4.2. Plant Species

The aerial parts of *S. dulcis* (2 kg) were collected at Cidade Universitária Dom Delgado at the Federal University of Maranhão (UFMA), city of São Luís (MA, USA) (Latitude: 02°31′47″S Longitude: 44°18′10″W and Altitude: 24 m) in April 2017 under similar conditions of climate and temperature [35,57]. The species was identified in the “Atico Seabra” herbarium at UFMA under exsiccate no. 7426.

4.3. Obtaining Extract from the Aerial Parts of Scoparia dulcis

The aerial parts of *S. dulcis* were oven dried with forced air circulation at 45 °C under constant weight and pulverized in a knife mill to obtain a moderately thick powder (666.66 g). The obtained powder which underwent an extraction process by maceration, was subjected to the drug/solvent ratio of 1:4 (*w*/*v*) with 2400 mL of ethanol for 48 h. The extractive solution was filtered and concentrated in a rotary evaporator under vacuum (40 °C) to obtain a 70% ethanol extract from the aerial parts of *S. dulcis* [35,57].

4.4. Experimental Protocol

Twenty animals were divided into four experimental groups with, 5 animals each: sadio group (GS): untreated and uninduced healthy animals for OA; salina group (GSAL): OA animals treated orally, with 1 mL/kg/day with 0.9% sodium chloride (NaCl); *Scoparia dulcis* group (GSD): animals with OA treated orally, with 500 mg/kg/day of crude extract of *S. dulcis*; meloxicam group (GM): animals with OA treated orally with 1 mg/kg/day of meloxicam.

4.5. Sodium MIA-Induced OA Model

For the induction of OA, the animals were anesthetized with intraperitoneal injections of 40 mg/kg of sodium thiopental. 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 of sodium MIA (diluted in a maximum volume of 25 µL) into the right knee through the patellar ligament [58,59].

4.6. Clinical Evaluations

4.6.1. Motor Activity Assessment—Forced Walking (Rotarod test)

The animals were placed in a Rotarod (IITC Life Science, Woodland Hill, CA, USA) at a rate of 16 revolutions per minute for a period of 300 s. Use of the affected limb was assessed through forced walking. Use of the homolateral paw for MIA induction was graded by a subjective measure that employed a numerical scale ranging from 5 to 1 (5 = normal paw use; 4 = mild limping; 3 = severe limping; 2 = intermittent disuse of affected paw; 1 = complete disuse of affected paw) [60].

4.6.2. Weight-Bearing Test/Weight Distribution Test on Hind Legs

The animals were placed in a glass bowl angled and positioned so that each hind leg lay 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 3 measurements [58].

4.6.3. Quantification of Mechanical Allodynia (*Von Frey Test*)

The evaluation of mechanical allodynia was performed using an electronic device (Model 1601C, Life Science, San Francisco, CA, USA), which consisted of a pressure transducer connected to a digital force counter expressed in grams (g) and calibrated to record a maximum force of 150 g. The animals were placed in individual transparent acrylic boxes on raised platforms to allow access to the lower part of their bodies. The holes in the platforms provided access to the transducer tip, allowing its contact with the animals′ paws. The frequency of the paw′s withdrawal response to the filament stimulus was measured in 5 applications, lasting 1s each, always performed by the same evaluator, and the final result was the mean of all measurements [60,61].

4.7. Mouse Grimace Scale (MGS)

The MGS a new method to evaluate spontaneous pain in animals in the laboratory through changes in facial expressions. To discriminate the subjective sensation of facial pain, the following evaluation criteria were adapted from Sotocinal et al. [62]: absent pain equals “0”; moderate pain equals “1”, and severe pain equals “2” through identification of eye changes, changes in nose/cheek protuberance, changes in ears and changes in mustache.

4.8. Edematogenic Evaluation

To evaluate the anti-edematogenic effect, a Starrent® brand caliper was used to quantify the knee joint diameter in millimeters. The pachymeter was positioned on the joint line to check the transverse diameter of the knee. The measurement was performed 3 times in each knee and the mean difference between the 2 limbs was used as the final result [63].

4.9. Cyclooxygenase (COX) Inhibition

The assay was performed according to the manufacturer′s recommendations (Cox colorimetric inhibitor screening—Cayman chemical®) in 96-well plates. To determine the percentage of inhibition of the extract, which was ascertained from the absorbance data and obtained by reading the plate at 590 nm, initially the BW mean value was subtracted from the absorbance means of A (A-BW) and absorbance of each extract at each concentration tested (Absorbance of extract sample [x]-BW). Then, the percentage of inhibition per se was determined by subtracting and dividing the value of each sample (crude extract of *S. dulcis*, at each concentration tested, subtracted the BW value) from the mean absorbance value of A (already subtracted BW), and multiplying by 100.

4.10. Chemical Analysis of Crude Extract of S. dulcis

The *S. dulcis* crude extract was analyzed by HPLC (LC-20AD Shimadzu, Kyoto, Japan) and a Phenomenex Luna C-18 (250 × 4.6 mm − 5 um) column. The mobile phases consisted of ultrapure water containing 0.1% formic acid (A) and methanol (B). The following linear gradient was applied: 0 min, 5% B; 1−60 min, 5−100% B; 60−70 min, 100% B at flow rate of 1 mL/min. The LC was coupled to a mass spectrometer (Amazon Speed ETD, Bruker, MA, USA) equipped with electrospray ionization (ESI) and an ion-trap (IT) type analyzer in negative mode, under the following conditions: capillary voltage at 4.5 kV, capillary temperature of 325 °C, entrainment gas (N2) flow at 12 L/min, and nitrogen nebulizer pressure at 27 psi. The acquisition range was *m*/*z* 100–1000, with 2 or more events.

4.11. In silico Assay

4.11.1. Predictive Models and Theoretical Calculations

The compounds identified in the crude extract of *S. dulcis* had their geometric, electronic and vibrational properties optimized using the Gaussian program 09 [64]. The GaussView 5.0.8 [65]

was used to obtain 3D structural models. Geometric optimization calculations were performed according to the functional density theory (DFT) method, which combine the functional hybrid B3LYP and the set of bases 6-31 ++ G (d, p).

4.11.2. Molecular Docking

All docking procedures utilized Autodock 4.2 package [66,67]. 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 [67]. Docking methodology described in the literature was used with modifications [11,59]. Gasteiger partial charges were calculated after adding all hydrogens. Non- polar hydrogens from COX-2 and *S. dulcis* compounds were subsequently merged. The dimensions of the cubic box in the X-, Y- and Z-axes were 120 Å × 120 Å × 120 Å, respectively, with a spacing of 0.375 Å between grid points. The grid box was centered on 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 *S. dulcis* compounds interactions were chosen using the criterion of lowest docking conformation of cluster with lowest energy combined with visual inspection.

4.12. Laboratory Analysis of Cytokines

A laboratory analysis of the synovial fluid to quantify IL-6, IL-10, TNF-α, and IFN-γ was performed using an enzyme-linked immunosorbent assay (ELISA, R&D Systems®, Minneapolis, MN, USA).

The synovial fluid samples were obtained on D28 by washing out the affected knee joint twice with 200 µL of phosphate-buffered solution (0.15 M, pH 7.4) containing 37.2 mg of ethylenediaminetetra-acetic acid (EDTA, 0.01 M).

4.13. Histopathological Analysis of Articular Cartilage

On day 21, the articular cartilage and subchondral bone of the knee of each animal were removed after euthanasia. The excised components were embedded in paraffin blocks and cut into 5 µm sections, and the proteoglycans of the organic cartilage matrix were specifically stained using 0.5% safranin-*O*.

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 0–6, 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 indicated morphologically intact cartilage, grade 1 indicated an intact surface with possible focal lesions or abrasion, grade 2 showed discontinuity in the articular surface, grade 3 showed vertical fissures, grade 4 presented erosion, grade 5 exhibited denudation with sclerotic bone or fibrocartilaginous tissue repair or both, and grade 6 showed remodeling and bone deformation with changes in the contour of the articular surface [68,69].

4.14. Statistical Analysis

A comparison of the means of the experimental groups was performed with a univariate analysis of variance (One-way ANOVA), followed by the multiple comparisons test. In the evaluation of 2 sources of variability, a bivariate variance analysis (2-way ANOVA) was used. Data were analyzed using GraphPadInstal® 7.0 software (GraphPad software, San Diego, CA, USA) and all analyses had a significance level of $p < 0.05$.

5. Conclusions

Oral treatment with the crude extract of *S. dulcis* reduced edema, spontaneous pain, and peripheral nociceptive activity in the right knee OA, attenuating the histological changes in the

synovial membrane and the articular cartilage with lower synovial levels of IL-6 and IFN-γ, and higher levels of IL-10, possibly due to its anti-inflammatory action.

