UNIVERSIDADE FEDERAL RURAL DO SEMIÁRIDO
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
CENTRO DE CIÊNCIAS AGRÁRIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL
DOUTORADO EM CIÊNCIA ANIMAL

RAFAEL FERNANDES DE QUEIROZ NETO

ATIVIDADE ANTI-INFLAMATÓRIA E ANTIOXIDANTE DO EXTRATO DA *CISSAMPELOS SYMPODIALIS* NA INFLAMAÇÃO PULMONAR INDUZIDA PELA FUMAÇA DO CIGARRO EM CAMUNDONGOS

MOSSORÓ–RN

2023

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Tese de Doutorado apresentada ao Programa de Pós-graduação em Ciência Animal da Universidade Federal Rural do Semiárido como requisito parcial para obtenção do título de Doutor em Ciência Animal.

Linha de pesquisa: Morfofisiologia e Biotecnologia Animal

Orientador: Prof. Dr. Moacir Franco de Oliveira
Co-orientador: Prof. Dr. Emanuel Kennedy Feitosa Lima

MOSSORÓ-RN

2023

Q469a Queiroz Neto, Rafael.
Atividade anti-inflamatória e antioxidante do extrato da Cissampelos sympodialis na inflamação pulmonar induzida pela fumaça do cigarro em camundongos / Rafael Queiroz Neto. - 2023.
90 f. : il.

Orientador: Moacir Franco de Oliveira.
Coorientador: Emanuel Kennedy Feitosa Lima.
Tese (Doutorado) - Universidade Federal Rural do Semi-árido, Programa de Pós-graduação em , 2023.

1. Cissampelos sympodialis. 2. Estresse oxidativo. 3. Inflamação. 4. Enfisema. I. Franco de Oliveira, Moacir, orient. II. Kennedy Feitosa Lima, Emanuel, co-orient. III. Título.

Ficha catalográfica elaborada por sistema gerador automáto em conformidade com AACR2 e os dados fornecidos pelo) autor(a).
Biblioteca Campus Mossoró / Setor de Informação e Referência
Bibliotecária: Keina Cristina Santos Sousa e Silva
CRB: 15/120

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Defendida em: **22/12/ 2023.**

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DADOS CURRICULARES DO AUTOR

RAFAEL FERNANDES DE QUEIROZ NETO nasceu no dia 06 de outubro de 1983, na cidade de Natal/RN. Cursou o ensino fundamental e médio no Colégio Nossa Senhora das Neves (em Natal), concluindo no ano de 2000. Iniciou o ensino superior em 2005, na Faculdade de Ciências da Saúde da Universidade do Estado do Rio Grande do Norte (FACS-UERN), onde graduou-se em Medicina, concluindo em 2011. Fez Residência Médica em Medicina de Família e Comunidade. Em 2014 assumiu concurso para Servidor Público Federal, cargo de médico e, em 2015, assumiu o concurso para o Magistério Superior na Universidade Federal Rural do Semi-Árido (UFERSA), aonde leciona no curso de Medicina. Ingressou em 2016 no Mestrado do Programa de Pós-Graduação em Ciência Animal da UFERSA, concluindo no ano de 2018. Em 2020 iniciou seu Doutorado no Programa de Pós-Graduação em Ciência Animal da UFERSA.

Ao Mestre dos Mestres,

Dedico

AGRADECIMENTOS

Foram muitas, as pessoas e parcerias, a contribuir direta ou indiretamente para o desenvolvimento dessa tese. Todo o suporte demandado nessa trajetória de quatro longos anos para a construção desse projeto foi fundamental para que agora esteja finalizado.

Em primeiro lugar gostaria de agradecer à minha família, principalmente aos meus pais Pedro Fernandes de Queiroz e Aldenora Tomaz da Silva Queiroz, que estiveram comigo durante todo o meu processo formativo, enfatizando sempre o poder transformador da educação. E também à minha filha Maria Tereza Boaventura Fernandes de Queiroz, a força motriz dos meus dias mais leves e, sobretudo, dos mais pesados.

Aos meus orientadores e amigos Moacir Franco de Oliveira e Emanuel Kennedy Feitosa Lima, muito obrigado por todo o suporte desde a gestação ao fim desse trabalho. Levarei comigo sempre os ensinamentos e o investimento de vocês na minha formação.

Muito obrigado aos meus amigos do laboratório Márcio Matheus Barros Moura, Deymisson D. Feitosa, Maria Joana Nogueira de Moura, Aline Gabrielle Gomes da Silva, João Augusto Rodrigues, Igor Renno Lopes por todo o apoio e convivência nesses anos. Todos foram determinantes para o desenvolvimento dessa tese; O trabalho tem a participação de vocês do início ao término. Obrigado também ao demais integrantes do laboratório, e aos professores Flávio Santos, Rodrigo Costa, Micássio Fernandes de Andrade e Diogo Paiva Cavalcanti por todos os auxílios.

Aos meus ex-alunos e colaboradores Priscila Aigner Menezes (a Bela) e Felipe Andrade, toda a gratidão de um professor orgulhoso.

Aos meus amigos e aos professores da pós, agradeço pelo privilégio de terem compartilhado tantos ensinamentos comigo.

“Se eu vi mais longe, foi porque estava sobre os ombros de gigantes”.

Sir Isaac Newton – 1675.

RESUMO

A Cissampelos sympodialis é uma espécie da flora brasileira endêmica em áreas de clima semiárido. Conhecida popularmente como “milona” suas folhas e raízes são empregadas na medicina popular no tratamento de doenças respiratórias, com capacidade comprovada em reduzir a inflamação das vias aéreas em modelo murino de asma. A inalação de fumaça do cigarro pode causar inflamação nos pulmões alterando sua morfofisiologia principalmente pela formação de espécies reativas ao oxigênio (ROS), resultando em estresse oxidativo. A presente tese teve como objetivo avaliar a atividade anti-inflamatória e antioxidante do extrato da *Cissampelos sympodialis* (ECsy) na inflamação pulmonar induzida por fumaça de cigarro em camundongos C57BL/6. Os animais foram agrupados em fumantes e não-fumantes (controle). O grupo fumante foi exposto durante cinco dias (fase aguda) ou 60 dias (fase crônica) a fumaça do cigarro e tratados com o ECsy (10 ou 100 mg/mL por nebulização) ou veículo. Durante a fase aguda o ECsy foi administrado durante os mesmos cinco dias de exposição a fumaça do cigarro, contudo, na fase crônica os animais foram tratados durante 60 dias após a exposição à fumaça e estabelecimento do enfisema pulmonar. Vinte e quatro horas após o término de cada tratamento foi feita a eutanásia dos animais para coleta do lavado broncoalveolar (modelo agudo) e remoção dos pulmões para análise histológica e análises bioquímicas e de biologia molecular. A exposição à fumaça do cigarro nos modelos agudo e crônico resultou em alterações morfológicas e inflamatórias, decorrentes do aumento do estresse oxidativo tecidual, com evolução para enfisema pulmonar no modelo crônico. O tratamento com ECsy foi capaz de reduzir o dano oxidativo e modular a resposta inflamatória em ambos os modelos. Assim, atenuando as alterações morfológicas no parênquima pulmonar e o estabelecimento do enfisema pulmonar (modelo crônico). Os resultados demonstram a ação antioxidante, anti-inflamatória e o potencial anti-enfisematoso do ECsy na lesão pulmonar crônica induzida pela fumaça do cigarro.

Palavras-chave: *Cissampelos sympodialis*. Estresse oxidativo. Inflamação. Enfisema.

ABSTRACT

Cissampelos sympodialis is a species of the Brazilian flora endemic to semi-arid climate areas. Popularly known as "milona," its leaves and roots are used in folk medicine to treat respiratory diseases, with proven efficacy in reducing airway inflammation in a murine model of asthma. Inhalation of cigarette smoke can cause inflammation in the lungs, altering their morphophysiology, mainly due to the formation of reactive oxygen species (ROS), resulting in oxidative stress. The present thesis aimed to evaluate the anti-inflammatory and antioxidant activity of the extract of *Cissampelos sympodialis* (ECsy) in cigarette smoke-induced lung inflammation in C57BL/6 mice. The animals were grouped into smokers and non-smokers (control). The smoker group was exposed to cigarette smoke for five days (acute phase) or 60 days (chronic phase) and treated with ECsy (10 or 100 mg/mL by nebulization) or vehicle. During the acute phase, ECsy was administered for the same five days of cigarette smoke exposure. However, in the chronic phase, the animals were treated for 60 days after smoke exposure and the establishment of pulmonary emphysema. Twenty-four hours after the end of each treatment, the animals were euthanized for bronchoalveolar lavage collection (acute model) and lung removal for histological analysis and biochemical and molecular biology analyses. Cigarette smoke exposure in acute and chronic models resulted in morphological and inflammatory changes, resulting from increased tissue oxidative stress, progressing to pulmonary emphysema in the chronic model. ECsy treatment was able to reduce oxidative damage and modulate the inflammatory response in both models, attenuating morphological changes in the lung parenchyma and the establishment of pulmonary emphysema in the chronic model. The results demonstrate the antioxidant, anti-inflammatory, and potential anti-emphysematous action of ECsy in cigarette smoke-induced chronic lung injury.

Keywords: *Cissampelos sympodialis*. Oxidative stress. Inflammation. Emphysema.

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LISTA DE ABREVIAÇÕES

AChE acetilcolinesterase

ALI lesão pulmonar aguda (do inglês, acute lung injury)

BALF fluído do lavado broncoalveolar (do inglês, bronchoalveolar lavage fluid)

BCI índice de broncoconstrição (do inglês, bronchoconstriction index)

Ca^{2+} íon cálcio

cAMP monofosfato cíclico de adenosina (do inglês, cyclic adenosine monophosphate)

CAT catalase

CS fumaça de cigarro (do inglês, cigarette smoke)

DPOC Doença Pulmonar Obstrutiva Crônica

ECsy extrato de *Cissampelos sympodialis*

EDS escore de dano epitelial (do inglês, epithelial damage score)

FVC capacidade vital forçada (do inglês, forced vital capacity)

GM-CSF fator estimulador de colônias de granulócitos e macrófagos (do inglês, granulocyte-macrophage colony-stimulating fator)

GOLD Iniciativa Global para Doença Pulmonar Obstrutiva Crônica (do inglês, Global Initiative for Chronic Obstructive Lung Disease)

GPx glutationa peroxidase

GSH glutationa reduzida

H&E hematoxilina e eosina

IgE imunoglobulina E

IL interleucina

IFN-γ interferon gama

iNOS óxido nítrico sintase induzida (do inglês, inducible nitric oxide synthase)

LABA beta-2 agonista de longa duração

LAMA antagonista muscarínico de longa duração

LIS escore de inflamação pulmonar (do inglês, lung inflammation score)

Lm diâmetro alveolar médio

MDA malonaldeído

MMP metaloprotease de matriz (do inglês, matrix metalloproteinase)

MPO mieloperoxidase

NAC: N-acetilcysteina

NADPH fosfato de dinucleótido de nicotinamida e adenina (do inglês, nicotinamide adenine dinucleotide phosphate)

NE elastase neutrofílica (do inglês, neutrophil elastase)

NFκB fator nuclear kappa B (do inglês, nuclear factor kappa B)

NO óxido nítrico (do inglês, nitric oxide)

Nrf2 fator nuclear eritroide 2 relacionado ao fator 2 (do inglês nuclear fator erythroid 2-related factor-2)

ROS espécies reativas de oxigênio (do inglês, reactive oxigen species)

RNS espécies reativas de nitrogênio (do inglês, reactive nitrogen species)

SOD superóxido dismutase

TGF-β fator de crescimento transformante beta (do inglês, transforming growth factor)

TNF-α fator de necrose tumoral alfa (do inglês, tumor necrosis factor alpha)

VEF1 volume expiratório forçado no primeiro segundo Vv: densidade de volume

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Capítulo III

Cissampelos sympodialis Extract Attenuates Pulmonary Emphysema Induced by Chronic Cigarette Smoke Exposure

INTRODUÇÃO

O uso de plantas com fins terapêuticos é uma prática milenar derivada das experiências do cotidiano que tiveram alguma aplicabilidade no tratamento das mais diversas moléstias em homens ou animais e foram assim transmitidas por meio das gerações, perpetuando-se na cultura popular (Lemos; Araújo, 2015).

Algumas dessas plantas despertam interesse progressivo por parte da comunidade científica devido às atividades biológicas presentes em seus compostos orgânicos. Neles são encontradas substâncias com potencial terapêutico, tais como flavonoides, alcaloides, terpenos, taninos, lignanas, entre outros, que passam a ser objetos de incessantes estudos, com o intuito de se comprovar suas ações farmacológicas e possíveis aplicações em situações que comprometem o estado de saúde (de Sousa Araújo et al., 2008).

A *Cissampelos sympodialis* Eichl., uma espécie da flora brasileira, pertencente à família Menispermaceae, é encontrada desde o Ceará até o norte de Minas Gerais, ocorrendo principalmente em áreas de clima semiárido. Essa espécie é conhecida popularmente como "milona", "jarrinha", "orelha-de-onça" e "abuteira", cujas folhas e raízes são empregadas na medicina popular no tratamento de doenças do aparelho respiratório, reumatismo e artrites (Cavalcanti et al., 2013; de Sales et al., 2018).

Estudos imunofarmacológicos com folhas de *C. sympodialis* demonstraram ação imunomoduladora, anti-inflamatória e broncodilatadora, com destaque para a warifteína, um dos principais alcaloides presentes no extrato da *C. sympodialis*, que atua inibindo a produção de leucotrienos, fosfodiesterase e estimulando a interleucina-10, demonstrando seu grande potencial para o tratamento da asma, uma doença inflamatória crônica caracterizada por obstrução ao fluxo aéreo, geralmente reversível com o uso de broncodilatadores (Vieira et al., 2018), característica que a diferencia clinicamente da Doença Pulmonar Obstrutiva crônica (DPOC).

Causada principalmente pelo tabagismo a fisiopatologia da DPOC se caracteriza pela diminuição do fluxo de ar nos pulmões em função da redução do calibre das pequenas vias aéreas, não totalmente reversível ao uso de broncodilatadores, com destruição e remodelamento do parênquima pulmonar, por consequência de uma resposta inflamatória crônica. Essas alterações manifestam-se clinicamente por meio de dispneia, tosse crônica, produção de muco e sibilância (Vogelmeier et al., 2017; Mourato et al., 2018).

A DPOC é um grave problema de saúde pública e, por ser uma das principais causas de morbimortalidade no mundo, torna pertinente a busca por alternativas terapêuticas como a

investigação das atividades biológicas do extrato da *Cissampelos sympodialis* na inflamação pulmonar induzida pela fumaça do cigarro, considerando o potencial farmacológico dessa espécie como agente adjuvante no tratamento de doenças respiratórias vinculadas ao tabagismo, a exemplo do enfisema pulmonar.

CAPÍTULO I

1 FUNDAMENTAÇÃO TEÓRICA

1.1 DOENÇA PULMONAR OBSTRUTIVA CRÔNICA

A doença pulmonar obstrutiva crônica (DPOC) é caracterizada pela limitação do fluxo aéreo nas vias respiratórias e a presença de sintomas respiratórios persistentes, como a tosse e dispneia, por exemplo (GOLD, 2022). Essa limitação crônica do fluxo aéreo se deve ao acometimento das pequenas vias aéreas, relacionado a um estado inflamatório crônico que leva a um estreitamento dessas vias, e destruição do parênquima, que pode levar ao enfisema pulmonar ou bronquite crônica, que são os dois fenótipos dessa doença (Vestbo et al., 2013).