The tested concentration of 50 µg/mL showed the highest inhibition for COX-1 and COX-2 in the crude extract. All compounds showed high parameter affinity with the COX-2 structure, highlighting suspensaside and nicotiflorin and performed interactions with important residues from the COX active site, such as Arg120 and Glu524 and with neighboring residues.

In the challenging search for drugs that modify the pathogenesis and history of OA progression, preclinical results from the present study are encouraging and support the potential use of the crude extract of *S. dulcis* as a promising therapeutic agent that may complement or serve as an alternative to drug treatment. Further studies should be performed evaluating other doses, extracts, and fractions, as well as toxicity and other possible mechanisms of action.

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Sample Availability: Samples of the compounds are not available from the authors

4.2 CAPITULO II – ARTIGO 2

Título: Therapeutic and antioxidant effect of *Scoparia dulcis* Linn chloroform fraction on the progression of experimental osteoarthritis

Revista: Fitoterapia (submetido – anexo 3, página 87)

Qualis: B1, Medicina I (Plataforma Sucupira)

Fator de impacto: 2.431

Normas da revista: Anexo 4 (página 88)

Therapeutic and antioxidant effect of *Scoparia dulcis* Linn chloroform fraction on the progression of experimental osteoarthritis

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Abstract: Pain is recognized as one of the main symptoms of 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 aim of this study is to evaluate the analgesic, anti-inflammatory and antioxidant effect of the chloroform fraction from *S. dulcis* in an experimental model of osteoarthritis. The experiment was performed with Wistar rats divided into 4 groups with 5 animals each: healthy groups, saline, chloroform fraction and meloxicam. Knee osteoarthritis was induced by intra-articular injection of sodium mono-iodoacetate. Clinical pain parameters were evaluated on days 0, 5, 10, 15 and 20 after induction. Synovial fluid cytokines were quantified. Polyphenol values and total flavonoid contents, antioxidant activity (DPPH •, FRAP and ABTS • + methods) were quantified. The enzymes superoxide dismutase, catalase and glutathione peroxidase were evaluated. A histopathological evaluation was performed on hematoxylin and eosin stained articular cartilage. Results showed that a 15-day treatment with chloroform fraction reduced edema, spontaneous pain, peripheral nociceptive activity and proinflammatory cytokines in synovial fluid. The chloroform fraction showed antioxidant activity. The chloroform fraction of *S. dulcis* has therapeutic potential for the treatment of osteoarthritis due to its antiinflammatory, antinociceptive and antioxidant action.

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

1. Introduction

Osteoarthritis (OA) is a common degenerative joint disease which leads to pain, stiffness, a reduced motion, swelling, crepitus, and disability [1]. OA mainly affects the joints that are given greater weight, such as the knees [2]. It is characterized by the progressive destruction of articular cartilage with joint-space narrowing, osteophyte formation, subchondral bone sclerosis and synovitis [3]. The a etiology and pathogenesis of OA remain poorly understood, but they have been associated with several risk factors such as aging, obesity, and traumatic injuries [4]. However, biochemical factors have been associated with the development of OA [1].

Medicinal plants and their derivatives represent a frequent alternative for the treatment of diseases [5]. Among these species, we highlight *Scoparia dulcis* (*S. dulcis*), a perennial herb

found in tropical and subtropical regions known as vassoarinha [6], has been used to alleviate discomfort caused by menstruation, menopause, pain at work of delivery, uterine inflammation and gastric lesions [2], although the exact mechanism of action remains uncertain [7]. Its isolated bioactive constituents have contributed to demonstrate the plant′s medicinal effect by the presence of flavonoids, terpenes, tannins, saponins and steroids in inflammatory, nociceptive and antioxidant processes [8-11].

No published data on the anti-nociceptive, anti-inflammatory and antioxidant action of the chloroform fraction have been found in the treatment of knee OA in rats.. Therefore, the objective of this research is to evaluate the analgesic, anti-inflammatory and antioxidant effect of the chloroform fraction of *S. dulcis*, in an experimental model of OA.

2. Materials and Methods

The study was conducted at the Experimental Laboratory for Study of Pain (LEED), after approval by the Ethics Committee on Animal Use of the Federal University of Maranhão - Brazil (CEUA-UFMA number 23115.018030/2014-94).

2.1. Animals

Twenty male approximately 60-day-old Wistar rats, Rattus norvegicus species (albinus variety) (200-300g) were used in the study which were procured from the Central Vivarium (Biotério Central) of Federal University of Maranhão (UFMA), São Luis, Brazil. Animals were placed in polyethylene boxes (n = 5 per box) and provided with free access to food and water in an environment with controlled temperature and 12/12 h light/dark cycle at 22 °C and were fed a standard ration and water ad libitum and maintained under controlled conditions of light and temperature.

2.2. Plant Species

The aerial parts of *S. dulcis* (2 kg) were collected at Cidade Universitária Dom Delgado at the Federal University of Maranhão (UFMA), city of São Luís (MA, USA) (Latitude: 02°31′47″S Longitude: 44°18′10″W and Altitude: 24 m) in April 2017 under similar conditions of climate and temperature [11, 12]. The species was identified in the “Atico Seabra” herbarium at UFMA under exsiccate no. 7426.

2.3. Obtaining Extract from the Aerial Parts of *Scoparia dulcis*

The aerial parts of *S. dulcis* were oven dried with forced air circulation at 45 °C under constant weight and pulverized in a knife mill to obtain a moderately thick powder (666.66 g). The obtained powder which underwent an extraction process by maceration, was subjected to the drug/solvent ratio of 1:4 (w/v) with 2400 mL of ethanol for 48 h. The extractive solution was filtered and concentrated in a rotary evaporator under vacuum (40 °C) to obtain a 70% ethanol extract from the aerial parts of *S. dulcis*. After obtaining the concentrated crude extract, it was partitioned with chloroform five times every 12 hours, obtaining the chloroform phase (1000 mL), being concentrated in rotary evaporator route at 30 ° C, obtaining the solid phase of the mixture. Chloroform fraction to be used in biological activity analysis [11, 12].

2.4. Experimental Protocol

Twenty animals were divided into four experimental groups with, 5 animals each: health group(HG): untreated and uninduced healthy animals for OA; saline group (SALG): OA animals treated orally, with 1 mL/kg/day with 0.9% sodium chloride (NaCl); chloroform fraction group (CF): animals with OA treated orally, with 500 mg/kg/day of chloroform fraction of *S. dulcis*; meloxicam group (GM): animals with OA treated orally with 1 mg/kg/day of meloxicam.

2.5. Sodium MIA-Induced OA Model

For the induction of OA, the animals were anesthetized with intraperitoneal injections of 40 mg/kg of sodium thiopental. 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 of sodium MIA (diluted in a maximum volume of 25 µL) into the right knee through the patellar ligament [13, 14].

2.6. Clinical Evaluations

2.6.1. Motor Activity Assessment—Forced Walking (Rotarod test)

The animals were placed in a Rotarod (IITC Life Science, Woodland Hill, CA, USA) at a rate of 16 revolutions per minute for a period of 300 s. Use of the affected limb was assessed through forced walking. Use of the homolateral paw for MIA induction was graded by a subjective measure that employed a numerical scale ranging from 5 to 1 (5 = normal paw use; 4 = mild limping; 3 = severe limping; 2 = intermittent disuse of affected paw; 1 = complete disuse of affected paw) [15].

2.6.2. Weight-Bearing Test/Weight Distribution Test on Hind Legs

The animals were placed in a glass bowl angled and positioned so that each hind leg lay 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 3 measurements [13].

2.6.3. Quantification of Mechanical Allodynia (*Von Frey Test*)

The evaluation of mechanical allodynia was performed using an electronic device (Model 1601C, Life Science, San Francisco, CA, USA), which consisted of a pressure transducer connected to a digital force counter expressed in grams (g) and calibrated to record a maximum force of 150 g. The animals were placed in individual transparent acrylic boxes on raised platforms to allow access to the lower part of their bodies. The holes in the platforms provided access to the transducer tip, allowing its contact with the animals′ paws. The frequency of the paw′s withdrawal response to the filament stimulus was measured in 5 applications, lasting 1s each, always performed by the same evaluator, and the final result was the mean of all measurements [15, 16].

2.7. Mouse Grimace Scale (MHG)

The MHG a new method to evaluate spontaneous pain in animals in the laboratory through changes in facial expressions. To discriminate the subjective sensation of facial pain, the following evaluation criteria were adapted from Sotocinal et al. [17]: absent pain equals “0”; moderate pain equals “1”, and severe pain equals “2” through identification of eye changes, changes in nose/cheek protuberance, changes in ears and changes in mustache.