A DPOC, no mundo, é causada principalmente pelo tabagismo, porém a exposição a gases nocivos, fatores individuais como genética, imunidade ou autoimunidade, estresse oxidativo e inflamação influenciam no acometimento individual e progressão da doença, interrelacionando-se no dano estrutural e funcional próprio da doença, que varia entre indivíduos afetados (Paschoal; Moreira, 2016).

A alta prevalência da DPOC a torna uma das principais causas de morbidade e mortalidade em todo o mundo. Adeloye et al., (2015), estimaram uma prevalência global de 384 milhões de casos, 15% de ocorrência nas Américas, ficando em terceiro lugar nas taxas de mortalidade, com cerca de 3,2 milhões de pacientes (Wang et al., 2016), com quase 90% das mortes ocorrendo em países de baixa e média renda (Burney et al., 2015).

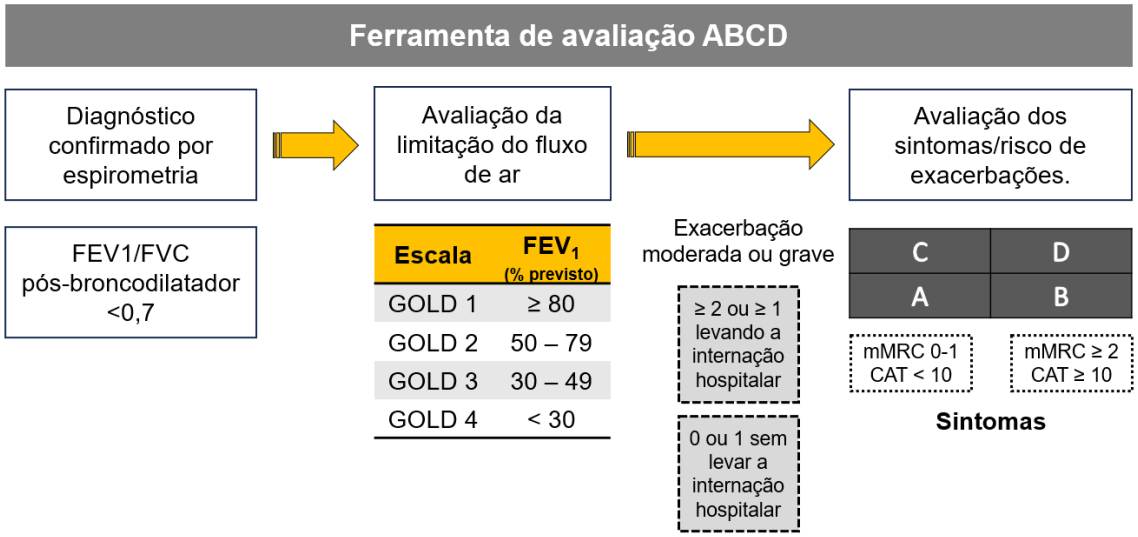
Os indivíduos fumantes com ou sem DPOC, em sua maioria, podem queixar-se de sintomas respiratórios, como a tosse e o excesso de expectoração, isso se deve à ação danosa da fumaça de cigarro sobre sistema respiratório, que provoca alteração do epitélio respiratório com consequente destruição das células ciliares e estímulo à produção de muco. Estruturas importantes na proteção das vias aéreas, que atuam evitando que partículas ou gases nocivos atinjam as vias aéreas inferiores, prejudicando a membrana de troca (Paschoal, Moreira; 2016).

Uma das hipóteses mais relevantes sobre o dano tecidual que ocorre na DPOC é o desequilíbrio entre a quantidade de proteases a antiproteases. O contato com substâncias nocivas, como a fumaça de cigarro ou outros gases tóxicos, leva a uma ativação de células de defesas liberando proteases. Essa liberação ocorre em quantidade superior ao sistema inibitório, levando a dano tecidual, remodelando a arquitetura pulmonar (Robertoni et al., 2015). O contínuo remodelamento pulmonar que ocorre na DPOC, devido ao intenso processo

de síntese e degradação de componentes da matriz extracelular, leva a ocorrência do enfisema pulmonar, que é a principal manifestação da doença. Nesse processo de remodelamento atuam enzimas pertencentes ao grupo das metaloproteinases (MMP) como elastases e colagenases, que degradam as fibras de elastina e colágeno na parede dos alvéolos (Robertoni et al., 2015; Lin; Thomas, 2010).

O diagnóstico da DPOC requer a demonstração espirométrica da limitação do fluxo de ar persistente, conforme definido por uma relação pós-broncodilatador FEV1/FVC < 70%, em pacientes com sintomas apropriados e histórico de exposição a estímulos nocivos (Ries et al., 2007). As diretrizes GOLD mais recentes classificam a gravidade da DPOC com base na porcentagem do FEV1 previsto. Separadamente, a gravidade da dispneia e o histórico de exacerbação são incorporados em uma grade 2x2 para formar quatro "grupos" A-D (Figura 1). Essa formação de grupos GOLD A-D forma a base das recomendações de tratamento mais recentes (Mirza et al., 2018).

Figura 1 - Ferramenta de Avaliação DPOC GOLD ABCD.



Fonte: Adaptado de Manian (2019).

O tratamento da DPOC tem como objetivos melhorar a qualidade de vida do paciente, controlar e prevenir exacerbações e diminuir o risco de outras complicações (hipertensão pulmonar, hipóxia crônica, tromboembolismo pulmonar, câncer de pulmão). Para atingi-los, o GOLD recomenda medidas não-farmacológicas e farmacológicas.

Dentre os tratamentos não-farmacológicos parar de fumar é fundamental para reduzir o declínio progressivo da função pulmonar ao longo do tempo, assim como as exacerbações e as

comorbidades relacionadas ao tabagismo (câncer de pulmão e doenças cardiovasculares), o que diminui a mortalidade em pacientes com DPOC (Anthonisen et al., 2005). Do mesmo modo, reduzir a poluição do ar em ambientes fechados utilizando um fogão em vez de cozinhar em fogo aberto pode levar ao declínio progressivo da função pulmonar de maneira semelhante à cessação do tabagismo (Romieu et al., 2009). Adicionalmente, aumentar a atividade física diária pode ser igualmente eficaz que parar de fumar, além de prevenir morbidade e mortalidade em pacientes com DPOC (Waschki et al., 2015).

A reabilitação pulmonar é uma estratégia de tratamento multidisciplinar eficaz para melhorar a dispneia, tolerância ao exercício e qualidade de vida relacionada à saúde (Spruit et al., 2013). Embora o programa clássico de exercícios com treinamento individualizado de resistência e fortalecimento ainda seja o pilar da reabilitação pulmonar, a educação, a promoção de mudanças comportamentais e o autogerenciamento também são considerados essenciais para qualquer intervenção bem-sucedida (Casaburi; Zuwallack, 2009).

Quanto ao tratamento farmacológico, a vacinação contra influenza e a vacinação pneumocócica geralmente são recomendadas para pacientes com DPOC, pois há alguma evidência de benefícios específicos de ambas as vacinas para pacientes com DPOC, como redução do risco de exacerbações ou internações hospitalares, conforme descreve Jae et al., (2010).

A farmacoterapia de manutenção em pacientes com DPOC está voltada para melhorar os sintomas, a qualidade de vida relacionada à saúde, a intolerância ao exercício e o risco de exacerbações. O Uso de Beta-2 agonistas de longa duração (LABAs) inalatórios e antagonistas muscarínicos de longa duração (LAMAs) têm efeitos positivos semelhantes na limitação do fluxo de ar, redução do aprisionamento aéreo e melhora da intolerância ao exercício (Decramer et al., 2013). Ademais, terapias combinadas fixas com LABA e LAMA, facilitam o gerenciamento do tratamento broncodilatador, de modo que os pacientes não precisam inalar vários dispositivos inaladores (Wedzicha et al., 2008). Alguns pacientes ainda se beneficiam do uso de corticosteroides inalados além da terapia de broncodilatadores, devido a diminuição das exacerbações quando os corticosteroides inalados fizeram parte da terapia de manutenção inalada (Watz et al., 2016).

Foram feitos esforços consideráveis na última década para encontrar novas terapias relativas à inflamação na DPOC, mas com pouco sucesso. Isso pode ser parcialmente resultado da complexidade da inflamação e dos fenótipos clínicos relacionados (Martinez; Donohue; Rennard, 2011). Brightling et al., (2014), descreve que o direcionamento de uma

única via ou mecanismo inflamatório pode não ser suficiente para suprimir persistentemente a inflamação em todos os pacientes com DPOC, aspecto que possivelmente dificulta o tratamento desta.

A mudança da formulação para que os pacientes inalem certos compostos, como inibidores de fosfodiesterase, pode ajudar a limitar os efeitos colaterais (Watz; Mistry; Lazaar, 2013). Enquanto a eficácia dos broncodilatadores pode ser demonstrada em estudos de curto prazo em um pequeno número de pacientes, estudos mais longos em subgrupos altamente selecionados são necessários para que os medicamentos possam direcionar a inflamação, o que torna o desenvolvimento de medicamentos mais difícil.

1.2 ENFISEMA PULMONAR

A DPOC causa o aumento irreversível dos espaços aéreos periféricos, obstrução do fluxo de ar para dentro e para fora dos pulmões, sintomas respiratórios persistentes e progressivos, e problemas respiratórios devido à destruição progressiva das paredes alveolares. Ela possui um espectro fisiopatológico com manifestações clínicas que variam entre a bronquite crônica e o enfisema pulmonar (Chaouat; Naeije; Weitzenblum, 2008).

A avaliação visual por tomografia computadorizada e autópsia indicaram que o enfisema pulmonar é dividido em três subtipos principais: enfisema centroacinar, parasseptal e panacinar. O enfisema centroacinar é a forma mais comum da doença e está relacionada com a inalação da fumaça de cigarro por longo período, atingindo a região central do bronquíolo principal e os seus ramos. A forma parasseptal tem extensão limitada, atingindo os sacos alveolares e os dutos pulmonares. No enfisema panacinar ocorre a destruição alveolar e dilatação dos espaços de forma permanente e está associado à deficiência da proteína alfa 1-antitripsina (Smith et al., 2014). A fisiopatologia da doença é multicausal. Algumas condições genéticas (por exemplo, deficiência de alfa-1 antitripsina) (de Serres; Blanco, 2014), crescimento pulmonar comprometido pré-natal ou durante a infância, infecção recorrente do trato respiratório na infância, poluição ambiental, exposição ocupacional, baixo status socioeconômico e exposição prolongada a produtos químicos, partículas nocivas e gases prejudiciais são fatores de risco, embora a fumaça do cigarro seja a principal causa da DPOC (Mahmood et al., 2017).

Em geral, o enfisema causa redução da elasticidade pulmonar, aumento da complacência pulmonar e aumento dos volumes pulmonares com redução das taxas de fluxo expiratório, que resulta em inflamação permanente nos pulmões e aumento acentuado dos

espaços aéreos com destruição gradual da área de troca capilar alveolar, que pode ser fixa ou apenas parcialmente reversível. As manifestações clínicas incluem dificuldade para respirar, tosse crônica com ou sem catarro e cansaço (Jankowich; Rounds, 2012).

1.2.1 Tabagismo e Estresse Oxidativo no Enfisema Pulmonar

A disfunção endotelial induzida por fumaça de cigarro manifesta-se inicialmente pelos efeitos tóxicos direcionados por fumaça de cigarro nas células endoteliais, seguido do aumento dos níveis de estresse oxidativo com redução da ativação de vias antioxidantes nas células endoteliais e, finalmente, pelo aumento de mediadores com atividades vasoconstritoras, pró-inflamatórias e remodeladoras (Polverino; Celli; Owen, 2018). As células epiteliais e os macrófagos ativam os fibroblastos por meio da liberação de mediadores, como TGF-β, levando a fibrose ao redor das pequenas vias aéreas e remodelamento (Barnes, 2017).

O ciclo inflamatório induzido pela DPOC nos pulmões ocorre quando as células epiteliais, os macrófagos e os neutrófilos são estimulados por agentes nocivos a liberar quimiocinas, como o fator de necrose tumoral-α (TNF-α) e interleucinas (IL-1, IL-6,IL-8), moléculas de sinalização celular que promovem ativação e recrutamento das células inflamatórias, induzindo a liberação de enzimas proteolíticas como metaloproteinases (MMPs) e elastase neutrofílica (NE), que causam degradação descontrolada de elastina, levando ao enfisema pulmonar (Bonvini, 2020).

Além da inflamação, o estresse oxidativo é um dos principais desencadeadores da patogênese e progressão da DPOC. Inúmeros estudos confirmaram que a exposição a irritantes nocivos provenientes de fontes exógenas e endógenas, especialmente o tabagismo e a poluição do ar, induz danos aos lipídios, proteínas e ácidos nucleicos, causando lesões diretas ou indiretas nos pulmões. O dano tecidual aumenta o recrutamento adicional de células inflamatórias nos pulmões, exacerbando o estresse oxidativo e o ciclo inflamatório (Kirkham; Barnes, 2013). Consequentemente, o número de fagócitos (como macrófagos, neutrófilos e eosinófilos) aumenta nos pulmões, resultando no aumento da produção de espécies reativas de oxigênio (ROS).

O excesso de ROS leva à diminuição dos antioxidantes, suprime as defesas antioxidantes e provoca a permeabilidade e destruição das células epiteliais pulmonares. O estresse oxidativo está envolvido na iniciação de respostas inflamatórias por meio da ativação de fatores de transcrição sensíveis ao redox, como o fator nuclear 2 relacionado ao fator

eritroide 2 (Nrf2; antioxidante) e o fator nuclear de cadeia leve kappa B (NF-κB) (pró-oxidante) tanto nos pulmões quanto em tecidos extra-pulmonares. A ativação do NF-κB leva à regulação positiva de mediadores pró-inflamatórios, resultando na indução de inflamação pulmonar e sistêmica (Fischer; Voynow; Ghio, 2015).

O NF-κB é um fator de transcrição clássico que induz a inflamação em resposta ao estresse oxidativo através da ativação de genes que codificam citocinas pró-inflamatórias e quimiocinas (Yu et al., 2018) e ou por inativar o Nrf2 (Liu; Qu; Shen, 2008; Schuliga, 2015). A interação entre Nrf2 e esses fatores inflamatórios estabelece um mecanismo pelo qual Nrf2 regula a inflamação (Suzuki et al., 2008).

Estudos em camundongos e seres humanos indicaram que o Nrf2 parece desempenhar um papel protetor contra o enfisema, e a perda da atividade do Nrf2 está correlacionada com a progressão da DPOC. Os camundongos tratados com elastase tiveram um aumento significativo na expressão de genes antioxidantes regulados por Nrf2 nos macrófagos alveolares. O transplante de medula óssea de camundongos selvagens para camundongos $Nrf2^{-/-}$ reduziu significativamente os sintomas de enfisema, indicando que a via antioxidante induzida pelo Nrf2 nos macrófagos alveolares desempenha um papel protetor contra a inflamação pulmonar e o enfisema (Ishii et al., 2017).

Vários produtos naturais ou compostos farmacológicos que podem simultaneamente ativar a via do Nrf2 e inibir a via do NF-κB mostraram potencial terapêutico contra a inflamação respiratória e a DPOC (Kennedy-Feitosa et al., 2019). A fisetina, um composto flavonóide de plantas, foi sugerida como uma terapia potencial para a DPOC, uma vez que pode ativar a via do Nrf2 e reduzir o estresse oxidativo, a inflamação e o dano pulmonar causados pela exposição à fumaça de cigarro em ratos (Hussain et al., 2019), demonstrando a importância do Nrf2 na proteção dos pulmões, ao mesmo tempo em que sugere o Nrf2 como um potencial alvo terapêutico no tratamento da DPOC.

1.3 PLANTAS MEDICINAIS E FITOTERÁPICOS

O uso crônico de corticosteroides e broncodilatadores pode ser ineficaz ou até mesmo estar associados a efeitos colaterais (Patel et al., 2019). Além desses efeitos indesejados, a resistência a corticosteroides ou antibióticos são outras limitações importantes das farmacoterapias atuais para a DPOC (Barnes, 2008). Assim, surge a necessidade de explorar novas opções terapêuticas para o tratamento da DPOC com maior eficácia, segurança e menos

efeitos adversos. É nesse contexto que o uso de plantas medicinais tem sido impulsionado como uma alternativa terapêutica à medicina alopática.

As propriedades antioxidantes e anti-inflamatórias dos fitoquímicos utilizados no tratamento de patologias pulmonares podem conferir proteção pulmonar e reduzir o risco de DPOC. Esses fitoquímicos podem ser usados como monoterapia ou em combinação com outros medicamentos anti-inflamatórios, permitindo a redução das reações adversas aos medicamentos e dos custos com aquisições. Ervas ou seus constituintes bioativos podem melhorar a inflamação e o enfisema ao restaurar o desequilíbrio redox, seguidos pela supressão da expressão de fatores pró-inflamatórios e proteases associados à DPOC (Singla; Dharwal; Naura, 2020).

Foi demonstrado que o uso de frutas e vegetais como fontes ricas de fitoquímicos em populações de alto risco pode reduzir o risco de DPOC na população (Baines et al., 2015; Kaluza et al., 2017). Compostos fenólicos, carotenoides e alcaloides impedem a metilação do DNA reduzindo o estresse oxidativo e a inflamação, prevenindo assim a progressão da DPOC. Existem dados estabelecidos que confirmam que as intervenções dietéticas precoces na vida podem proteger e preservar a função pulmonar ao longo de toda a vida (Zhai et al., 2018).

Os fitoquímicos modulam o estresse oxidativo reduzindo a atividade de fatores como MDA, iNOS, MPO e aumentando a atividade de SOD, GSH, GPx, CAT, NADPH (Bellik et al., 2013), também reduzindo a expressão IL-2, IL-1β, IL-6, IL-8, TNF-α, além de diminuir a atividade dos leucócitos, o que reduz a inflamação (Fu et al., 2012). Em conjunto, os fitoquímicos podem atuar em alguns dos mecanismos mencionados da DPOC, logo, podem desempenhar papel benéfico à prevenção ou melhoria dos sintomas associados ao enfisema pulmonar.

1.3.1 *Cissampelos sympodialis*

Cissampelos sympodialis é uma espécie do gênero *Cissampelos* endêmica do Brasil, sendo encontrada no Sudeste e Nordeste, entre os estados de Minas Gerais e Ceará (da Silva Mendes et al., 2020). A principal característica distintiva para identificar este gênero é a forma deltoide de suas folhas glabras (Figura 2A). Também na inflorescência, há a presença de 3-6 flores pistiladas por fascículo; fruto do tipo drupáceo, obovado e com mudança de cor de vermelho para laranja quando maduro (Barbosa-Filho et al., 1997). A espécie é conhecida popularmente como “milona” ou “jarrinha”, habita regiões abertas, atinge cerca de 80 a 100 cm de altura e apresenta frutos de pequeno diâmetro e avermelhados, quando maduros (Figura

2B). As raízes e folhas da *Cissampelos sympodialis* são usadas para os fins medicinais (Barbosa-Filho et al., 1997; da Silva Mendes et al., 2020), popularmente no tratamento de doenças de origem inflamatória, como asma e outras doenças respiratórias, artrite e doenças geniturinárias (Batisita-Lima et al., 2001; Piuvezam et al., 2012).

Figura 2. Folhas de *Cissampelos sympodialis* Eichl. (A). Frutos maduros *C. sympodialis* (B).

Fonte: Adaptado de Cavalcanti et al,. (2013).

O gênero *Cissampelos* apresenta uma ampla variedade de alcaloides e níveis moderados de compostos não alcaloides (Semwal et al., 2014). A quantidade e os grupos de metabólitos secundários desse gênero são influenciados por fatores como sazonalidade, ritmo circadiano, estágio de desenvolvimento e idade, temperatura, disponibilidade de água, radiação UV, nutrientes do solo, altitude, composição atmosférica e danos aos tecidos (Gobbo-Neto; Lopes, 2007).

Estudos químicos realizados com a *C. sympodialis* resultaram no isolamento de diferentes alcaloides (Barbosa-Filho et al., 1997). A partir dos extratos da raiz de *C. sympodialis*, várias análises fitoquímicas foram realizadas, resultando na detecção de diversos grupos de alcaloides, como warifteína, metilwarifteína, milonina e roraimina (Bezerra-Santos et al., 2006). Cavalcanti et al. (2014) realizaram a caracterização fitoquímica desta planta e

detectaram majoritariamente a presença de alcaloides, seguidos de compostos fenólicos como flavonoides e saponinas.

Dentre os interesses por estudos com plantas medicinais, destacam-se as pesquisas voltadas a identificar as atividades biológicas de seus componentes estruturais, na busca por agentes com atividades de interesse médico ou nutricional, entre outros. Pesquisas neste sentido, relatam que o extrato etanólico das partes aéreas de *C. sympodialis* aumentou os níveis de GSH, que atua como um antioxidante endógeno por meio da eliminação de radicais livres, que são os causadores de danos à mucosa gástrica, conferindo à *C. sympodialis* uma possível atividade gastroprotetora relacionada a mecanismos antioxidantes (de Sales et al., 2018). A fração alcaloide encontrada na *C. sympodialis* é uma fonte de alcaloides com potencial atividade antioxidante e atividade inibitória da acetilcolinesterase (AChE) in vitro (Ramasamy; Anandakumar; Kathiresan, 2022).

Quanto à estudos envolvendo a atividade anti-inflamatória da *C. sympodialis* verificou-se em estudos com fração aquosa do extrato etanólico obtido das folhas dessa planta, que a mesma inibe a migração de neutrófilos, provavelmente, pelo fato de provocar a diminuição da produção de TNF-α, IL-1, IL-6 e IL-8 por macrófagos, inibição da via do Ca^{2+} e estímulo da produção de IL-10, esta última, uma citocina anti-inflamatória (Batista-Lima et al., 2001). Extratos das folhas também diminuíram a produção de NO e citocinas inflamatórias (TNF-α, IL-1, IL-6 e IL-8) envolvidas na patogênese da psoríase e aumentaram a produção de citocinas anti-inflamatórias (IL-10), sugerindo que o extrato das folhas de *C. sympodialis* poderia representar uma adição nova e segura ao tratamento da psoríase (Feily; Namazi, 2009).

Os extratos de partes aéreas da *C. sympodialis* também tiveram eficácia comprovada em experimentos com o intuito de pesquisar atividades antidepressiva (Almeida et al., 2005), cardioprotetora (de Freitas et al., 1995), imunomoduladora e antialérgica (Bezerra-Santos et al., 2006), analgésica e antipirética (Ramasamy; Anandakumar; Kathiresan, 2022), antimotilidade e antidiarreica, antiúlcera (de Sales et al., 2018), antimicrobiana (Cunha et al., 2019), anticâncer (da Silva Mendes et al., 2020), broncodilatadora e relaxante muscular (Thomas; Barbosa-Filho; Agra, 1997).

2 HIPÓTESES

a) O extrato da *Cissampelos sympodialis* possui atividade anti-inflamatória sobre pulmões inflamados de camundongos expostos à fumaça do cigarro.

b) O extrato da *Cissampelos sympodialis* possui atividade antioxidante sobre os pulmões inflamados de camundongos expostos à fumaça do cigarro.

c) O extrato da *Cissampelos sympodialis* é capaz de modular o desenvolvimento do enfisema e o remodelamento pulmonar causados pela inflamação decorrente da inalação da fumaça do cigarro.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Caracterizar a atividade anti-inflamatória e antioxidante do extrato da *Cissampelos sympodialis* na inflamação pulmonar induzida por fumaça de cigarro em camundongos C57BL/6.

3.2 OBJETIVOS ESPECÍFICOS

a) Avaliar a atividade anti-inflamatória do extrato da *Cissampelos sympodialis* na inflamação pulmonar aguda e crônica induzida pela exposição à fumaça do cigarro;

b) Caracterizar a atividade do extrato da *Cissampelos sympodialis* como modulador do desequilíbrio redox em pulmões de camundongos expostos a fumaça do cigarro;

c) Identificar as possíveis vias através das quais o extrato da *Cissampelos sympodialis* apresenta seus efeitos biológicos na inflamação pulmonar aguda e crônica induzidas pela fumaça do cigarro;

d) Investigar a eficácia do extrato da *Cissampelos sympodialis* sobre o enfisema e o remodelamento pulmonar em pulmões de camundongos expostos à fumaça do cigarro.

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CAPÍTULO II

Revista Brasileira de Farmacognosia

(Bazilian Journal of Pharmacognosy)

Manuscript Number: RBFA-D-23-01066

Cissampelos sympodialis Extract Modulates Pulmonary Macrophage Activity in Acute Lung Injury Induced by Cigarette Smoke in Mice

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ABSTRACT

Introduction: Cigarette smoke can induce acute lung injury (ALI) by increasing inflammatory cells and the release of mediators that cause tissue damage. The extract of *Cissampelos sympodialis* (ECsy) has been used in the treatment of respiratory diseases due to its anti-inflammatory and antioxidant effects. Here, we show that ECsy reduces cigarette smoke-induced ALI by modulating the activity of pulmonary macrophages. **Methods:** CEUA (16/2020). Mice were divided into groups: Control, ALI, ALI + 10, ALI + 100, and ALI + Dexa. The ALI groups were exposed to 12 cigarettes/day and treated with saline (ALI), ECsy (10 or 100 mg/mL; 15 min/day), or dexamethasone (0.4 mg/mL) for 5 days. The control was exposed to room air and treated with saline. After 5 days, lung and bronchoalveolar lavage fluid (BALF) were collected for morphological, redox, and inflammatory evaluations. A significant difference was considered when $p < 0.05$. **Results**: Lm and lung inflammation score decreased in the ALI + 100 and ALI + Dexa groups compared to the ALI group. The bronchoconstriction index decreased in the ALI + 10, ALI + 100, and ALI + Dexa compared to ALI. Epithelial height decreased in ALI compared to ALI + 100 and ALI + Dexa. Epithelial damage decreased in the ALI + 100 compared to ALI. Cellularity and protein concentration in BALF decreased in the treated groups compared to the ALI group. Macrophages decreased in number and size in the ALI + 100 and ALI + Dexa compared to the ALI. TNF-α and IL-1β levels decreased in the ALI + 100 and ALI + Dexa compared to the ALI. ROS increased in ALI compared to the other groups. Myeloperoxidase, catalase, and superoxide dismutase activities decreased in the ALI + 100 and ALI + Dexa compared to ALI. MDA levels decreased in ALI + 10, ALI + 100, and ALI + Dexa compared to ALI. Immunohistochemistry showed higher Nrf2 factor expression in the ALI + 100 and ALI + Dexa compared to the ALI. **Conclusion:** The results demonstrate the anti-inflammatory and antioxidant potential of ECsy in cigarette smoke-induced acute lung injury.

Keywords: Cigarette smoke. *Cissampelos*. Macrophage function.

1 INTRODUCTION

The term acute lung injury (ALI) was defined to describe a clinical syndrome characterized by non-cardiac pulmonary inflammation and edema (Bernard et al., 1994). ALI is associated with endothelial and epithelial dysfunction resulting from the loss of alveolar-capillary membrane integrity, with the migration of cells such as macrophages and neutrophils to the alveolar space, as well as an increase in proinflammatory/cytotoxic mediators (Matuschak; Lechner, 2010; Ware, 2006).

Cigarette smoke is one of the main risk factors for the development of ALI, and when inhaled through the airways, it recruits and activates inflammatory cells such as neutrophils, lymphocytes, and especially macrophages (Lugg et al., 2022; Schuliga, 2015). Once recruited, these cells activate cellular mechanisms that lead to the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS), linking smoking to oxidative stress and inflammation (Schuliga, 2015).

Macrophages play a crucial role in this response, as they act as the first line of defense against harmful agents and enhance the inflammatory response by orchestrating the cytokine cascade. They act in the innate response, phagocytizing particulate matter and releasing IL-1β, IFNγ, and TNF-α. Thus, they promote the conversion of monocytes and the recruitment of neutrophils and other macrophages (Aggarwal; King; D'alessio, 2014).

Research on secondary metabolites of plant origin has shown anti-inflammatory and antioxidant activities in acute lung injury, and this is why they are believed to contribute to the therapy of inflammatory lung diseases (Magalhães et al., 2010). *Cissampelos sympodialis* Eichl. is a species of the Brazilian flora known as "milona" in popular usage, and its leaves and roots are used in traditional medicine to treat respiratory diseases (Cavalcanti et al., 2014; De Fátima Agra; De Freitas; Barbosa-Filho, 2007). It has shown great potential in immunopharmacological studies for the treatment of asthma, such as reducing leukotrienes, phosphodiesterase, and increasing IL-10, a cytokine with anti-inflammatory potential (Piuvezam et al., 1999; Vieira et al., 2013).

However, there are no studies on the hydroalcoholic extract of *C. sympodialis* (ECsy) for the treatment of acute lung injury induced by cigarette smoke, or its effects on lung function. Therefore, the purpose of this study was to investigate whether ECsy could attenuate the pathophysiological changes in mice with cigarette smoke-induced ALI by modulating the activity of pulmonary macrophages.

2 METHODS

2.1 Plant Material and Extract Preparation

Leaves of *Cissampelos sympodialis* Eichl were collected from the Botanical Garden of the Pharmaceutical Technology Laboratory at the Federal University of Paraiba (voucher sample Agra 1456). After drying in an oven at 50°C, the leaves were ground into a powder using a knife mill. The powder was extracted with ethanol and water (80/20 v/v) at 25-30°C for 5 days. The dry extract was obtained after removing the solvent using a rotary evaporator at 60°C (Piuvezam et al., 1999).

2.2 Animals

Male C57BL/6 mice (18–22 g), obtained from CEMIB/UNICAMP, were fed a commercial diet (Presence) and had free access to water in a controlled environment (22 - 24°C and a 12/12 h light/dark cycle). The research was approved by the Committee on Ethics in Animal Use (CEUA/UFERSA) (Certificate No. 16/2020).