2.8. Edematogenic Evaluation

To evaluate the anti-edematogenic effect, a Starrent® brand caliper was used to quantify the knee joint diameter in millimeters. The pachymeter was positioned on the joint line to check the transverse diameter of the knee. The measurement was performed 3 times in each knee and the mean difference between the 2 limbs was used as the final result [18, 19].

2.9. Chemical Analysis of chloroform fraction of *S. dulcis*

2.9.1. Determination of Total Phenolic Content (TPC) in *S. dulcis* chloroform fraction

The total polyphenol contents in the extracts were determined using Folin–Ciocalteau reagent and 20% sodium carbonate (NaCO3). The reaction mixture was kept in the dark for 2h at room temperature and absorbance was measured at 760 nm using Lambda 35 UV–Vis spectrophotometer (Perkin Elmer, Inc., Waltham, MA, USA). The TPC was calculated from a gallic acid calibration curve (2,5 – 40.0 µg/mL) and expressed as gallic acid equivalent (%). Analyzes were performed in triplicate and the mean value was calculated for each sample [20].

2.9.2. Determinations of Total Flavonoid Concentration (TFC) in *S. dulcis* chloroform fraction

For total flavonoid concentration we used photocolorimetric method with 5% methanolic aluminum chloride solution (AlCl3). The mixture was kept in the dark. for 30 min at room temperature and absorbance was measured at 425 nm in UV–Vis Lambda 35 spectrophotometer (Perkin Elmer, Inc., Waltham, MA, USA). The concentration was calculated from the calibration curve constructed with standard quercetin solution (Merck, Germany) (1–30.00 µg/mL) and expressed as quercetin equivalent (%). The analyses were performed in triplicate [21].

2.9.3. Identification of compounds in *S. dulcis*

The *S. dulcis* chloroform fraction was analyzed by HPLC (LC-20AD Shimadzu, Kyoto, Japan) and a Phenomenex Luna C-18 (250 × 4.6 mm − 5 um) column. The mobile phases consisted of ultrapure water containing 0.1% formic acid (A) and methanol (B). The following linear gradient was applied: 0 min, 5% B; 1−60 min, 5−100% B; 60−70 min, 100% B at flow rate of 1 mL/min. The LC was coupled to a mass spectrometer (Amazon Speed ETD, Bruker, MA, USA) equipped with electrospray ionization (ESI) and an ion-trap (IT) type analyzer in negative mode, under the following conditions: capillary voltage at 4.5 kV, capillary temperature of 325 °C, entrainment gas (N2) flow at 12 L/min, and nitrogen nebulizer pressure at 27 psi. The acquisition range was m/z 100–1000, with 2 or more events.

2.10. Determination of Antioxidant Activity

2.10.1. DPPH• Radical Scavenging Activity

The antioxidant activity of *S. dulcis* chloroform fraction was evaluated by using the DPPH• free radical scavenging assay as described by Brand–Willians et al., [22] with modifications from Dutra et al., [21]. The samples were diluted on methanol at different concentrations (30–480 µg/mL) and added to a methanol solution of DPPH• (40.0 µg/mL). After 30 min of reaction at room temperature in the dark, the absorbance of each solution was read at 517 nm in a Lambda 35 UV− vis spectrophotometer (Perkin–Elmer, Inc., USA). Methanol was used as the control, and DPPH• solution was used as the blank. The percent inhibition was calculated using the formula:

DPPH• scavenging activity (%)
100 – [(Asample – Ablank) X 100/ Acontrol]

where Asample = absorbance of the sample after 30 min of reaction, Ablank = absorbance of the blank, and Acontrol = absorbance of the control.

The percentage of scavenging activity was plotted against the sample concentration to obtain the IC50, defined as the concentration of sample necessary to cause 50% inhibition. All experiments were done in triplicate.

2.10.2. Ferric Reducing Antioxidant Power Assay (FRAP)

This protocol was used to determine the antioxidant activity based on iron reduction using the FRAP assay. FRAP measures the ferric–reducing ability of a sample in acid medium (pH 3.6), forming an intense blue color as the ferric tripyridyltriazine (Fe3+−TPTZ) complex is reduced to the ferrous (Fe2+) form. The test was performed according Benzie et al., [23] with modifications from Dutra et al.,[21]. The samples were diluted on methanol at different concentrations (12,5 – 200 µg/mL). The absorbance of the reaction mixture was read at 593 nm in a Lambda 35 UV−vis spectrophotometer (Perkin–Elmer, Inc., USA) using FRAP solution as a blank. The results were expressed as millimoles of Fe2+ per gram of sample. All experiments were done in triplicate.

2.10.3. ABTS•+ Assay

The ABTS solution was prepared in water and potassium persulfate and kept in the dark room for 16 hours before testing for the complete oxidation of ABTS•+ and the generation of the highly stable chromophore cation radical 2,2′–azino–bis(3 ethylbenzothiazoline–6–sulfonic acid) (ABTS•+) [24] with [25] modifications. The ABTS•+ solution was diluted with 70% ethanol/water (70:30, v/v) until the absorbance at 734 nm reached 0.7 ± 0.02. ReadinHG were performed by

reacting 1000–20 µg/mL of *S. dulcis* samples with the ABTS•+ solution. All studies were performed at least in triplicate monitoring the decrease in absorbance for 6 min; results reported corresponded to the % of remaining chromophores compared to conditions in the absence of antioxidants. The IC50 values were determined to each sample, using the formula: Scavenging ability (%) = (1 − Asample/Ablank) × 100

2.10.4. Determination of the enzyme superoxide dismutase (SOD)

Superoxide dismutase activity was determined according to [26]. The assay was performed over a 5 minutes period. 15µl of serum and 1485µl of working solution containing 50mM potassium phosphate buffer pH 7.8, 1.3mM riboflavin, 13mM methionine, 63mM NBT and 0.1mM EDTA were added. This procedure contains 2 whites, both of which have 15µL of 50mM Potassium Phosphate Buffer pH 7.8 and 1485µL of Working Solution, one reacting with light incidence which causes the formation of blue formazana blue compound and the other in the dark. Absorbance was obtained by spectrophotometer at 560nm. In the calculation of the enzyme activity the dark white absorbance value was subtracted from the absorbance values of the samples. Results were expressed in unit / mg protein.

2.10.5. Catalase enzyme determination (CAT)

Catalase activity is directly proportional to the decomposition rate of hydrogen peroxide. In quartz cuvette 955µL of phosphate buffer and 30µL of serum sample were added, this cuvette was placed in a spectrophotometer and discounted against a blank of phosphate buffer. Afterwards, 35µL of hydrogen peroxide were added to the tissue and 15µL to the serum sample and the decrease in absorbance was monitored over a period of 5 minutes at a wavelength of 240nm. Results were expressed in pmoles / mg protein [8].

2.10.6. Determination of glutathione peroxidase (GPx) enzyme

The enzyme glutathione peroxidase (GPx) is an 80Kda protein that converts H2O2 to H2O. In this process, the enzyme reduces 2 reduced glutathione (GSH) generating an oxidized glutathione (GSSG) with water as a byproduct [27].

The activity of GPx was determined by the in serum that uses terbutyl peroxide as a reaction substrate. The oxidation of GSH by GPx occurs, generating GSSG, which is converted to GSH by glutathione reductase, consuming in this reaction a molecule. NADPH, which can be accompanied by spectrophotometer at 340nm. The reaction medium was composed of 50mM buffer (K2HPO4) pH 7.0, 1mM EDTA, 0.1U / ml glutathione reductase, 1mM GSH and 0.2mM NADPH. The reaction was started with the addition of 0.5mM terbutyl peroxide and followed for 5 minutes.

2.11. Cytokines Analysis

A laboratory analysis of the synovial fluid to quantify IL-6, IL-10, TNF-α, and IFN-γ was performed using an enzyme-linked immunosorbent assay (ELISA, R&D Systems®, Minneapolis, MN, USA).

The synovial fluid samples were obtained on D21 by washing out the affected knee joint twice with 200 µL of phosphate-buffered solution (0.15 M, pH 7.4) containing 37.2 mg of ethylenediaminetetra-acetic acid (EDTA, 0.01 M).

2.12. Histopathological Analysis of Articular Cartilage

On day 21, the articular cartilage and subchondral bone of the knee of each animal were removed after euthanasia. The excised components were embedded in paraffin blocks and cut into 5 µm sections, and the proteoglycans of the organic cartilage matrix were specifically stained using hematoxylin and eosin (H&E) staining.