2.3 Experimental Design

Exposure and Cigarette Smoke Treatment The animals were divided into five experimental groups (n=7 each), as described in Figure 1. The groups (ALI, ALI + 10, ALI + 100, and ALI + Dexa) were exposed to the smoke from 12 commercial cigarettes (Marlboro Red, containing 10 mg tar, 0.9 mg nicotine, and 10 mg carbon monoxide) per day for 5 days using an inhalation chamber. The control group was exposed to ambient air (Kennedy-Feitosa et al., 2019). At the end of each day, the animals were treated with ECsy at 10 mg/mL, 100 mg/mL, dexamethasone (0.4 mg/mL i.p.), or the respective vehicle for each group. ECsy or the vehicle was administered via nebulization (15 minutes/day/once a day) in a chamber attached to a nebulizer (RespiraMax) (Bastos et al., 2011). Dexamethasone was administered intraperitoneally (i.p.).

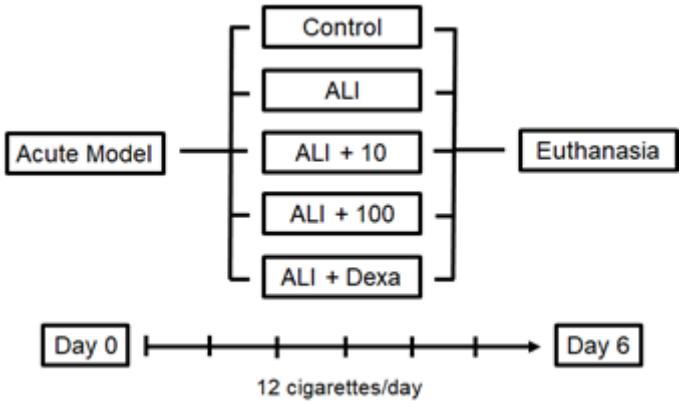

Figure 1. Experimental design. Acute Lung Injury Model: Control, Acute Lung Injury (ALI), Acute Lung Injury + 10 mg/mL extract (ALI + 10), Acute Lung Injury + 100 mg/mL extract (ALI + 100), and Acute Lung Injury + Dexamethasone 0.4 mg/mL (ALI + Dexa).

2.4 Experimental Protocols

2.4.1 Tissue Processing and Histological Analysis

Twenty-four hours after the end of the fifth day of the experimental procedure, the animals were anesthetized by inhalation of sevoflurane and euthanized by cervical dislocation. Bronchoalveolar lavage fluid (BALF) was collected by lavaging the lungs with buffered saline solution (3 × 0.5 mL) and used for the determination of the total number of leukocytes in a Neubauer chamber and protein quantification (Bradford, 1976).

The lungs were perfused with saline to remove blood. The right lung was homogenized for biochemical analyses and cytokine measurement by ELISA, while the left lung was collected and transferred to 4% paraformaldehyde fixative solution (pH 7.4; 4°C) for histological processing. Sections of 5 µm were obtained and stained using the Hematoxylin and Eosin (H&E) technique.

2.4.2 Morphological and Morphometric Analysis

Histopathological analysis was performed using an adaptation of the inflammation score (Bao et al., 2015). Images of 10 non-coincident fields of lung parenchyma at 40x magnification were captured and analyzed by three investigators using the following criteria for scoring lung inflammation: normal tissue (zero); minimal inflammatory change (presence of inflammatory cells without architectural changes) (one); minimal, non-obvious damage to lung architecture (two); thickening of alveolar septa (three); formation of nodules or areas of pneumonitis distorting normal architecture (four); and total field obliteration (five).

The mean alveolar diameter (Lm) was calculated using the equation Lm = Ltot/Li from 10 photomicrographs of random and non-coincident fields free of bronchi and vessels, captured using a light microscope (Leica MD750) with a 40x objective. Ltot corresponds to the total length of lines in the microscopic field, and Li is the number of intersections of alveolar structures with the lines of the grid. The result was expressed in micrometers (Valença; Porto, 2008; Weibel, 1963).

The airway perimeter was evaluated by determining the bronchoconstriction index (BCI) from 10 images of different bronchi captured on each H&E-stained slide (40x). BCI was quantified using the number of intercepts crossing the basal membrane (NI) and the number of points reaching the lumen of the airways (L) via the M42 grid. BCI was calculated using the equation IBC = NI/√L, following the method of Sakae et al. (1994). For this, only bronchi with a ratio of the major to minor diameter of the ellipse that described their lumen equal to or less than 1.25 were considered.

The height of the bronchial epithelium (μm) was obtained by measuring between the basal membrane and the apical membrane at least four different points on the same bronchus from 10 images of different bronchi captured on each H&E-stained slide (40x) using ImageJ software. Epithelial damage was assessed semi-quantitatively through images of 10 different bronchi captured at 40x magnification on each H&E-stained slide. Three examiners independently assessed the damage and applied the following staging system: intact epithelium (zero); absent cilia (one); eroded upper cell layer and intact basal cell layer (two); eroded epithelium (three) (Michaudel et al., 2018).

Alveolar macrophages were estimated by counting in 10 random and non-coincident fields of lung sections using a 100x objective. Two investigators performed morphometry by counting coded sections. Similarly, the areas of these cells were measured in μm^2 using ImageJ software. The population of neutrophils was estimated based on myeloperoxidase (MPO) activity, measured using hydrogen peroxide, cetyltrimethylammonium bromide (HTAB), and tetramethylbenzidine (TMB) with a wavelength of 630 nm (Suzuki et al., 1983).

2.4.3 Cytokine Measurement

TNF-α and IL-1β levels were measured in homogenized lung by ELISA. The assay followed the manufacturer's instructions (Peprotech, Rocky Hill, New Jersey, USA). The values were expressed in pg/mg of protein.

2.4.4 Oxidative Stress

Analysis Superoxide dismutase (SOD) activity was evaluated by monitoring the inhibition of adrenaline auto-oxidation at a wavelength of 480 nm (Bannister; Calabrese, 1987). Catalase (CAT) activity was determined by monitoring the rate of hydrogen peroxide concentration decrease at a wavelength of 240 nm (Aebi, 1984). ROS levels were assessed by the nitroblue tetrazolium blue salt (NBT) reaction at 630 nm (Choi et al., 2006). The concentration of malondialdehyde (MDA) was measured using the thiobarbituric acid-reactive substance (TBARS) assay at 532 nm (Draper; Hadley, 1990).

2.4.5 Immunohistochemistry

To detect the presence and distribution of the nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor in lung epithelium, immunohistochemical reactions were performed. To do so, 5μm sections were deparaffinized and hydrated to perform endogenous peroxidase blocking. Subsequently, they were incubated with the primary polyclonal anti-Nrf2 antibody (1:100; Sigma-Aldrich) overnight, followed by incubation with a secondary antibody (1:100 Sigma-Aldrich). Diaminobenzidine was used as the chromogen, and the nuclei were stained with Delafield's Hematoxylin. Negative controls were performed by substituting the primary antibody with non-immune serum (Da Hora; Valença; Porto, 2005).

2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software (San Diego, CA, USA). All data were expressed as means ± S.E.M. (standard error of the mean). Analysis of variance (one-way ANOVA) was used, followed by Pearson multiple comparison method when appropriate. Results considered statistically significant had a null hypothesis probability of less than 5% ($p < 0.05$).

3 RESULTS

3.1 Morphology and Morphometry

The histological evaluation of the ALI group showed a slight but significant alteration in lung architecture, with some septal ruptures (arrows), resulting in an increase in mean alveolar diameter (Lm) when compared to the control group ($p < 0.05$). The groups that received ECsy exhibited reduced Lm compared to the ALI group (Figure 2A). The ALI group

displayed leukocyte infiltration (arrowheads) in alveoli and parenchyma. The ALI + 100 and ALI + Dexa groups showed a similar pattern in pulmonary parenchyma analysis, with reduced leukocyte infiltration compared to ALI (arrowheads). These analyses reflect the results of the pulmonary inflammation score, which was higher in the ALI group compared to the control group ($p < 0.05$), while the ALI + 100 ($p < 0.01$) and ALI + Dexa ($p < 0.001$) groups showed a reduction in the score compared to the ALI group, which was not observed in the ALI + 10 (Figure 2B). The mean values are described in Table 1.

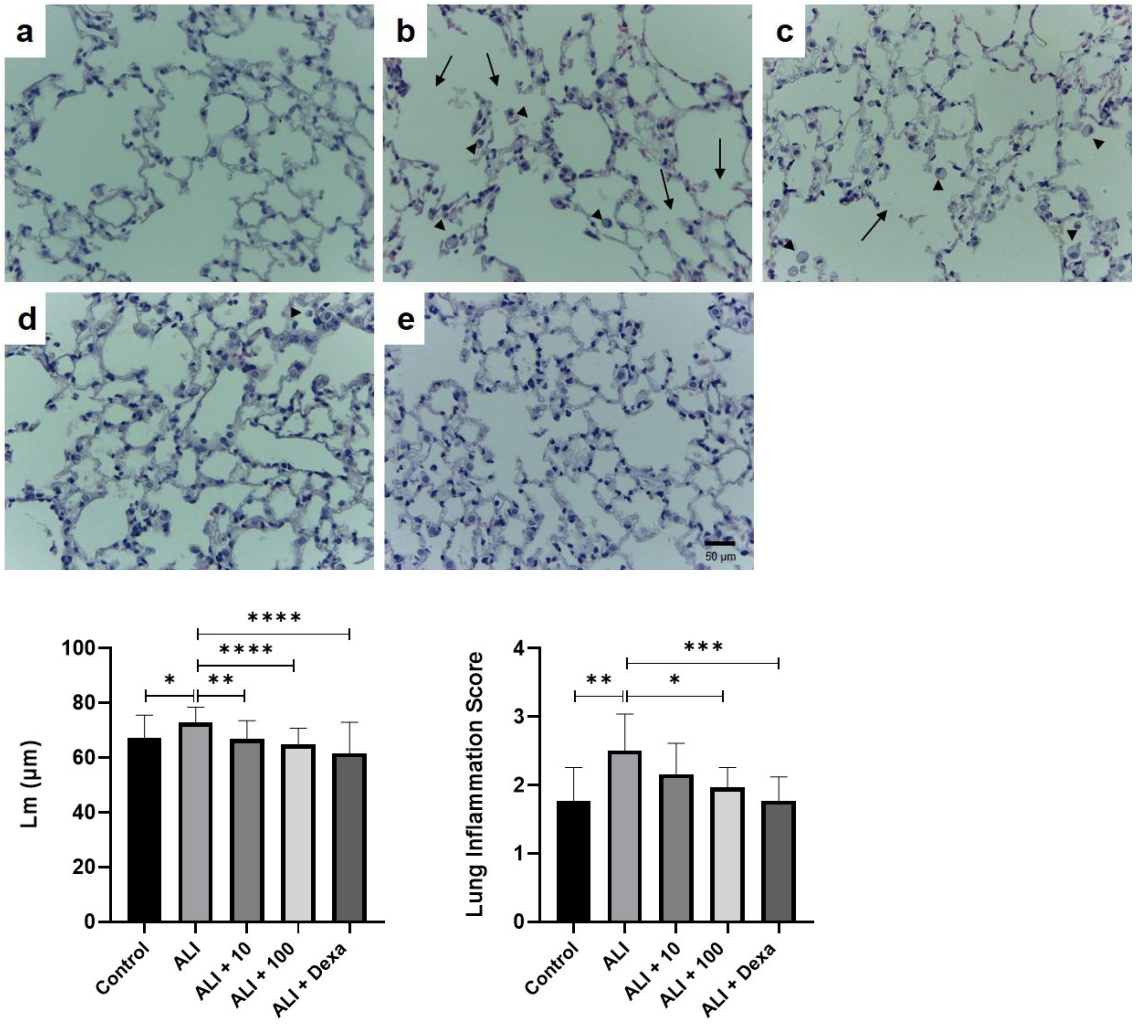

Figure 2. Photomicrographs of morphological analysis of C57BL/6 mouse lung parenchyma. Arrows represent septal ruptures, and arrowheads represent alveolar leukocytes. Hematoxylin and Eosin, 40X objective, Section 5 µm, Scale bar 50 µm. (A) Mean alveolar diameter (Lm); (B) Lung Inflammation Score (LIS). Groups: (a) Control; (b) ALI; (c) ALI + 10; (d) ALI + 100; (e) ALI + Dexa. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Values for all measurements are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

The bronchoconstriction index (BCI) was higher in the ALI group compared to the control group ($p < 0.0001$). The ALI+10 and ALI+100 groups exhibited reduced BCI compared to the ALI group ($p < 0.0001$) (Figure 3A). The height of the bronchial epithelium in the ALI group showed a significant reduction compared to the control group ($p < 0.0001$). In contrast, the ALI + 100 ($p < 0.01$) and ALI + Dexa ($p < 0.05$) groups demonstrated recovery in epithelial height compared to the ALI group (Figure 3B). The assessment also revealed less epithelial damage in the ALI + 100 ($p < 0.05$) compared to the ALI group (Figure 3C). The mean values are described in Table 1.

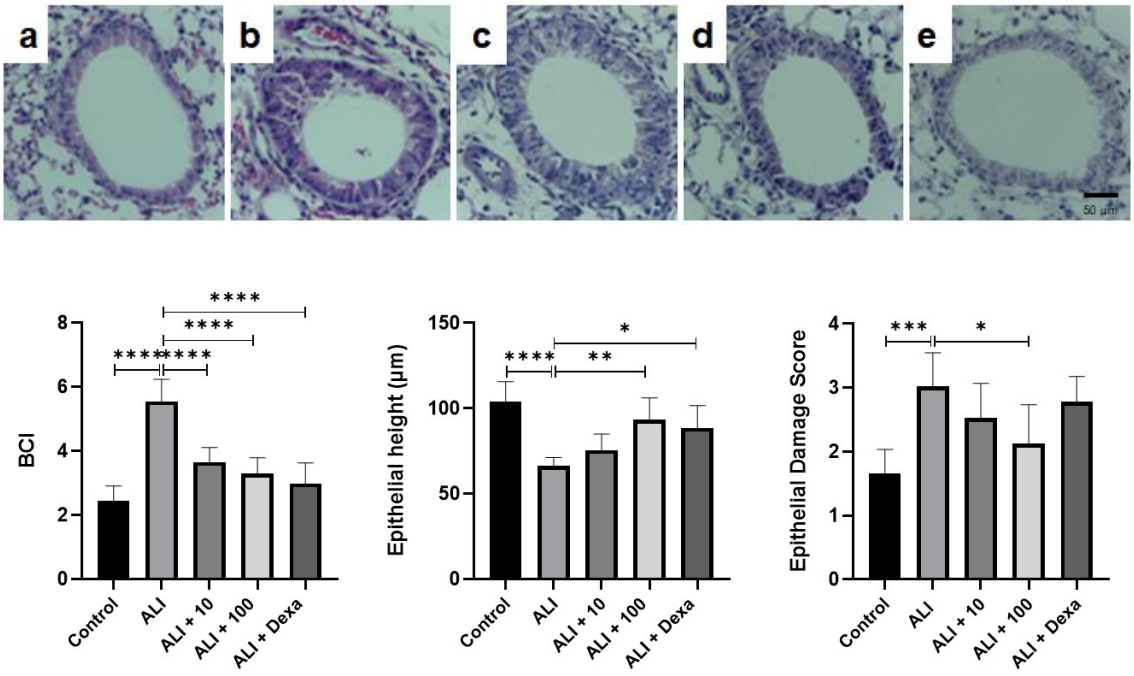

Figure 3. Photomicrographs of morphological analysis of C57BL/6 mouse bronchi. Hematoxylin and Eosin, 40X objective, Section 5 µm, Scale bar 50 µm. (A) Bronchoconstriction Index (BCI); (B) Epithelial Height; (C) Epithelial Damage Score (EDS). Groups: (a) Control; (b) ALI; (c) ALI + 10; (d) ALI + 100; (e) ALI + Dexa. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Values for all measurements are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

Table 1. Morphology and morphometry

Groups	LIS	Lm (μm)	BCI	Epithelial height (μm)	EDS
Control	1,77 ± 0,14	67,2 ± 1,31	2,43 ± 0,18	103 ± 4,46	1,65 ± 0,14
ALI	2,51 ± 0,16	72,8 ± 0,88	5,54 ± 0,26	66,1 ± 2,07	3,0 ± 0,2
ALI + 10	2,16 ± 0,13^{ns}	66,9 ± 0,93	3,65 ± 0,18	75,4 ± 4,26^{ns}	2,52 ± 0,22^{ns}
ALI + 100	1,96 ± 0,08	64,7 ± 0,85	3,29 ± 0,2	93,2 ± 4,83	2,1 ± 0,23
ALI + Dexa	1,77 ± 0,09	61,6 ± 1,8	2,98 ± 0,26	88,4 ± 5,87	2,78 ± 0,16^{ns}

ns: non-significant results

3.2. Inflammatory Markers

Cigarette smoke induced variation in leukocyte infiltrates in the bronchoalveolar lavage fluid (BALF) in all exposed groups. In the ALI group, cigarette smoke led to an increase in leukocyte infiltrates compared to the control group ($p < 0.0001$). In contrast, the ALI + 10, ALI + 100, and ALI + Dexa groups exhibited a reduction in infiltrates compared to the ALI group ($p < 0.0001$) (Figure 4A). The concentration of protein in BALF significantly increased in the ALI group compared to the control group ($p < 0.0001$). Similarly, it was also observed that the ALI + 10, ALI + 100, and ALI + Dexa groups had reduced protein levels in BALF compared to the ALI group (Figure 4B). The population of neutrophils was estimated based on myeloperoxidase (MPO) activity. MPO showed higher enzymatic activity in the ALI group compared to the control group ($p < 0.01$). The ALI + 100 ($p < 0.0001$) and ALI + Dexa ($p < 0.05$) groups exhibited lower MPO enzymatic activity when compared to the ALI group (Figure 4C). As for the number of macrophages, the ALI group showed a significant increase in the number of these cells compared to the control group ($p < 0.05$). In contrast, the ALI + 100 and ALI + Dexa groups showed a reduction in the number of macrophages compared to the ALI group ($p < 0.05$) (Figure 4D). The mean values are described in Table 2.

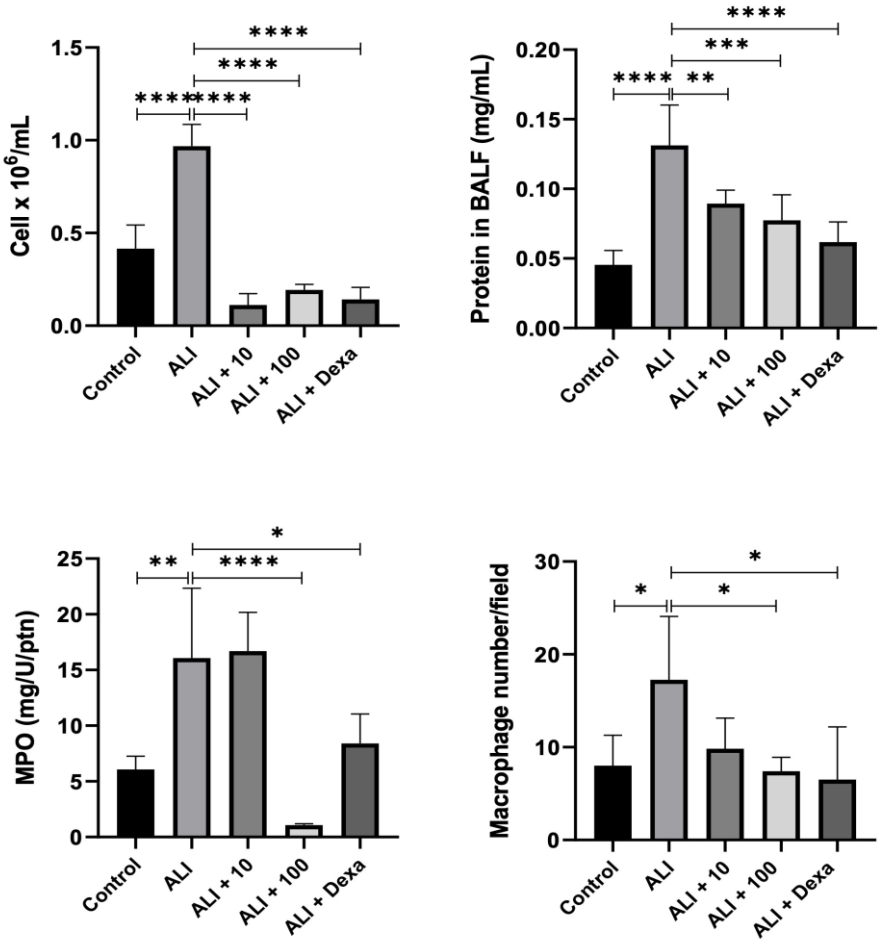

Figure 4. Inflammatory markers. (A) Leukocyte count (10^6/mL); (B) Protein concentration in BALF; (C) Myeloperoxidase activity (MPO); (D) Macrophage count. Groups: (a) Control; (b) ALI; (c) ALI + 10; (d) ALI + 100; (e) ALI + Dexa. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Values for all measurements are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

Table 2. Inflammatory markers

Groups	Cells (10^6 /mL)	Protein (mg/mL)	Macrophage number/field	MPO (mg/U/ptn)
Control	0,41 ± 0,04	0,04 ± 0,004	8,00 ± 1,34	6,08 ±0,59
ALI	0,96 ± 0,04	0,12 ± 0,014	17,2 ± 3,42	16,0 ± 2,56
ALI + 10	0,11 ± 0,02	0,08 ± 0,007	9,8 ± 1,35^{ns}	16,7 ± 1,55^{ns}
ALI + 100	0,19 ± 0,01	0,07 ± 0,01	7,40 ± 0,68	1,06 ± 0,07
ALI + Dexa	0,14 ± 0,02	0,06 ± 0,006	6,50 ± 2,84	8,41 ±1,07

ns: non-significant results

3.3 Macrophage Activity

To investigate the effect of ECsy on macrophage activity in this model, macrophages were also evaluated for their dimensions. It was observed that the area of macrophages increased in the ALI group compared to the control group ($p < 0.0001$). This area was reduced in the ALI+10, ALI+100, and ALI + Dexa groups when compared to the ALI group (Figure 5A).

The levels of tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1β) were increased in the group exposed to cigarette smoke only (ALI) when compared to the control group ($p < 0.01$) (Figures 5B and C). Reduced levels of TNFα were detected in the ALI + 100 ($p < 0.01$) and ALI + Dexa ($p < 0.01$) groups compared to the ALI group (Figure 5B). IL-1β levels were reduced in the ALI + 10 ($p < 0.05$), ALI + 100 ($p < 0.05$), and ALI + Dexa ($p < 0.05$) groups compared to the ALI group (Figure 5C). The mean values are described in Table 3.

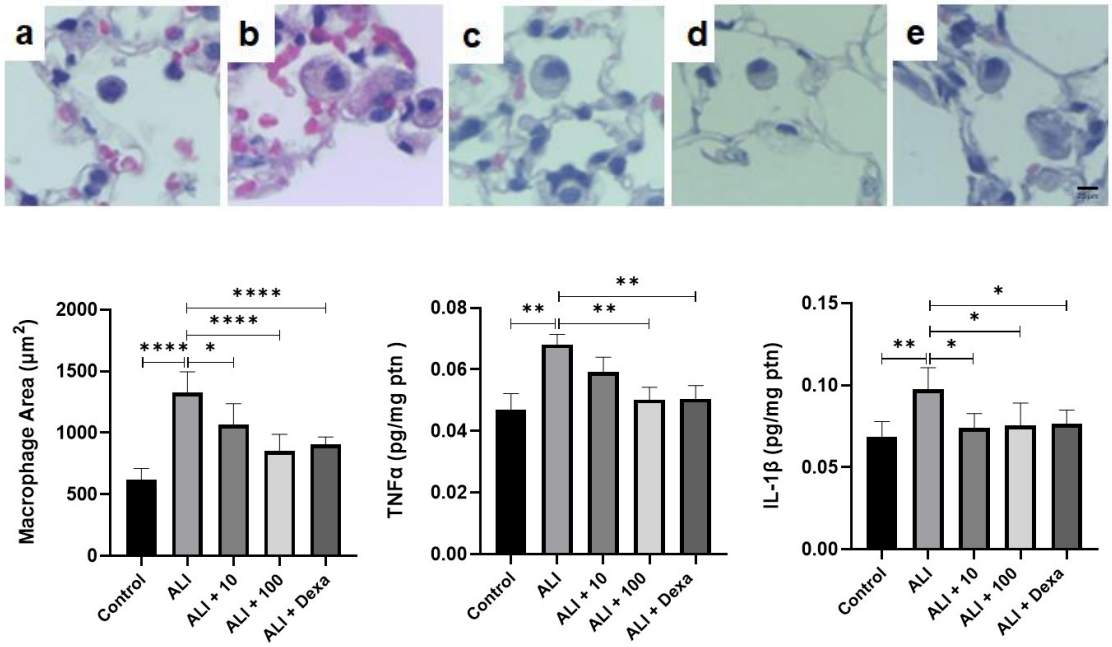

Figure 5. Photomicrographs of morphometric analysis of C57BL/6 mouse macrophages. Hematoxylin and Eosin, 100X objective, Section 5 µm, Scale bar 25 µm. (A) Macrophage area; (B) Tumor Necrosis Factor (TNF-α); (C) Interleukin-1 (IL-1β). Groups: (a) Control; (b) ALI; (c) ALI + 10; (d) ALI + 100; (e) ALI + Dexa. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Values for all measurements are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

Table 3. Inflammatory markers – macrophage activity			
Groups	Macrophage área (μm^2)	TNF-α	IL-1β
Control	619,9 ± 34,5	0,047 ± 0,003	0,068 ± 0,003
ALI	1325 ± 69,45	0,068 ± 0,002	0,097 ± 0,006
ALI + 10	1064 ± 65,1	$0,059 \pm 0,002^{ns}$	0,074 ± 0,004
ALI + 100	850 ± 51,6	0,050 ± 0,002	0,075 ± 0,006
ALI + Dexa	966 ± 24,85	0,050 ± 0,002	0,076 ± 0,004

ns: non-significant results

3.4 Oxidative Stress

When evaluating ROS levels, it was found that the highest mean values were observed in the ALI group compared to the control group ($p < 0.0001$). It was also noted that the ALI + 10, ALI + 100, and ALI + Dexa groups had reduced ROS levels compared to the ALI group ($p < 0.0001$) (Figure 6A). The activities of SOD and catalase increased in the ALI group compared to the control group ($p < 0.001$ and $p < 0.0001$, respectively). The ALI + 100 ($p < 0.05$) and ALI + Dexa ($p < 0.01$) groups exhibited reduced SOD activity compared to the ALI group (Figure 6B), and these groups also showed reduced catalase activity, with the ALI + 100 ($p < 0.05$) and ALI + Dexa ($p < 0.0001$) groups compared to the ALI group (Figure 6C). MDA levels were higher in the ALI group compared to the control group ($p < 0.0001$). On the other hand, the ALI + 10 ($p < 0.05$), ALI+ 100 ($p < 0.01$), and ALI + dexa ($p < 0.001$) groups exhibited reduced MDA levels compared to the ALI group (Figure 6D).

The transcription factor Nrf2 was more expressed specifically in the bronchial epithelium region. The ALI group showed lower Nrf2 expression compared to the control group ($p < 0.0001$). The ALI + 100 ($p < 0.0001$) and ALI + Dexa ($p < 0.05$) groups exhibited higher expression of the factor compared to the ALI group (Figure 7). The mean values are described in Table 4.

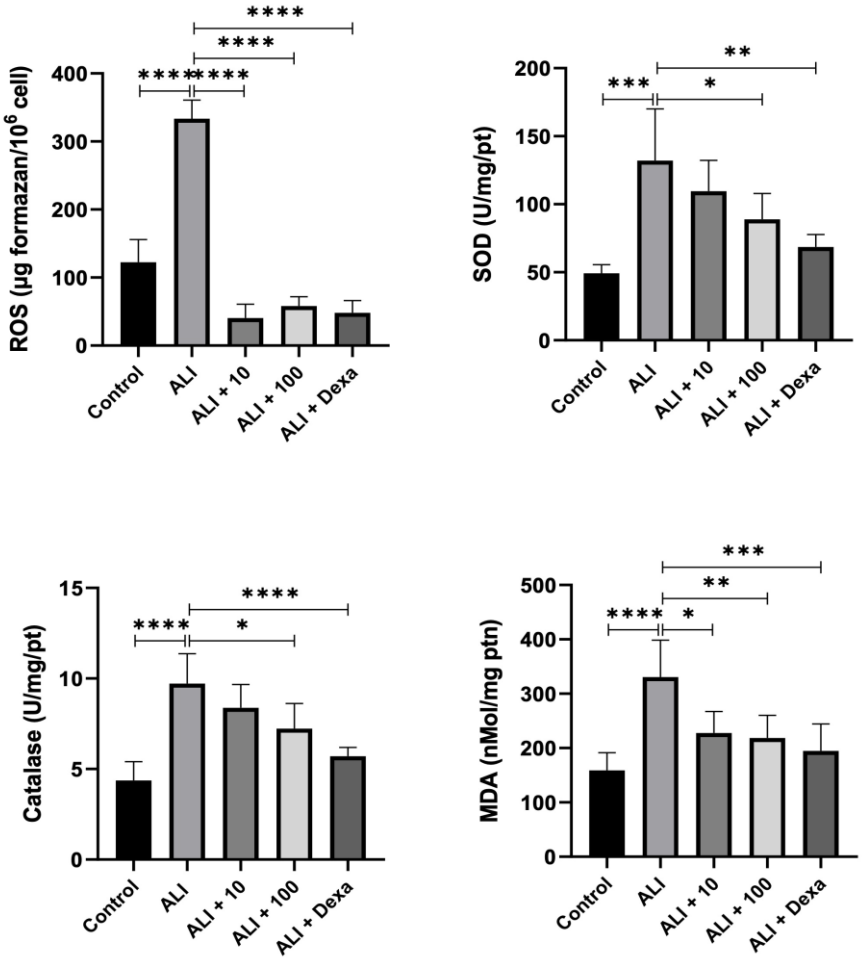

Figure 6. Oxidative stress. (A) Reactive Oxygen Species (ROS) levels; (B) Superoxide Dismutase (SOD) activity; (C) Catalase (CAT) activity; (D) Malondialdehyde (MDA) equivalent concentration. Groups: (a) Control; (b) ALI; (c) ALI + 10; (d) ALI + 100; (e) ALI + Dexa. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Values for all measurements are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