For the synovial membrane, sections were blindly assessed and classified for cell infiltration, vascularization, intimal hyperplasia, subintimal edema, and subintimal fibrosis.

The histopathological evaluation was performed according to the guidelines of the Osteoarthritis Research Society International (OARSI). For the synovial membrane, sections were blindly evaluated and classified for cell infiltration, vascularization, intimal hyperplasia,

subintimal edema, and subintimal fibrosis according to the classification proposed by Kuyinu et al. [28].

2.13. Statistical Analysis

A comparison of the means of the experimental groups was performed with a univariate analysis of variance (One-way ANOVA), followed by the multiple comparisons test. In the evaluation of 2 sources of variability, a bivariate variance analysis (2-way ANOVA) was used. Data were analyzed using GraphPad® 7.0 software (GraphPad software, San Diego, CA, USA) and all analyses had a significance level of $p < 0.05$.

3. Results

Twenty animals were divided into four experimental groups, five animals each: healthy group (HG): untreated healthy animals not induced for OA; saline group (SALG): OA animals treated orally at 1 mL/kg/day with 0.9% sodium chloride (NaCl); chloroform fraction group (CF): OA animals treated orally with 500 mg/kg/day of chloroform fraction of *S. dulcis*; meloxicam group (GM): OA animals orally treated with 1 mg/kg/day meloxicam reported in the results below.

3.1. Evaluation of Mechanical Allodynia—Von Frey

Induction of OA reduced the nociceptive threshold of paw withdrawal in all groups in a similar way. However, after 10 days, nociceptive threshold increase and decrease of mechanical allodynia occurred in the CF and GM groups, which presented similar values ($p = 0.7672$) after 20 days (Figure 1).

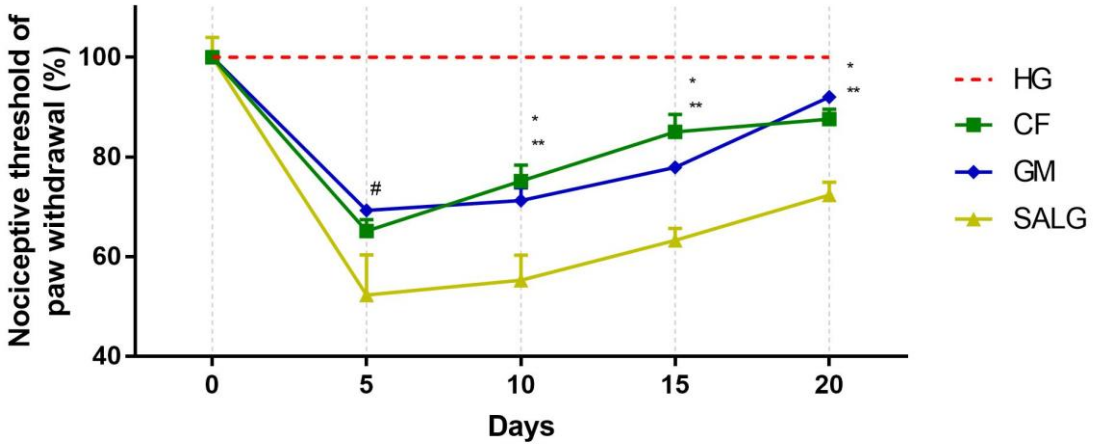

Fig. 1. Effect of the chloroform fraction of *S. dulcis* on the tactile sensory evaluation using the Von Frey test. Results were expressed as mean ± standard mean error; # difference between healthy group (HG) and saline group (SALG) in D5 ($p < 0.0001$), HG and chloroform fraction group (CF) in D5 ($p < 0.0001$), HG and meloxicam group (GM) in D5 ($p < 0.0001$); * difference between CF and SALG in D10 ($p < 0.0001$), D15 ($p < 0.0001$) and D20 ($p = 0.0009$); ** difference between GM and SALG in D10 ($p = 0.0004$), D15 ($p = 0.0016$) and D20 ($p < 0.0001$), using 2-way ANOVA with the multiple comparisons test ($p < 0.05$).

3.2. Assessment of Motor Activity/Forced Walking—Rotarod test

The induction of OA reduced gait score in all groups in a similar way. However, from the 15th to the 20th day the CF showed a gradual increase in its GM motor score for motor activity (Figure 2).

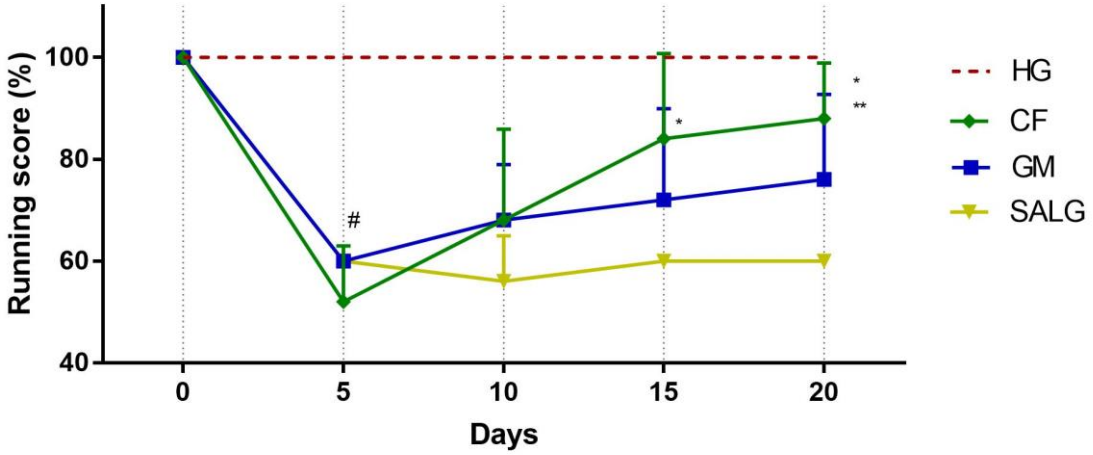

Fig. 2. Effect of the chloroform fraction of *S. dulcis* on the evaluation of motor activity/forced ambulation using the Rotarod test. Results were expressed as mean ± standard mean error; # difference between HG and SALG in D5 ($p < 0.0001$), HG and CF in D5 ($p < 0.0001$), HG and GM in D5 ($p < 0.0001$); * difference between the CF and SALG in the D15 ($p = 0.0005$) and D20 ($p < 0.0001$); ** difference between GM and SALG in the D20 ($p < 0.0472$), using 2-way ANOVA with the multiple comparisons test ($p < 0.05$).

3.3. Functional Incapacity—Weight Bearing Test

Induction of OA reduced the nociceptive threshold of paw withdrawal in all groups in a similar way. However, after 15 days there was an increase in functional capacity (CF). At the end of 20 days GM is equal to HG (Figure 3).

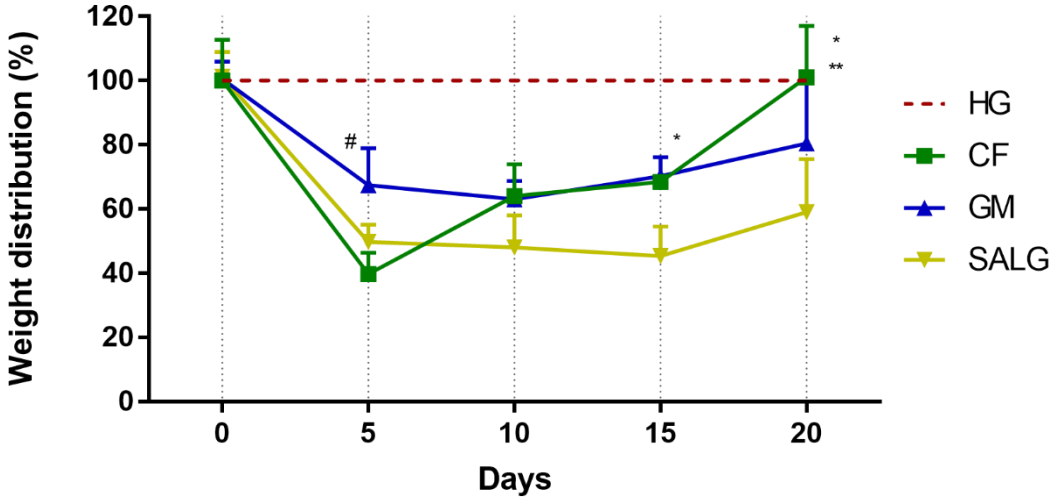

Fig. 3. Effect of the chloroform fraction of *S. dulcis* on the assessment of the weight distribution in the hind paws using the weight bearing test. Results were expressed as mean ± standard mean error; # difference between HG and SALG in D5 ($p < 0.0001$), HG and CF in D5 ($p < 0.0001$) and HG and GM in D5 ($p < 0.0001$); * difference between CF and SALG in D15 ($p = 0.0062$) and D20 ($p = 0.0148$); ** difference between GM and SALG in D20 ($p<0.0001$), using 2-way ANOVA with the multiple comparisons test ($p < 0.05$).