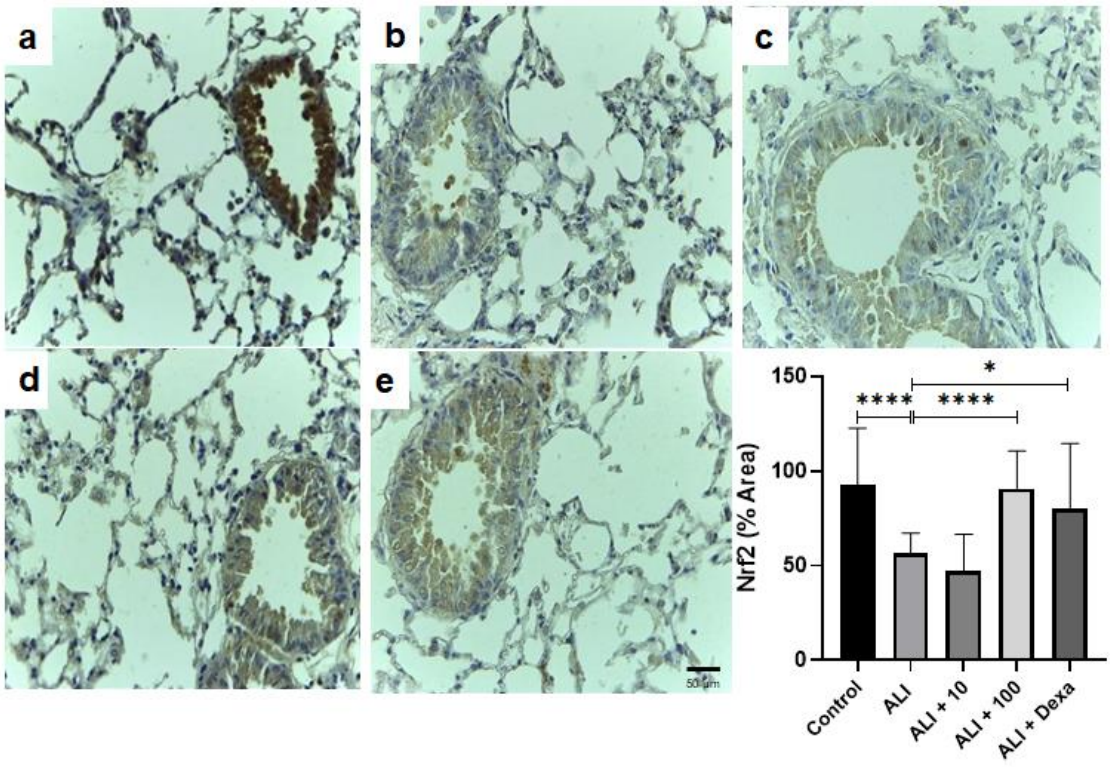

Figure 7. Photomicrographs of immunohistochemical analysis of C57BL/6 mice. Objective 40X, Sections 5 µm, Scale bar 50 µm. Nrf2: Groups: (a) Control; (b) ALI; (c) ALI + 10; (d) ALI + 100; (e) ALI + Dexa. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Values for all measurements are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

Table 4. Oxidative stress

Groups	ROS	SOD	CAT	MDA	Nrf2
Control	122,3 ± 16,76	49,3 ± 3,13	4,37 ± 0,39	158,9 ± 14,6	92,6 ± 5,9
ALI	333,5 ± 13,67	132,1 ± 14,3	9,71 ± 0,625	330,7 ± 30,3	56,5 ± 2,52
ALI + 10	40,6 ± 8,99	109,6 ± 10,1^{ns}	8,38 ± 0,48^{ns}	227,8 ± 16,0	47,3 ± 3,9^{ns}
ALI + 100	58,07 ± 5,18	88,8 ± 8,56	7,61 ± 0,42	218,5 ± 15,7	90,7 ± 3,69
ALI + Dexa	48,1 ± 7,39	68,59 ± 4,09	5,7 ± 0,185	194,8 ± 18,7	79,7 ± 7,6

ns: non-significant results

4 DISCUSSION

Immunopharmacological studies with *C. sympodialis* extract (ECsy) have demonstrated immunomodulatory and anti-inflammatory action in lung diseases such as asthma and bronchitis (Bezerra-Santos et al., 2012; Vieira et al., 2013). These activities can be attributed to the phenolic and alkaloid compounds present in the extract, as these compounds exhibit anti-allergic, anti-inflammatory, and antioxidant properties (Adamski et al., 2020; Cavalcanti et al., 2014; Vieira et al., 2018; Zhang et al., 2022). In our research, we have shown for the first time the anti-inflammatory and antioxidant action of ECsy in cigarette smoke-induced acute lung injury.

Inhalation of cigarette smoke can provoke inflammatory changes in the lungs, ranging from leukocyte recruitment to histopathological alterations, such as alveolar septal ruptures or thickening, areas of inflammatory infiltrate, alveolar hemorrhage, and airway epithelial changes (Da Hora; Valença; Porto, 2005; Valença; Porto, 2008). Moreover, damage to the respiratory epithelium induced by cigarette smoke compromises the integrity of the alveolar-capillary barrier, reducing the height of this epithelium and exposing the body to harmful agents that increase airway responsiveness, resulting in bronchoconstriction (Bastos et al., 2011; Matuschak; Lechner, 2010; Ware, 2006).

In our model, we believe that ECsy treatment directly impacted pulmonary morphological parameters such as Lm and inflammation score, resulting in less damage to the bronchial epithelium. This, in part, explains the reduced bronchoconstriction observed through the assessment of BCI. We found that this is due to the preservation of the alveolar-capillary barrier provided by ECsy treatment. These effects on lung morphology are supported by the results obtained in another study using an ALI model in which animals were treated with milonine, an alkaloid from ECsy, which showed less disruption in lung architecture and less inflammatory infiltrate (Bernardo et al., 2022). The effects on bronchoconstriction are supported by the fact that ECsy reduces airway contractions in an asthma model through increased cAMP, which, in turn, reduces intracellular Ca^{2+}, a factor involved in the muscle contraction mechanism (Cavalcanti et al., 2013; Lima et al., 2014; Thomas; Barbosa Filho; Agra, 1997).

In the presence of cigarette smoke, defense cells, particularly macrophages and neutrophils, are recruited and activated, increasing their presence in the BALF and releasing chemotactic and oxidative agents, thus contributing to tissue oxidative stress (Laskin; Malaviya; Laskin, 2019; Murray; Wynn, 2011). This was also observed in our model.

However, ECsy treatment modified the cellular profile in the BALF, reducing the total number of leukocytes, proteins, macrophages, and lowering MPO activity as a neutrophil marker (Vieira et al., 2013).

The process of lung inflammation is accompanied by ROS production, resulting in oxidative stress (Barnes, 2020; Laskin; Malaviya; Laskin, 2019). This state of oxidative stress is responsible for a significant portion of DNA, membrane, and protein damage (Avery, 2011; Neofytou et al., 2012). Balancing this process are the enzymes SOD and CAT, which are part of the cellular and tissue defense antioxidant system. SOD converts superoxide anions into hydrogen peroxide and water, while CAT converts hydrogen peroxide into water and oxygen (Aebi, 1984; Bannister; Calabrese, 1987). In this study, ECsy treatment was effective in reducing this state of oxidative stress, demonstrating its antioxidant capacity. This is likely due to the decrease in ROS levels, resulting in lower superoxide anion and hydrogen peroxide levels, and consequently, reduced SOD and CAT activities, leading to lower lipid peroxidation and a decrease in MDA concentrations, a cytotoxic product of this peroxidation (Cavalcante; Bruin, 2009). Although there are no descriptions in the literature about ECsy's activity on ROS, SOD (Amresh; Rao; Singh, 2007) and CAT (Surendran et al., 2011), studies using extracts from *Cissampelos pareira*, a plant from the same genus, showed a reduction in these oxidative stress biomarkers in ALI models.

Nrf2 regulates multiple antioxidant genes involved in the synthesis of catalase (CAT) and superoxide dismutase (SOD), and its expression is reduced in lung disease (Mizumura et al., 2020; Suzuki et al., 2008), supporting our results. There was a positive regulation of the transcription factor with ECsy treatment, which was also observed by Yu et al., (2018) in a study with a natural flavonoid.

Dewhurst et al., (2017) attributed the increase in alveolar macrophage size to cigarette smoke. The enlargement of these cells is associated with their activation state, and when activated, macrophages produce granulocyte-macrophage colony-stimulating factor (GM-CSF), which initiates the inflammatory process by stimulating the production of TNF-α and IL-1β (Vlahos et al., 2010). Increased TNF-α production is associated with macrophage activation since these cells are the major producers and responders to this factor (Parameswaran; Patial, 2010). In our model, cigarette smoke-induced injury showed an increase in macrophage size as well as increased TNF-α release, corroborating previous studies. The reduction in macrophage activity is related to lower TNF-α (Yu et al., 2018) and IL-1β (Di Carlo; Häcker; Gentle, 2022) release, which results from the neutralization of

granulocyte-macrophage colony-stimulating factor (GM-CSF). This mechanism may explain the reduction in macrophage size and activation through ECsy treatment. Similar results were obtained by Bernardo et al., (2022) in another LPS-induced ALI model, where milonine treatment reduced pro-inflammatory cytokines such as TNF-α and IL-1β.

In a pro-inflammatory state with oxidative stress and increased ROS, a reduction in Nrf2 expression via GM-CSF may occur (Barnes, 2020). Likewise, an increase in Nrf2 expression can help control inflammation by reducing ROS. This is what we observed with ECsy treatment, as there was an increase in the expression of this factor and a reduction in oxidative stress parameters.

Therefore, the function of macrophages in cigarette smoke-induced ALI is influenced by Nrf2 activation, as observed in our model. Thus, the reduced macrophage activity marked by smaller cell size is associated with increased Nrf2 expression. This is supported by the fact that Nrf2 expression is reduced in lung macrophages and bronchiolar epithelium of smokers and patients with COPD (Suzuki et al., 2008), as seen in our study.

5 CONCLUSION

Hence, the study observed that ECsy has antioxidant and anti-inflammatory properties, as it mitigates cigarette smoke-induced ALI by modulating macrophage activity, especially through Nrf2 activation. Therefore, we suggest that ECsy is a compound with pharmacological activity for the treatment of ALI and has the potential to be used in the therapy of patients with a clinical history associated with this type of lung injury.

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CAPÍTULO III

Revista Brasileira de Farmacognosia

(Bazilian Journal of Pharmacognosy)

Manuscript Number: RBFA-D-23-01067

Cissampelos sympodialis Extract Attenuates Pulmonary Emphysema Induced by Chronic Cigarette Smoke Exposure

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ABSTRACT

Introduction: Continuous inhalation of cigarette smoke can induce lung injury and lead to the development of emphysema. The *C. sympodialis* extract (ECsy) has been used in respiratory disease treatment due to its anti-inflammatory and antioxidant effects. Here, we demonstrate that ECsy reduces inflammation and oxidative stress in mice using a cigarette smoke-induced pulmonary emphysema model. **Methods:** CEUA (16/2020). Male C57BL/6 mice were divided into six groups: Control, CS, CS + 10, CS + 100, CS + NAC, and CS + Dexa. CS groups were exposed to 12 cigarettes/day for 60 days and subsequently treated with saline (CS), ECsy (10 or 100 mg/mL), NAC (100 mg/mL via nebulization, 15 min/day), or dexamethasone (0.4 mg/mL i.p) for an additional 60 days. The control group was exposed to ambient air and treated with saline. After 120 days, lungs were collected for morphological evaluation, redox profiles, and inflammation. When p-value <0.05, the difference was considered statistically significant. **Results:** Lm and peribronchiolar collagen deposition were reduced in all groups compared to CS. The CS group also showed reduced food consumption and weight gain compared to the other groups. An increase in ROS and superoxide dismutase activity was evident in the CS group compared to the other groups, except CS + 10. Catalase activity and MDA levels were reduced in CS + 10, CS + 100, CS + NAC, and CS + Dexa compared to CS. TNF-α and IL-1β levels were reduced in CS + 100 and CS + Dexa groups when compared to the CS group. **Conclusion:** The results demonstrate the anti-emphysematous potential of ECsy in chronic lung injury induced by cigarette smoke.

Keywords: Antioxidant. Anti-inflammatory. Lung injury. Weight gain.

1 INTRODUCTION

Lung diseases pose a significant challenge to global health. Chronic obstructive pulmonary disease (COPD) is a serious public health issue that affects respiratory function and quality of life, primarily impacting morbidity and mortality (Momtazmanesh et al., 2023; Wisnivesky; De-Torres, 2019). Mainly caused by smoking, its pathophysiology is characterized by decreased lung airflow due to the narrowing of small airways, not fully reversible with bronchodilator use. This is followed by the destruction and remodeling of lung parenchyma as a consequence of chronic inflammatory response. Clinically, these changes manifest through symptoms such as dyspnea, chronic cough, mucus production, and wheezing (Mirza et al., 2018).

Among the numerous therapeutic approaches available for COPD, there is a growing focus on alternative treatments such as medicinal plants, with *Cissampelos sympodialis* emerging as a potential natural solution (De Fátima Agra; De Freitas; Barbosa-Filho, 2007). Traditional uses of this plant include the treatment of inflammatory and allergic diseases (Batista-Lima et al., 2001; Bezerra-Santos et al., 2006; De Fátima Agra; De Freitas; Barbosa-Filho, 2007; Vieira et al., 2018). Initial research suggests that the plant extract possesses anti-inflammatory, bronchodilator, and antioxidant properties (Cavalcanti et al., 2013; De Sales et al., 2018; Silva et al., 2017), which could be beneficial in treating various health conditions like asthma and allergies (Bernardo et al., 2022; Vieira et al., 2018).

The extract is rich in antioxidant compounds (Cavalcanti et al., 2014), helping combat damage caused by free radicals, reducing oxidative stress in lung cells, and modulating damage progression while preventing the perpetuation of the inflammatory process (Avery, 2011; Barnes, 2020; Neofytou et al., 2012), as observed in diseases like asthma and COPD.

COPD is a result of continuous activation of inflammatory pathways, and due to its high prevalence, it is pertinent to explore therapeutic alternatives. Investigating the *Cissampelos sympodialis* extract biological activities in cigarette smoke-induced chronic lung inflammation is relevant given its pharmacological potential as an adjunct agent for treating smoking-related respiratory diseases such as pulmonary emphysema.

2 METHODS

2.1 Plant Material and Extract Preparation

Cissampelos sympodialis Eichl leaves were collected from the Botanical Garden of the Pharmaceutical Technology Laboratory at the Federal University of Paraiba (sample Agra 1456). After drying in an oven at 50°C, the leaves were pulverized using a knife mill. A solution of ethanol and water (80/20 v/v) was used for powder extraction at 25 to 30°C for 5 days. The dry extract was obtained after removing the solvent using a rotary evaporator at 60°C (Piuvezam et al., 1999).

2.2 Animals

Male C57BL/6 mice (18–22 g) from CEMIB/UNICAMP were fed with a commercial diet (Presence) and had free access to water in a controlled environment (22 to 24°C and 12/12 h light/dark cycle). The research was approved by the Ethics Committee on Animal Use (CEUA/UFERSA) (Opinion No. 16/2020).

2.3 Experimental Design

The animals were divided into six experimental groups (n=7 each) (Figure 1). The groups (CS, CS + 10, CS + 100, CS + NAC, and CS + Dexa) were exposed to an air-smoke mixture from 12 commercial cigarettes (10 mg tar, 0.9 mg nicotine, and 10 mg carbon monoxide – Marlboro red) per day, for 60 days, through an inhalation chamber to assess the effect of *Cissampelos sympodialis* on pulmonary emphysema. The control group was exposed to ambient air (Kennedy-Feitosa et al., 2019).

After establishing pulmonary emphysema, the animals were treated with the *Cissampelos sympodialis* extract (ECsy) via inhalation at doses of 10 and 100mg/mL, dexamethasone (0.4 mg/mL i.p), NAC (100 mg/mL v.i), or vehicle, according to each group, for an additional 60 days. ECsy, NAC, or vehicle were administered via nebulization (15 minutes/day, 1x day) in a chamber attached to a nebulizer (RespiraMax) (Bastos et al., 2011). Dexamethasone was administered intraperitoneally (i.p.). The persistence of emphysema was assessed by evaluating two parallel groups for morphological and stereological aspects: CS60d (euthanized on day 61, without undergoing any form of treatment) and CS120d (exposed to cigarette smoke for 60 days and euthanized on day 121, without undergoing any form of treatment).

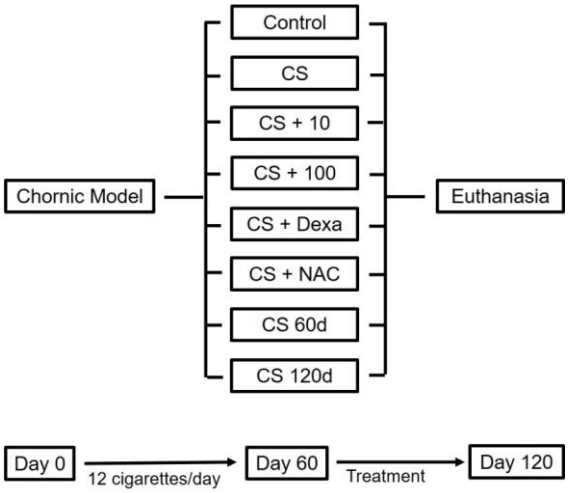

Figure 1. Experimental Model of Pulmonary Emphysema Induction and Treatment with C. sympodialis Extract: Control, Chronic Lung Injury (CS), Chronic Lung Injury + extract 10 mg/mL (CS + 10), Chronic Lung Injury + extract 100 mg/mL (CS + 100), Chronic Lung Injury + N-acetylcysteine 100 mg/mL (CS + NAC), and Chronic Lung Injury + Dexamethasone 0.4 mg/mL (CS + Dexa). Chronic Lung Injury + euthanized on day 61 (CS60d) and Chronic Lung Injury + euthanized on day 121 (CS120d).

2.4 Experimental Protocols

2.4.1 Tissue Processing and Histological Analysis

Twenty-four hours after the completion of the one hundred and twenty days of the experimental procedure, animals were anesthetized by inhalation with sevoflurane and euthanized by cervical dislocation. Lungs were perfused with saline to remove blood. The right lung of each animal was homogenized for biochemical analyses and cytokine measurement by ELISA, while the left lung was transferred to a 4% paraformaldehyde fixative solution (pH 7.4; 4°C) for histological processing. 5 µm sections were stained using Hematoxylin and Eosin (H&E) or Masson's Trichrome.

2.4.2 Morphological and Morphometric Analysis

The mean alveolar diameter (Lm) was calculated using the equation Lm = Ltot/Li from 10 photomicrographs of random and non-coincident fields free of bronchi and vessels, captured with a light microscope (Leica MD750) with a 40x objective. Ltot corresponds to the total length of lines in the microscopic field and Li is the number of intersections between the alveolar structures with the lines of the lines of the microscope grid. The result was expressed in micrometers (Valença; Porto, 2008; Weibel, 1963).

Peribronchiolar collagen was quantified by ImageJ software version 1.46m (NIH, Bethesda, MD) on Masson's Trichrome stained slides. Using 40x objective lenses, thirty fields were randomly selected per slide (60 fields per lung) and displayed on a color monitor. The image analysis program was set to detect areas of blue-stained collagen within each field. The fraction of the blue-stained area for each field was expressed as a percentage of the total tissue area (excluding air spaces) (Ayala et al., 2018).

The body mass and food consumption of the animals were measured daily to obtain a weekly average. One hundred grams of commercial feed were placed in each box daily, and the individual daily consumption of animals was calculated by averaging the consumption (excluding left over feed) divided by the number of animals per box (Yang et al., 2014).

2.4.3 Oxidative Stress Analysis

Reactive oxygen species (ROS) levels were assessed by the nitroblue tetrazolium salt (NBT) reaction at 630 nm (Choi et al., 2006). Superoxide dismutase (SOD) activity was evaluated by monitoring the inhibition of adrenaline auto-oxidation at a 480 nm wavelength (Bannister; Calabrese, 1987). Catalase (CAT) activity was measured by monitoring the decrease rate in hydrogen peroxide concentrations at a 240 nm wavelength (Aebi, 1984). Malondialdehyde (MDA) concentration was quantified by the thiobarbituric acid reactive species (TBARS) assay at 532 nm (Draper; Hadley, 1990).

2.4.4 Cytokine Measurement

TNF-α and IL-1β levels were determined in lung homogenates by the ELISA method, following the manufacturer's instructions (Peprotech, Rocky Hill, New Jersey, USA). Values were expressed in pg/mg of protein.

2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software (San Diego, CA, USA). All data were expressed as means ± S.E.M. (standard error of the mean). One-way analysis of variance (ANOVA) was applied followed by Pearson method for multiple comparisons, when appropriate. Results considered statistically significant had a null hypothesis probability of less than 5% ($p < 0.05$).

3 RESULTS

3.1 Morphology and Morphometry

Histological evaluation of the CS group showed significant alteration in lung architecture, with septal ruptures (arrows), resulting in an increase in mean alveolar diameter (Lm) compared to the control group ($p < 0.0001$). Groups treated with ECsy, NAC, and Dexa showed reduced Lm compared to the CS group ($p < 0.0001$) (Figure 2A). The CS 60d and CS 120d groups did not show differences in mean alveolar diameters compared to the CS group, indicating the persistence of emphysema for a group not subjected to any treatment. Table 1 describes the average values.

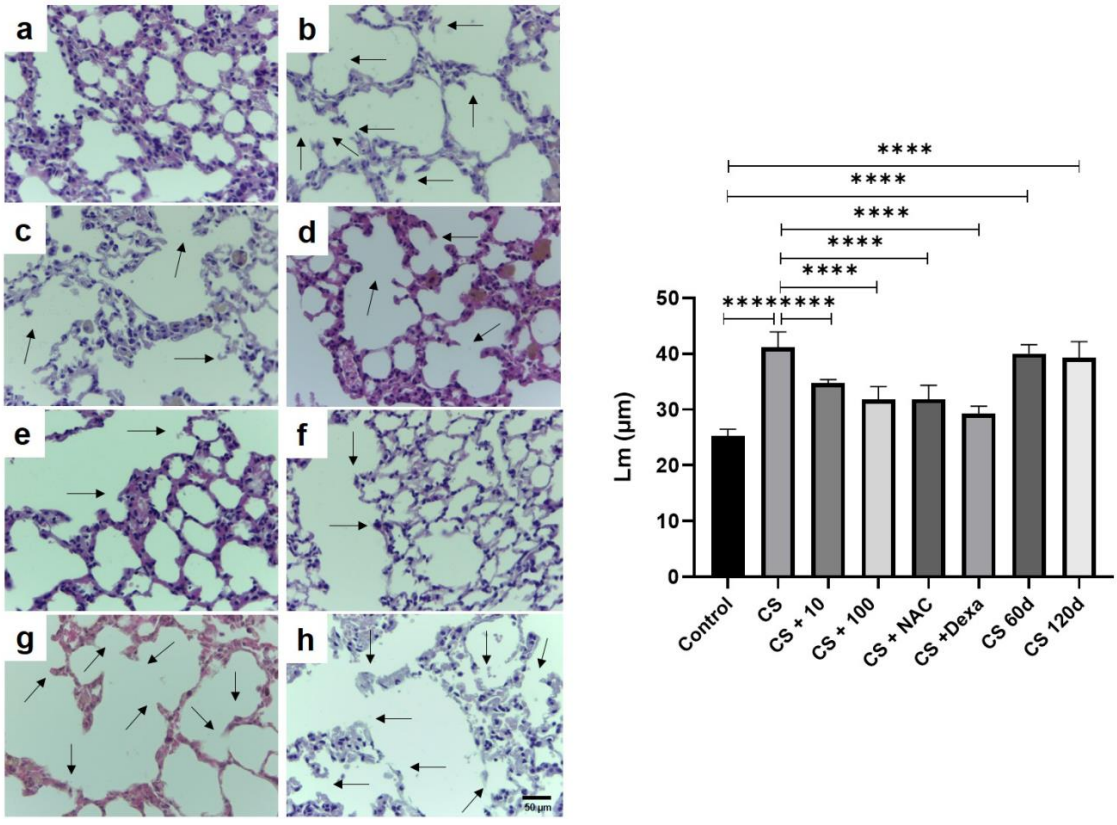

Figure 2. Photomicrographs of Morphological Analysis of C57BL/6 Mice Lung Parenchyma and Mean Alveolar Diameter (Lm). Arrows represent septal ruptures. Hematoxylin and Eosin, 40X Objective, 5 µm Section, Scale Bar 50 µm. Groups: (a) Control; (b) CS; (c) CS + 10; (d) CS + 100; (e) CS + NAC; (f) CS + Dexa; (g) CS 60d; (h) CS 120d. **** $p < 0.0001$. Values for all measurements are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

The CS group exhibited higher collagen deposition in the peribronchiolar region compared to the control group ($p < 0.001$) (Figure 3b and 3a, respectively). Groups CS + 10 ($p < 0.01$), CS + 100 ($p < 0.05$), CS + NAC ($p < 0.0001$), and CS + Dexa ($p < 0.001$) demonstrated a similar pattern in the collagen distribution analysis, with lower deposition around the bronchi (Figure 3c, 3d, 3e, and 3f) compared to the CS group. Table 1 describes the average values.

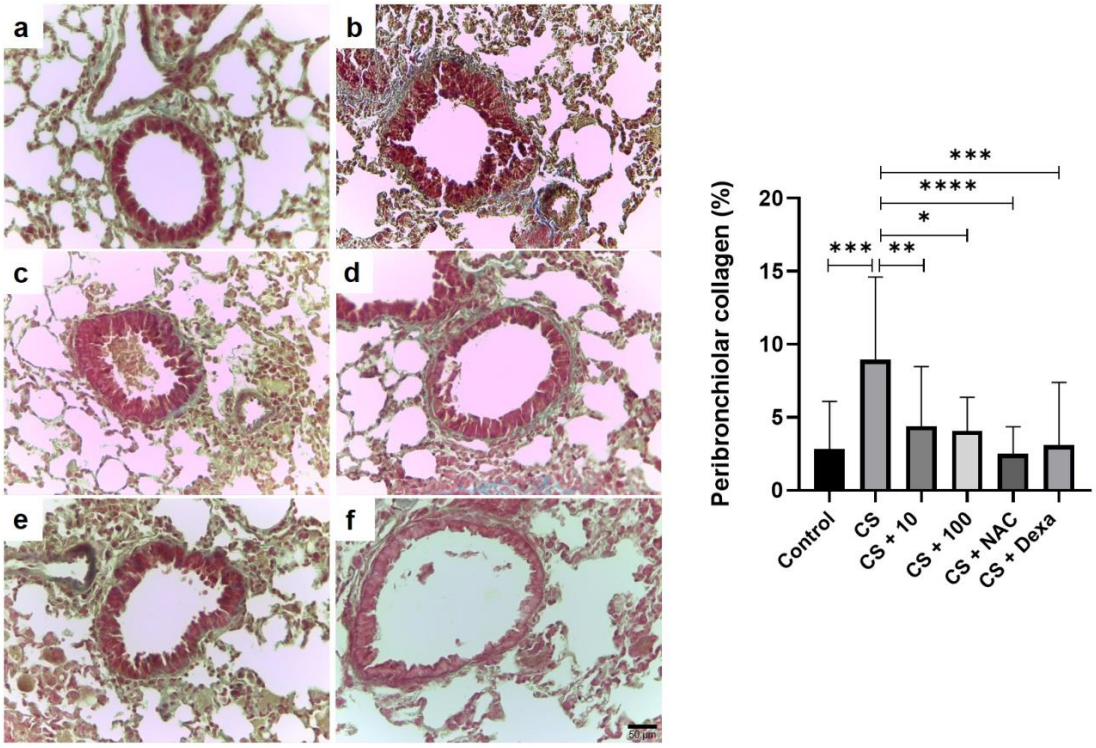

Figure 3. Photomicrographs of Peribronchiolar Fibrosis Analysis in C57BL/6 Mice and Peribronchiolar Collagen (%). Masson's Trichrome, 40X Objective, 5 µm Sections, Scale Bar 50 µm. Groups: (a) Control; (b) CS; (c) CS + 10; (d) CS + 100; (e) CS + NAC; (f) CS + Dexa. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and **$p < 0.0001$. Values for all measurements are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

Table 1. Morphology and morphometry of the mouse lung C57BL/6		
Groups	Lm (μm)	Peribronchiolar collagen (%)
Control	25,29 ± 0,49	2,84 ± 0,76
CS	41,1 ± 1,06	8,95 ± 1,2
CS + 10	34,7 ± 0,26	4,4 ± 0,9
CS + 100	31,8 ± 1,05	4,06 ± 0,8
CS + NAC	31,8 ± 1,1	2,5 ± 0,45
CS + Dexa	29,2 ± 0,54	3,09 ± 1,1
CS 60d	40,0 ± 0,61	-
CS 120d	39,28 ± 1,1	-

Body mass and food consumption were analyzed to obtain a weekly average. In the first 60 days, the CS group showed a significant reduction in food consumption ($p < 0.05$) concerning the experiment time, not noticed in the control group evaluation (Figure 4A). During the treatment period (day 61 to 120), a significant increase in food consumption was observed in all groups concerning the experiment time, except for the control group (Figure 4B). Body mass also varied over the experiment time. Both control ($p < 0.0001$) and CS ($p < 0.01$) groups showed significant mass gain concerning the experiment time (Figures 4C and D). There was also a significantly higher weight gain when comparing the control group with the CS group ($p < 0.05$) during the initial 60 days of exposure (Figure 4C). During treatment, a significant body mass increase was observed in all groups, except the CS + Dexa group, similar to what occurred in food consumption. Average values are shown in Table 2.