3.4. Edematogenic Evaluation

The induction of OA increased the circumference of the right knee characterizing edema in all groups in a similar way. However, after 10 days the edema reduction occurred in the treated groups (CF and GM), which presented values similar to HG at the end of 20 days (Figure 4).

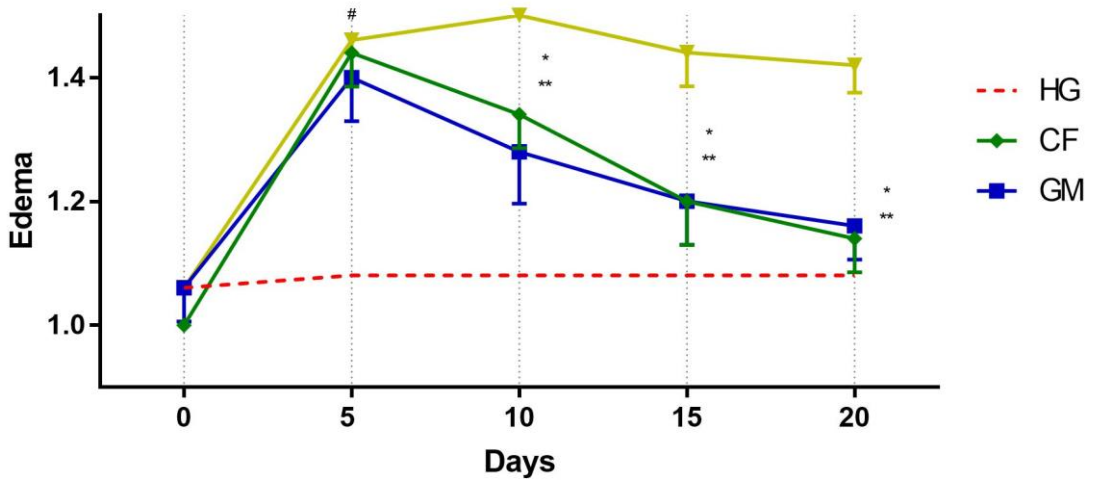

Fig. 4. Effect of the chloroform fraction of *S. dulcis* on the edematogenic evaluation of the circumference of the knee by the pachymeter. Results were expressed as mean ± standard mean error; # difference between HG and SALG in D5 ($p < 0.0001$), HG and CF in D5 ($p < 0.0001$) and HG and GM in D5 ($p < 0.0001$); * difference between CF and SALG in D10 ($p = 0.0003$), D15 ($p < 0.0001$) and D20 ($p < 0.0001$); ** difference between GM and SALG in D10 ($p < 0.0001$), D15 ($p < 0.0001$) and D20 ($p < 0.0001$), using 2-way ANOVA with the multiple comparisons test ($p < 0.05$).

3.5. Rating by Mouse Grimace Scale

The induction of OA increased spontaneous pain in all groups of the same magnitude. However, after 10 days the scores decreased in the treated groups (CF and GM) (Figure 5).

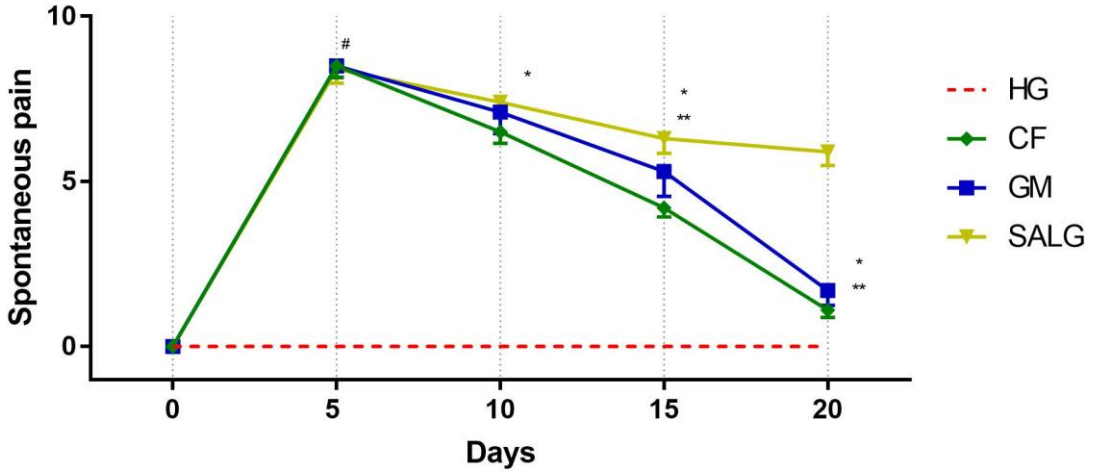

Fig. 5. Effect of the chloroform fraction of *S. dulcis* on the evaluation of spontaneous pain by Mouse Grimace Scale. Results were expressed as mean ± standard mean error; # difference between HG and SALG in D5 ($p < 0.0001$), HG and CF in D5 ($p < 0.0001$), HG and GM in D5 ($p < 0.0001$); * difference between CF and SALG in D10 ($p = 0.0014$), D15 ($p < 0.0001$) and D20 ($p < 0.0001$); ** difference between GM and SALG in D15 ($p = 0.0003$) and D20 ($p < 0.0001$), using 2-way ANOVA with the multiple comparisons test ($p < 0.05$).

3.6. Chemical Analysis and Determination of Antioxidant Activity

3.6.1. Polyphenols and total flavonoids; Activity antioxidant (DPPH• , FRAP and ABTS$^{\bullet+}$ methods)

The Total Phenolic Content, Total Flavonoids Content and Antioxidant Activity (DPPH•, FRAP and ABTS•+ methods) are present on Table 1. The TPC and TFC was 27,3% and 8,5%, respectively. Regarding the antioxidant activity, the DPPH IC50 found was 65.4 µg/mL, from ABTS was 31.04 µg/mL and the ferric reducing antioxidant power was close to Trolox.

Table 1. Values of polyphenols and total flavonoids contents, activity antioxidant (DPPH• , FRAP and ABTS$^{•+}$ methods) from chloroform fraction from *S. dulcis*.

Sample	TPC (%) [a,b]	TFC (%) [a,c]	DPPH• IC_{50} (µg/mL)	FRAP (mmol Fe^{2+}/g)	ABTS$^{•+}$ IC_{50} (µg/mL)
CFSD	27.36 ± 0.31	8.49 ± 0.43	65.41 ± 3.27^{c}	6.81 ± 0.73^{b}	31.04 ± 0.06^{c}
Trolox[1]	-	-	3.05 ± 0.61^{a}	8.74 ± 0.13^{c}	3.42 ± 0.41^{a}

Values represent the mean of triplicate measurements ± standard deviation. Different letters in the same column indicate a significant difference (Tukey. p <0.05). CFSD – Chloroform fraction from *S. dulcis*; (a) Results expressed as means ± standard deviation of quantitative evaluation tests for total polyphenols and total flavonoids in *S. dulcis* chloroform fraction (n: 5). (b) expressed as gallic acid equivalent; (c) expressed as quercetin equivalent. DPPH• 2.2-diphenyl-1-picrylhydrazyl radical; FRAP. ferric reducing antioxidant power; ABTS$^{•+}$. 2.2′-azinobis-3-ethylbenzotiazoline-6-sulfonic acid. (-) unrealized. [1] previously published by Lopes et al. [20].

3.6.2. Evaluation of catalase, superoxide dismutase and glutathione activity in the chloroform fraction of *S. dulcis*

The animals treated with the chloroform fraction and meloxicam had a reduction of the catalase enzyme when compared to the saline group. For the superoxide dismutase enzyme there was an increase in the chloroform fraction group when compared to the saline group. The group treated with the chloroform fraction showed an increase in glutathione enzyme when compared to the saline group, as shown in table 2

Table 2. Catalase, superoxide dismutase and glutathione activity in the chloroform fraction of *S. dulcis*

Enzymes	CF	GM	SALG	HG
Catalase	5.79 ± 2.11 (p<0.0001)*	5.54 ± 1.19 (p<0.0001)**	50.69 ± 3.43	41.09 ± 7.74
Superoxide Dismutase	163.4 ± 25.15 (p=0,0210)*	93.4 ± 29.62 (p=0.8387)	113.8 ± 8.78	16.58 ± 12.1
Glutathione	1.29 ± 0.16 (p<0.0001)*	1.40 ± 0.18 (p<0.0001)**	0.35 ± 0.09	1.35 ± 0.11

* p<0.05, difference between CF and SALG for catalase, superoxide dismutase and glutathione activity; ** p<0.05, difference between GM and SALG for catalase, superoxide dismutase and glutathione activity using One-way ANOVA with the multiple comparison test (p <0.05).