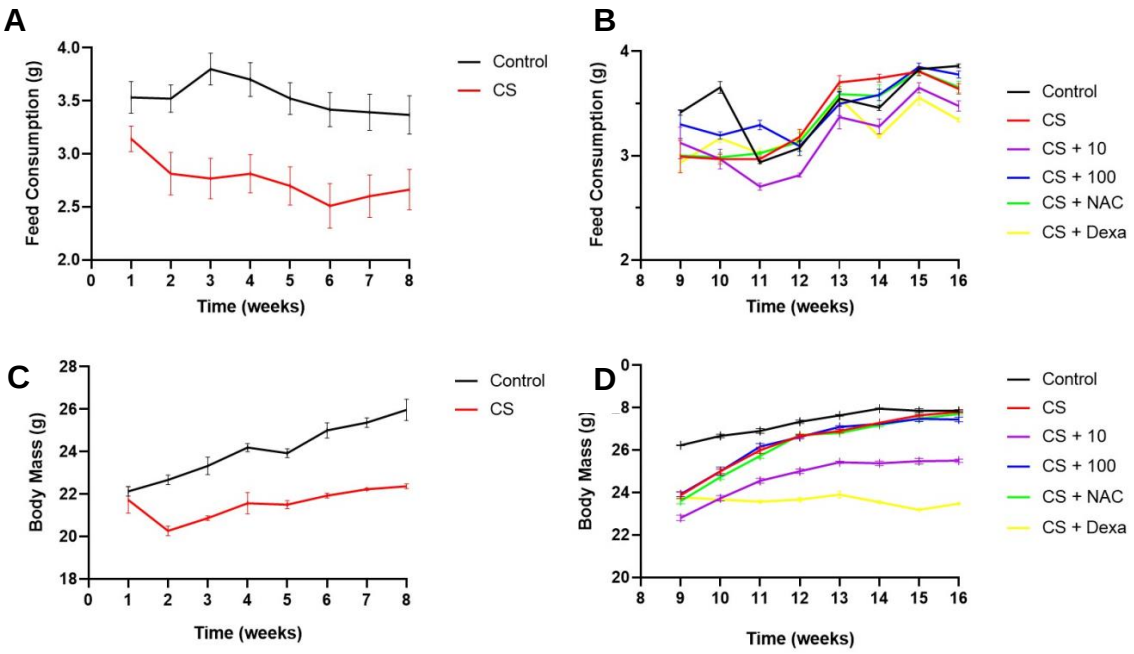

Figure 4. Food Consumption and Body Mass for Experimental Animal Groups. (A) Average weekly food consumption up to 60 days; (B) Average weekly food consumption from day 61 to 120; (C) Average weekly body mass up to 60 days; (D) Average weekly body mass from day 61 to 120. Groups: (a) Control; (b) CS; (c) CS + 10; (d) CS + 100; (e) CS + NAC; (f) CS + Dexa. ***$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and **$p < 0.0001$. Values for all measurements are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

Table 2. Morphometry – weight gain of mice C57BL/6

Groups	Feed Consumption (g)	Body Mass (g)
(Average 1 to 60 day)		
Control	3,53± 0,15	24,0 ± 0,29
CS	2,75 ± 0,18	21,5 ± 0,24
(Average 61 to 120 day)		
Control	3,47 ± 0,10	27,3 ± 0,16
CS	3,37 ± 0,16	26,4 ± 0,22
CS + 10	3,17 ± 0,19	24,7 ± 0,28
CS + 100	3,44 ± 0,16	26,3 ± 0,29
CS + NAC	3,34 ± 0,14	26,2 ± 0,22
CS + Dexa	3,24 ± 0,14	23,6 ± 0,17

3.2. Oxidative Stress

ROS levels were elevated in the CS group compared to the control group ($p < 0.0001$). ROS levels were decreased in the CS + 100 ($p < 0.01$), CS + NAC ($p < 0.05$), and CS + Dexa ($p < 0.0001$) groups compared to the CS group (Figure 5A). SOD and catalase enzymatic activity increased in the CS group compared to the control group ($p < 0.0001$). SOD activity decreased in the CS + 100 ($p < 0.001$), CS + NAC ($p < 0.01$), and CS + Dexa ($p < 0.001$) groups compared to the CS group (Figure 5B). Similarly, catalase activity decreased in the CS + 10, CS + 100, CS + NAC, and CS + Dexa groups ($p < 0.0001$) compared to the CS group (Figure 5C). MDA levels increased in the CS group compared to the control group ($p < 0.0001$). On the other hand, these levels decreased in the CS + 10 ($p < 0.001$), CS + 100, CS + NAC, and CS + Dexa ($p < 0.001$) groups compared to the CS group ($p < 0.0001$) (Figure 5D). Average values are detailed in Table 3.

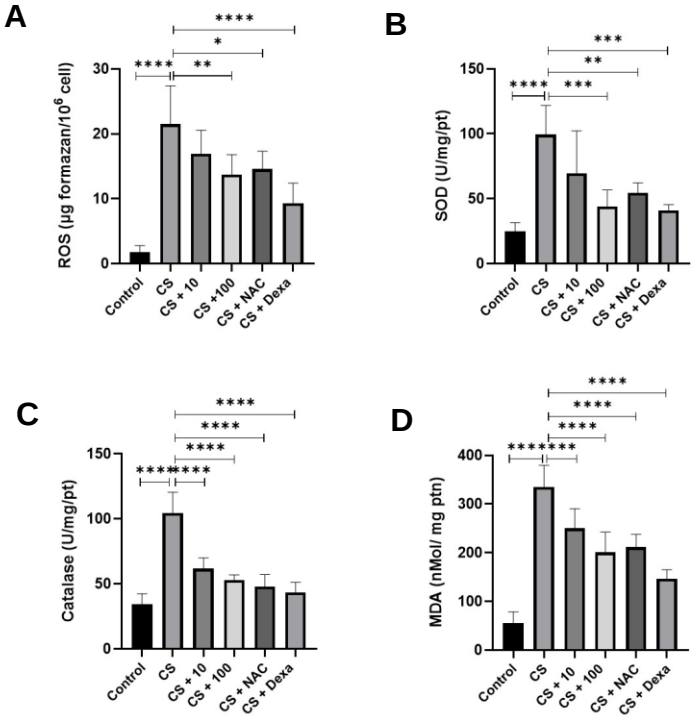

Figure 5. Oxidative Stress. (A) Reactive oxygen species (ROS) levels; (B) Superoxide dismutase (SOD) activity; (C) Catalase (CAT) activity; (D) Malondialdehyde (MDA) equivalent concentration. Groups: Control; CS; CS + 10; CS + 100; CS + NAC; CS + Dexa. ***$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and **$p < 0.0001$. Values for all measurements are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

Table 3. Oxidative stress				
Groups	ROS	SOD	CAT	MDA
Control	1,8 ± 0,37	24,96 ± 2,71	34,0 ± 3,1	55,37 ± 8,77
CS	21,48 ± 2,64	99,5 ± 9,95	104,4 ± 6,0	334 ± 18,3
CS + 10	16,9 ± 1,5^{ns}	69,56 ± 14,6^{ns}	61,6 ± 3,15	249,8 ± 15,4
CS + 100	13,7 ± 1,26	44,0 ± 5,7	52,9 ± 1,46	200,6 ± 15,7
CS + NAC	14,56 ± 1,22	54,57 ± 3,44	47,9 ± 3,46	211,6 ± 9,8
CS + Dexa	9,33 ± 1,17	41,0 ± 1,95	43,3 ± 2,9	146,2 ± 7,2

ns: non-significant results

3.3. Inflammatory Markers

When evaluating the levels of tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1β), the CS group showed the highest mean values compared to the control group ($p < 0.05$) (Figure 6). Decreased levels of TNF-α were found in the CS + 100 ($p < 0.05$) and CS + Dexa ($p < 0.05$) groups compared to the CS group (Figure 6A). IL-1β levels were decreased in the CS + 100 ($p < 0.05$) and CS + Dexa ($p < 0.05$) groups compared to the CS group (Figure 6B). Average values are described in Table 4.

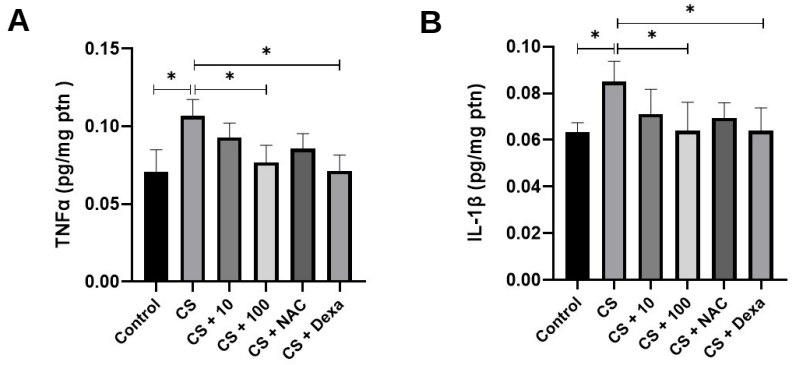

Figure 6. Inflammatory Markers. (A) Tumor necrosis factor (TNF-α); (B) Interleukin-1 (IL-1β). Groups: Control; CS; CS + 10; CS + 100; CS + NAC; CS + Dexa. *$p < 0.05$. Values for all measurements are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

Table 4. Inflammatory activity		
Groups	TNF-α	IL-1β
Control	0,07 ± 0,008	0,06 ± 0,002
CS	0,10 ± 0,006	0,08 ± 0,004
CS + 10	0,09 ± 0,004^{ns}	0,07 ± 0,005^{ns}
CS + 100	0,07 ± 0,006	0,06 ± 0,005
CS + NAC	0,08 ± 0,005^{ns}	0,07 ± 0,003^{ns}
CS + Dexa	0,07 ± 0,006	0,06 ± 0,004

ns: non-significant results

4 DISCUSSION

Chronic exposure to cigarette smoke induces continuous oxidative stress and indiscriminate activation of inflammatory pathways, involving respiratory epithelial cells and recruitment of defense cells, notably neutrophils and macrophages. These cells, through cytokines and proteolytic enzymes, cause histopathological changes such as alveolar septal breaks, alterations in respiratory epithelium, and increased collagen production by fibroblasts, resulting in septal thickening and peribronchiolar deposition (Lin; Thomas, 2010). This condition can lead to the destruction of lung parenchyma, airflow limitation, and tissue remodeling, ultimately resulting in chronic obstructive pulmonary disease (COPD) (Da Hora; Valença; Porto, 2005; Vestbo et al., 2013).

The aggression resulting from inhaling this smoke is followed by reactive oxygen species (ROS) production, responsible for oxidative damage (Barnes, 2020; Laskin; Malaviya; Laskin, 2019), degrading cell membranes, extracellular matrix proteins and DNA (Avery, 2011; Neofytou et al., 2012). In response, enzymes such as superoxide dismutase (SOD) and catalase (CAT) act as part of the antioxidant system, converting reactive oxygen species into non-toxic products (Aebi, 1984; Draper; Hadley, 1990). Our study confirmed the antioxidant action of *Cissampelos sympodialis* extract (ECsy), as it effectively reduced oxidative stress by decreasing ROS levels and subsequently lowering SOD and CAT activities, resulting in reduced malondialdehyde (MDA) concentrations— a cytotoxic product of lipid peroxidation (Cavalcante; Bruin, 2009). While there is limited literature on ECsy and its effects on ROS, SOD, and CAT, studies on *Cissampelos pareira* extract, belonging to the same genus as *C. sympodialis*, have shown a decrease in these oxidation markers (Amresh; Rao; Singh, 2007; Surendran et al., 2011) in acute lung injury models, suggesting a similar action for ECsy.

Research states that oxidative stress leads to an increase in defense cells in BALF (Bronchoalveolar Lavage Fluid) of COPD patients (Da Hora; Valença; Porto, 2005; Valença; Porto, 2008). Macrophages produce granulocyte-macrophage colony-stimulating factor (GM-CSF), which activates the NF-κB transcription factor pathway, furthering the inflammatory process by stimulating the production of TNF-α (Parameswaran; Patial, 2010; Vlahos et al., 2010) and IL-1β (Di Carlo; Häcker; Gentle, 2022; Wang et al., 2016), the production of matrix metalloproteinases (MMPs) (Grzela et al., 2016; Trojanek et al., 2014), and the production of TGF-β (Gorowiec et al., 2012; Shin et al., 2017; Wang et al., 2016). In this study, we observed that cigarette smoke caused increased release of TNF-α and IL-1β, in agreement with studies by Valença and Porto (2008) and Di Carlo et al (2022), respectively. These findings lead us to suggest that the reduction in inflammatory activity was caused by ECsy treatment as a result of the lower release of TNF-α (Yu et al., 2018) and IL-1β (Di Carlo; Häcker; Gentle, 2022), resulting from the reduced activation of the NF-κB neutralization factor (Wang et al., 2016) by the modulation of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Vlahos et al., 2010). This is also seen in a study using a milone, an alkaloid isolated from *C. sympodialis*, which reduced pro-inflammatory cytokines such as TNF-α and IL-1β in an acute lung injury model induced by LPS (Bernardo et al., 2022) and in a COPD model using a natural flavonoid (Yu et al., 2018).

TNF-α is also an important factor causing anorexia. When elevated, it causes appetite loss and, consequently, weight loss (Vlahos et al., 2010). This fact is corroborated in this study, as the period of smoke inhalation resulted in a decrease in food consumption and a lack of weight gain (Chen et al., 2005; De Carlos et al., 2014). After the start of treatment, as well as the cessation of smoke inhalation, both food consumption and body weight increased. This fact can be justified by the reduction of TNF-α in the groups treated with ECsy, demonstrating the association between inflammation and body weight.

In our model, chronic inhalation of cigarette smoke directly influenced morphological parameters for the establishment of pulmonary emphysema, such as mean alveolar diameter (Lm) and peribronchiolar collagen deposition, similar to the work of Zou et al., (2014) ECsy treatment was able to reduce Lm, collagen fiber density, and lung remodeling. We believe this is due to the response obtained by ECsy treatment, which provided containment of the chronic inflammatory process, reducing the activity of these proteolytic enzymes responsible for parenchymal and collagen and elastic fiber destruction (Da Hora; Valença; Porto, 2005; Valença; Porto, 2008) and reducing lung remodeling by fibroblasts through the reduction of

TGF-β (Gorowiec et al., 2012; Shin et al., 2017; Wang et al., 2016). These effects on lung morphology and collagen deposition are supported by results obtained by Bezerra-Santos et al., (2012) when evaluating ECsy in remodeling in an asthma model and de De Medeiros et al., (2020), who used ECsy in a model of diabetic rats.

5 CONCLUSION

ECsy has antioxidant and anti-inflammatory properties that significantly mitigate emphysema development and pulmonary remodeling. This conclusion is drawn from reduced lung tissue damage, resulting from lower release of pro-inflammatory cytokines (TNF-α and IL-1β) and decreased oxidation markers in ECsy-treated groups.

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CAPÍTULO IV

1 CONSIDERAÇÕES FINAIS

Pesquisas sobre doenças inflamatórias respiratórias, mais especificamente sobre a Doença Pulmonar Obstrutiva Crônica, e o desenvolvimento de novas terapias são de grande relevância dada a prevalência dessa doença na população, impacto nos índices de morbimortalidade e custos de saúde.

As evidências de que o extrato da *Cissampelos sympodialis* atua na DPOC, baseiam-se nos dois tratamentos experimentais desenvolvidos neste estudo, a partir de análises bioquímicas e da análise histológica dos pulmões de camundongos, em que o extrato da *Cissampelos sympodialis* demonstrou potencial anti-inflamatório e antioxidante, sendo capaz de atenuar a lesão pulmonar provocada pelo tabagismo e a progressão do enfisema pulmonar.

Observados os resultados obtidos neste estudo, inferimos que o extrato da *C. sympodialis* representa uma alternativa terapêutica e um recurso promissor no combate aos efeitos deletérios provocados pela DPOC.

Estudos que busquem isolar nesse extrato os compostos fitoquímicos com essas atividades farmacológicas e ainda visando definir os mecanismos de ação destes compostos são importantes para a confecção de uma futura droga derivada da *Cissampelos sympodialis* a fim de direcionar a um sítio de ação específico, aumentando assim a eficácia e diminuindo os possíveis efeitos colaterais ainda não avaliados.