3.6.3. Identification of compounds in *S. dulcis*

The HPLC-ESI-MS analysis exhibit 18 secondary metabolites in the *S. dulcis* chloroform fraction (Figure 6), highlighting flavonoids kaempferol derivates. Sixteen molecules were elucidated compounds (Table 3).

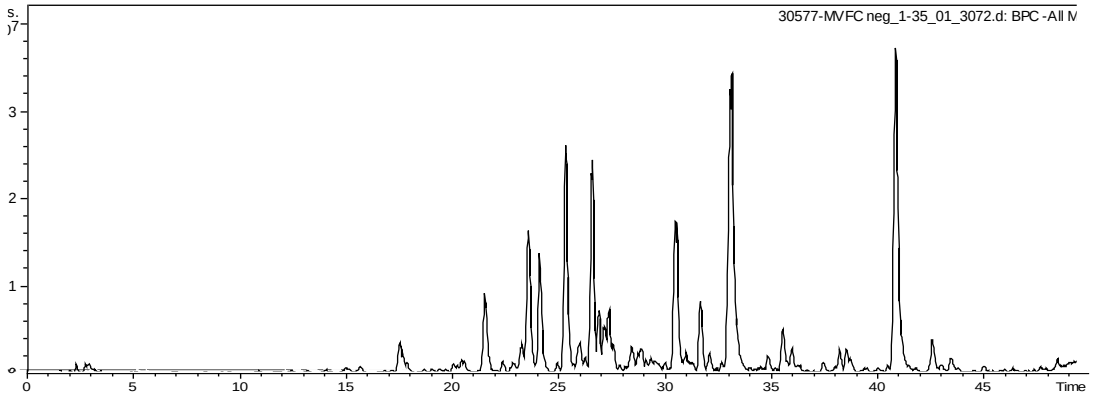

Fig. 6. Total Ion Chromatogram chloroform fraction from *S. dulcis* acquired by LC-ESI-MS (*m*/*z*: 100–1500 Da).

Table 3. Identification of compounds in *S. dulcis* chloroform fraction by LC-ESI/MS.

Peak	Tr (min)	$[M - H]^-$	MS^n fragments	Compound	Reference
1	18.0	417	285; 241; 152	kaempferol-3-*O*-pentoside	[2, 29]
2	20.0	327	164; 136	gonzalitosin I	[2, 30]
3	22.3	593	575; 473; 353	nicotiflorin	[2, 31]
4	22.3	639	621; 529; 459	suspensaside	[2, 32]
5	24.0	563	545; 473; 353	apigenin 6-C-pentosyl-8-C-hexoside	[2, 33]
6	24.1	631	563, 479	myricetin-3-*O*-(2″-*O*-galloyl) glucoside	[2, 34]
7	24.4	1127	563	acteoside dimer	[2, 35]
8	26.2	623	563	acteoside (verbascoside)	[2, 35]
9	26.2	623	461; 315	scoparin 7-*O*-glucoside	[2, 36]
12	27.4	791	445; 283	biochanin A *O*-hexoside-*O*-hexoside	[2, 33]
13	31.5	683	637; 313	Ginsenoside F1	[2, 37]
15	32.6	797	621; 475	N.I*	
17	42.1	313	297; 283	Diosmetin	[2, 38]
18	44.1	327	291; 229; 211; 171	trihydroxyoctadecadienoic acid	[2, 39]

* Not identified.

3.7. Determination of Cytokines (in Synovial Fluid)

The animals treated with the chloroform fraction showed a reduction of the cytokines IFN-γ and IL-6 when compared to the saline group that was statistically significant. The animals treated with the meloxicam showed a reduction of the cytokine IL-6. As for interleukin 10, the chloroform fraction and meloxicam groups presented a statistically significant increase when compared to the saline group (Table 4).

Table 4. Effect chloroform fraction of *S. dulcis* on cytokines.

Cytokines	CF	GM	SALG	HG
IFN-γ	1191 ± 116.2 ($p < 0.0001$)*	1559 ± 117.9 ($p = 0.3103$)	1748 ± 195.7	1302 ± 157.8
IL-6	31.93 ± 5.21 ($p = 0.0098$)*	32.19 ± 11.54 ($p = 0.0106$)**	55.91 ± 8.08	24.73 ± 9.92
IL-10	382 ± 60.021 ($p = 0.0049$)*	390.5 ± 22.62 ($p = 0.0019$)***	276.8 ± 46.76	373.2 ± 31.17

* CF and SALG for IFN-γ ($p < 0.0001$), IL6 ($p = 0.0098$) and IL10 ($p = 0.0049$); ** GM and SALG for IL6 ($p = 0.0106$) and IL10 ($p = 0.0019$), using One-way ANOVA with the multiple comparisons test ($p < 0.05$).

3.8. Microscopic Classification Synovial Membrane Histology in Osteoarthritis

Histopathological evaluation using the OARSI hematoxylin and eosin scoring system revealed that for the group treated with chloroform fraction there are histological differences for cell infiltration and edema in relation to the saline group. Meloxicam presented difference only for edema. For the analysis of vascularization, hyperplasia and fibrosis there was no difference between these groups (CF and GM) and the saline group (Table 5 and Figure 7).

Table 5. Effect of chloroform fraction of *S. dulcis* on synovial membrane.

Histology (H&E)	CF	GM	SALG	HG
Cellular infiltration	0.80 ± 0.44 (p<0.0068)*	1.2 ± 0.44 (p=0.0743)	2.2 ± 1.09	0 ± 0
Vascularization	1.4 ± 0.54 (p=0.4858)	2 ± 1.22 (p=0.9628)	2.4 ± 1.14	0 ± 0
Hiperplasia	0 ± 0 (p=0.2313)	0 ± 0 (p=0.2313)	2.4 ± 1.44	0 ± 0
Edema	1 ± 0 (p=0.0035)*	1.4 ± 0.89 (p=0.0352)**	2.6 ± 0.89	0 ± 0
Fibrosis	1.2 ± 0.44 (p=0.3807)	1.6 ± 1.44 (p=0.8031)	2.2 ± 0.44	0 ± 0

* Difference between CF and HG for histology of synovial membrane; ** Difference between GM and HG for histology of synovial membrane, using One-way ANOVA with the multiple comparisons test ($p < 0.05$).

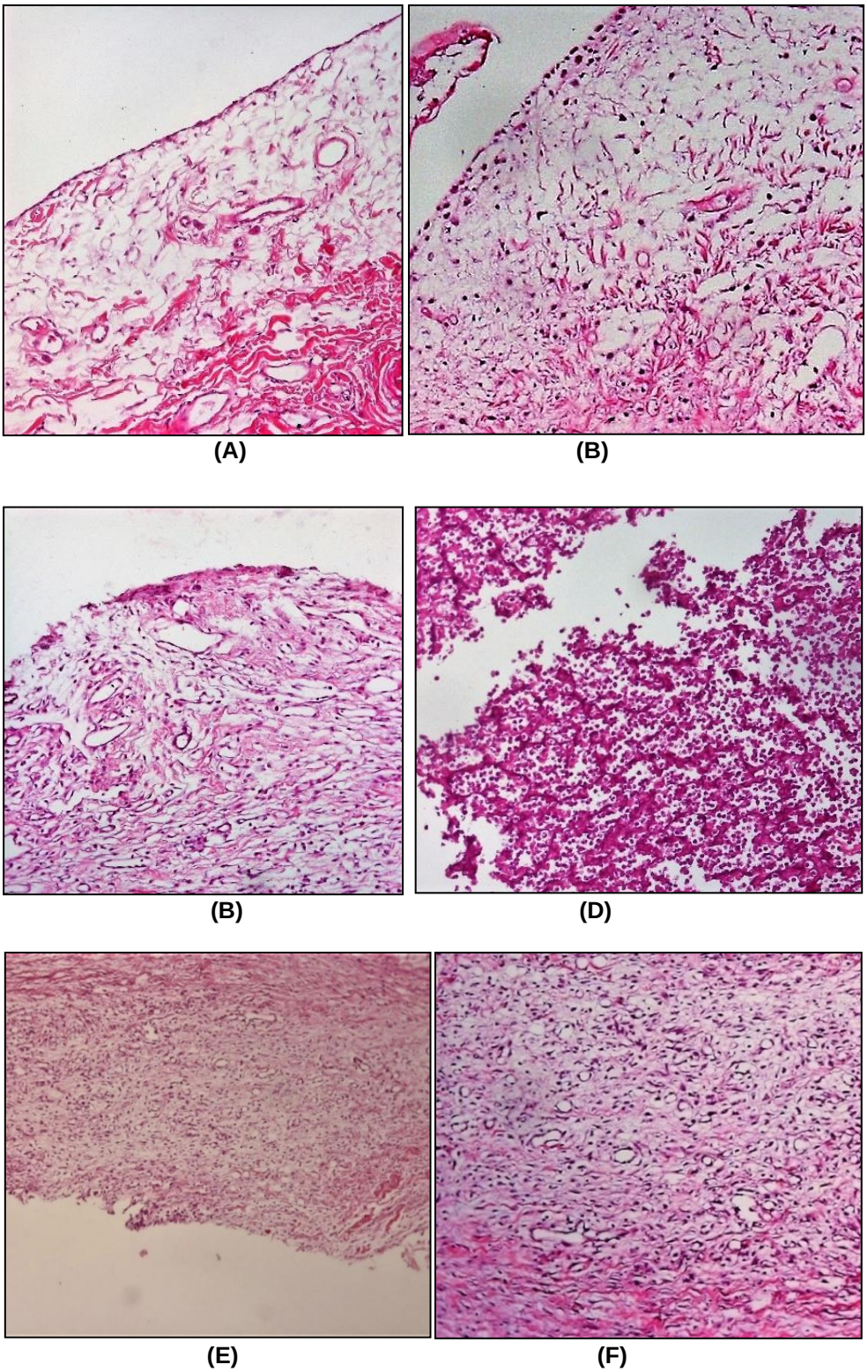

Figure 7. Effect of the chloroform fraction of *S. dulcis* for the histological classification of the synovial membrane. For cellular infiltration with score 0 (HG), no inflammatory cells are observed within the section (Figure 7A). For score 1 (FG), some inflammatory infiltrates are detected and consist of mononuclear and segmented cells (Figure 7B). For score 2 (HGD), the moderate presence of mononuclear cells is observed in 25% of the section (Figure 7C). For score 4 (SALG) there is a high concentration of mixed inflammatory infiltrates found within the section (Figure 7D). For edema, the score 0 (HG) represents the section without edema (Figure 7A). Score 1 (CF) represents slight edema detected in section (Figure 7E). Score 2 (SALG) shows areas of the edema section recognized by the blue appearance of the tissue and the lack of subintimal organization (Figure 7F).

4. Discussion

This study aimed to evaluate the anti-nociceptive, anti-inflammatory and antioxidant effect of the chloroform fraction of *S. dulcis* in an experimental model of knee OA in rats, with meloxicam as the positive control. It is the first study to evaluates the effect of chloroform fraction of *S. dulcis* on pain, inflammation and oxidative stress in knee OA in rats. It is the continuation of the study published by Lima et al. [2] where the anti-nociceptive and anti-inflammatory effects of crude extract *S. dulcis* in OA were evaluated with similar results.

Throughout our study, the following markers were observed: improvement in gait score; the reduction of mechanical allodynia; the distribution of weight discharge on the hind legs; signs of spontaneous pain and edema; reduction in inflammation; an increase in peripheral nociceptive threshold; decreased proinflammatory cytokines, inflammation and edema by synovial membrane histology; as well as an antioxidant activity.

There is a growing interest from researchers in the development of antioxidant substances from natural plant products [2], because experimental and clinical studies suggest that oxidative stress is considered one of the major causative factors in OA pathogenesis [1].

Free radicals have been implicated as mediators of tissue damage in arthritis, in conjunction with proinflammatory cytokines, as reactive oxygen species can lead to joint cartilage degeneration and osteoclast activation [40], may be implicated in the pathogenesis of inflammatory synovitis. Cells present in the inflamed joint, such as macrophages, neutrophils, lymphocytes and endothelial cells, once isolated and stimulated are capable of producing oxygen free radicals [41].

The chloroform fraction of *S. dulcis* in the study showed antioxidant activity by the tested methods, possibly due to the presence of polyphenols and flavonoids that sequester and / or inactivate free radicals in these compounds and may have attenuated the histological changes in the synovial membrane, articular cartilage and decreased inflammation. and edema due to possible inactivation of free radicals present in the affected joint [42]. Secondary metabolites isolated from the plant fraction have a peripheral mechanism of action similar to non-steroidal anti-inflammatory agents such as indomethacin and diclofenac sodium [43]. Non-steroidal anti-inflammatory drugs, besides inhibiting prostaglandin production, reduce the production of oxygen radicals, especially superoxide [44].

The enzymatic defense system includes the enzymes catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) [1]. These enzymes act by means of prevention mechanisms, preventing and / or controlling the formation of free radicals and non-radical species, involved in the initiation of chain reactions that culminate in the propagation and amplification of the process, with the occurrence of oxidative damage [45].

The antioxidant effect on CAT, GPx and SOD modulation in animals may be associated by the presence of polyphenols and flavonoids in the chloroform fraction of *S. dulcis*, presenting analgesic and anti-inflammatory action [7-10, 46, 47] and inhibition of free radical production [7, 48].

The decrease in CAT modulation in the study suggests that the inflammatory process caused by the injection of MIA into the chloroform fraction of *S. dulcis* could have caused this enzyme to be exhausted due to intense oxidative stress [49].

The superoxide dismutase is an enzyme that protects against cell membrane degeneration, representing the first intracellular barrier of the antioxidant defense system, and its decrease may lead to mitochondrial dysfunction and consequent chondrocyte apoptosis [50]. Its activity may increase to compensate for the excessive production of superoxide radicals, suggesting that its increase in the study for the chloroform fraction of *S. dulcis* group is an adaptive response of cellular and tissue protection of the organism to possible damage due to radicals. free of oxygen that causes oxidative stress [41].

Coulibaly et al. [48] evaluated different *S. dulcis* extracts by successive extraction with hexane, chloroform and methanol. These extracts showed significant antioxidant capacity in several enzyme models. The antioxidant activity of the extracts was related to their phytochemical composition especially that of polyphenols and carotenoids content. The chloroform extract was rich in phytochemicals and presented the highest antioxidant activity in the different systems tested. All extracts showed less than 50% inhibition of xanthine oxidase, but more than 50% inhibition of lipid peroxidation and lipoxygenase. The extracts strongly inhibited lipoxygenase-mediated lipid peroxidation, suggesting that other mechanisms not studied in this research may be involved.

During the inflammatory process, there is an increase in vascular permeability, capillary leakage and cell migration [51]. Distinct synovitis involving activated B cell infiltration and T lymphocytes, as well as overexpression of proinflammatory mediators is a common finding on histological examination as shown in the study. Elevated levels of IL-1α, IL-1β and TNF-α can be found in synovial membrane of OA patients [52].

Thus, a classic sign of the inflammatory process is the formation of edema that can continue for days, reducing joint mobility [53]. In addition, mechanical overload, static or dynamic cyclic pressure, and compression during gait can lead to the diffusion of oxygen free radicals into all joint compartments, increasing joint cartilage exposure to the inflammatory process and leading to degeneration [52].

The antioxidant action associated with the anti-inflammatory action of the chloroform fraction may have led to less misalignment, stress and joint friction through a possible protective action on articular cartilage, which would justify a progressive improvement of mechanical allodynia, functional disability, mechanical ambulation and disability of pain in the study.

Reduction of the inflammatory infiltrate in the synovial membrane and, consequently, reduction of edema due to the reduction of proinflammatory cytokines would be another possibility for these results, since synovial inflammation in OA is usually found in pathologically damaged areas with bone and cartilage and may release. proteinases and cytokines capable of accelerating joint destruction [54] by activating the nuclear transcription factor NF-kB, implying an inflammatory response in vascular endothelium and type A lining synovial cells through radical oxygen species that can trigger a possible mechanism of transition from membrane synovial inflammation to a process of joint degeneration [52] by signaling pathways of tissue mechanical loads that may stimulate proinflammatory cytokines in the chondrocytes [55]

The results for ambulation and weight discharge showed better results due to the probable concentration of secondary metabolites that may be higher in the chloroform fraction, leading to a decrease in oxidative stress in the joint when compared to the study by Lima et al. [2], which used crude extract of *S. dulcis.*

Corroborating our results, the study by Lima et al. [2] with *S. dulcis* crude extract using molecular coupling suggested that Suspensaside and Nicotiflorina were the most likely compounds to have spontaneous and favorable interaction with COX-2 generating anti-inflammatory [56] and antioxidant [57] activity in the chloroform fraction of *S. dulcis*.

Since these secondary metabolites were also identified in the chloroform fraction, it is suggested that the anti-inflammatory and antioxidant activity may be related to the presence of these compounds, since in low magnitudes the biomechanical signs of compression, traction, shear inhibit nuclear translocation. NF-kB transcription factors and act as potent inhibitors of proinflammatory cytokines, PGE_2 in the affected joints [58].

To date, OA treatment is based on drugs that control the pain and inflammation associated with synovitis but do not reduce the destruction of articular cartilage, so a herbal medicine with potential to protect this tissue has excellent clinical relevance [59]. Histopathological analysis of the synovial membrane showed encouraging results for the chloroform fraction of *S. dulcis*, suggesting the possible efficacy in the treatment of OA at the tested dose, suggesting a possible protective effect on the joint, which may justify the translation of its use for this purpose.

Nagy et al. [60] in a rat model of MIA-induced osteoarthritis reports that high doses of meloxicam (1mg / kg) had a chondroprotective effect and, at high dose, also protected against subchondral bone lesions, suggesting the interruption of the low inflammatory pathway. degree accompanied by chronic cartilage deterioration, as shown, the possible effect of the chloroform fraction in the study.

The effects of the chloroform fraction of *S. dulcis* on the histological progression, pain behavior, inflammatory process and antioxidant in OA presented in the study are impressive, however, this work had some limitations that need to be highlighted, such as not evaluating the antioxidant system. (glutathione, ascorbic acid, alpha-tocopherol and carotinoids) [1], different doses and possible toxic effects.

Oral treatment with the *S. dulcis* chloroform fraction reduced edema, spontaneous pain, peripheral nociceptive activity, and oxidative stress in right knee OA, attenuating histological changes in the synovial membrane with lower IL-6 and synovial levels. IFN-γ and higher levels of IL-10, possibly due to its anti-inflammatory and antioxidant action.

In the challenging search for drugs that modify the pathogenesis and history of OA progression, the preclinical results of this study through oral treatment with the *S. dulcis* chloroform fraction encourage us to support its potential use as a promising agent.

complementary therapy that may be an alternative to drug treatment. Other studies should be performed evaluating other doses, fractions, toxicity and other possible mechanisms of action.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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5. CONSIDERAÇÕES FINAIS

O tratamento oral com o extrato bruto e fração clorofórmica (500mg/Kg) de *S. dulcis* reduziu o edema, a dor espontânea e a atividade nociceptiva periférica no joelho direito com OA, atenuando as alterações histológicas na membrana sinovial e a cartilagem articular com menores níveis sinoviais de IL-6 e IFN-γ, e níveis mais elevados de IL-10, possivelmente devido à sua ação anti-inflamatória.

A concentração testada de 50 µg/mL mostrou a maior inibição para COX-1 e COX-2 no extrato bruto. Todos os compostos mostraram afinidade de alto parâmetro com a estrutura da COX-2, destacando suspensaside e nicotiflorin e, realizaram interações com importantes resíduos do sítio ativo da COX, como Arg120 e Glu524 e com resíduos vizinhos no extrato bruto de *S. dulcis*.

O extrato bruto e a fração clorofórmica apresentaram efeito antioxidante através das enzimas superóxido dismutase, catalase e glutationa peroxidase, bem como, através dos métodos de DPPH, FRAP e ABTS.

Na busca desafiadora de fármacos que modifiquem a patogênese e a história de progressão da OA, os resultados pré-clínicos do presente estudo, são encorajadores e impressionantes, pois apoiam o uso potencial do extrato bruto e da fração clorofórmica de *S. dulcis* como promissores agentes terapêuticos que pode complementar ou servir de alternativa para o tratamento medicamentoso da OA na articulação do joelho. Novos estudos devem ser realizados avaliando outras doses, extratos, frações, toxicidades e outros possíveis mecanismos de ação anti-inflamatórios periféricos e centrais, bem como, a ação antioxidante por meio do sistema antioxidante não enzimático.

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ANEXOS

ANEXO 1: Comprovante de aceite do artigo da Revista Molecules

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ANEXO 2: Dados não publicados sobre a atividade antioxidante do extrato bruto

Table 1. Values of polyphenols and total flavonoids contents, activity antioxidant (DPPH• , FRAP and ABTS$^{•+}$ methods) from crude extract from *S. dulcis*.

Sample	Total Phenolic Content (%)[a,b]	Total Flavonoid Concentration (%)[a,c]	DPPH• IC_{50} (µg/mL)	FRAP (mmol Fe^{2+}/g)	ABTS$^{•+}$ IC_{50} (µg/mL)
Crude extract	24.93 ± 0.74	5.84 ± 0.25	58.11 ± 0.03[b]	4.65 ± 0.06[a]	14.82 ± 0.02[b]
Trolox[1]	-	-	3.05 ± 0.61[a]	8.74 ± 0.13[c]	3.42 ± 0.41[a]

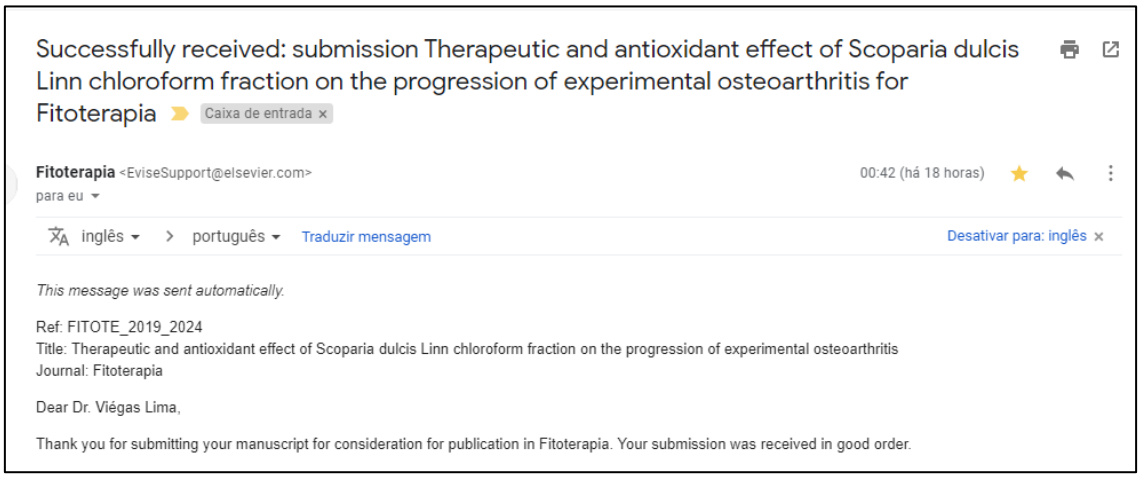
Values represent the mean of triplicate measurements ± standard deviation. Different letters in the same column indicate a significant difference (Tukey. p <0.05). (a) Results expressed as means ± standard deviation of quantitative evaluation tests for total polyphenols and total flavonoids in *S. dulcis* hydroalcoholic extract (n: 5). (b) expressed as gallic acid equivalent; (c) expressed as quercetin equivalent. DPPH• . 2.2-diphenyl-1-picrylhydrazyl radical; FRAP. ferric reducing antioxidant power; ABTS$^{•+}$. 2.2′-azinobis-3-ethylbenzotiazoline-6-sulfonic acid. (-) unrealized. [1] previously published by Lopes et al.. 2019.

Table 2. Catalase, superoxide dismutase and glutathione activity in the crude extract group (GSD) of *S. dulcis*

Enzymes	GSD	GM	SALG	HG
Catalase	7.87 ± 2.46 (p<0.0001)*	5.54 ± 1.19 (p<0.0001)**	50.69 ± 3.43	41.09 ± 7.74
Superoxide Dismutase	160.4 ± 27.16 (p=0.0310)*	93.4 ± 29.62 (p=0.8387)	113.8 ± 8.78	16.58 ± 12.1
Glutathione	1.34 ± 0.14 (p<0.0001)*	1,40 ± 0.18 (p<0.0001)**	0.35 ± 0.09	1.35 ± 0.11

* p<0.05, difference between GSD and SALG for catalase, superoxide dismutase and glutathione activity; ** p<0.05, difference between GM and SALG for catalase, superoxide dismutase and glutathione activity using One-way ANOVA with the multiple comparison test (p <0.05).

ANEXO 3: Comprovante de submissão da Revista Fitoterapia



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Article critically reviewing the historical, clinical and legal status of medicinal plants and accounts on topical issues are also welcomed.
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