UNIVERSIDADE FEDERAL DE VIÇOSA

Oxinflammation affects transdifferentiation to myofibroblasts, and *Trichilia silvatica* extract controls the oxinflammatory response: A preclinical analysis

Leonardo Lopes Silveira
Doctor Scientiae

VIÇOSA - MINAS GERAIS
2025

LEONARDO LOPES SILVEIRA

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Thesis submitted to the Cell and Structural Biology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Reggiani Vilela Goncalves

Co-advisers: Mariáurea M. S. Souza
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Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

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S587o
2025

Silveira, Leonardo Lopes, 1977-
Oxinflammation affects transdifferentiation to myofibroblasts, and *Trichilia silvatica* extract controls the oxinflammatory response: a preclinical analysis / Leonardo Lopes Silveira. – Viçosa, MG, 2025.
1 tese eletrônica (117 f.): il. (algumas color.).

Texto em inglês.
Orientador: Reggiani Vilela Gonçalves.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Biologia Animal, 2025.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2025.432
Modo de acesso: World Wide Web.

1. Histologia. 2. Cicatrização de ferimentos. 3. Estresse oxidativo. I. Gonçalves, Reggiani Vilela, 1979-. II. Universidade Federal de Viçosa. Departamento de Biologia Animal. Programa de Pós-Graduação em Biologia Celular e Estrutural. III. Título.

CDD 22. ed. 571.5

Bibliotecário(a) responsável: Euzébio Luiz Pinto CRB-6/3317

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APPROVED: April 28, 2025.

Assent:

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A minha filha Ana Júlia, presente de DEUS em minha vida. Ao meu amor, Pâmella, ao meu saudoso pai e a toda a nossa família.

ACKNOWLEDGMENTS

This work has been sponsored by the following Brazilian research agencies: Coordination for the Improvement of Higher Education Personnel (CAPES; Financing code 001), Minas Gerais State Foundation for Research Aid (FAPEMIG) and National Council of Scientific and Technological Development (CNPq).
Agradeço a Deus em primeiro lugar, pela força e por me ajudar a realizar esse sonho e por sempre estar presente ao meu lado, nas pequenas coisas, em cada benção enviada a mim. Sem ELE e suas bênçãos diárias nada seria possível. À Universidade Federal de Viçosa, essa renomada Instituição, que sempre fez parte dosmeus sonhos e onde fui recebido com muito carinho e atenção, juntamente com o programa de Pós-graduação em Biologia Celular e Estrutural pela oportunidade de cursar o doutorado e pela infraestrutura fornecida para a execução deste trabalho. O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES). À minha orientadora Reggiani Vilela Gonçalves, por quem sempre terei respeito e admiração, pela oportunidade, pela sabedoria, respeito, conselhos, didática no ensino e orientação. Sem o seu vasto conhecimento eu não conseguiria concluir esse estudo. Às minhas coorientadoras Mariáurea Matias Sarandy Souza e Manoela Maciel dos Santos Dias, pela sabedoria, conhecimentos, orientação e por me ajudarem sempre que precisei. À professora Vera Almeida, da Fundação Ezequiel Dias, por fornecer o extrato analisado e por enriquecer esse estudo com os ricos dados enviados e por ceder seu vasto conhecimento para que essa pesquisa fosse concluída. Aos professores Rômulo Dias Novaes, Giuseppe Valacchi, Mônica Moraes, Leandro Licursi de Oliveira, por todo o auxílio durante o desenvolvimento da pesquisa. Aos amigos do laboratório que contribuíram diretamente com esse estudo, em especial a Néia de Paula, Silvânia Pelinsari e Manoela Maciel dos Santos. À minha amiga Patrícia Matosinhos, que não mediu esforços para me ajudar nesse estudo, sempre com educação, respeito, dedicação e competência. Aos meus Pais, Consuelo e Edite, pelas orações, apoio, por entender meus sonhos e pelo exemplo de luta. Aos meus queridos irmãos Ricardo e Consuelo, fonte de apoio, amizade e inspiração. Minhas cunhadas Paola, Denise, Tatiana e a Bete pelo carinho e amizade. Meus sobrinhos Thales, Larissa, Maria Eduarda, Carolina, Sarah e Esther, vocês fazem tudo ficar mais leve e me enchem de orgulho. À minha

querida esposa Pâmella, pela paciência, companheirismo e amor e também por entender o quanto esse sonho era importante para nós. Minha querida filha Ana Júlia, por sua alegria, amor e carinho. E por dar valor a dedicação do papai à pesquisa e ao ensino. Seu sorriso é meu alimento diário. Aos todos os colegas do grupo de pesquisa do Laboratório de Patologia Experimental (LAPEX), pelo apoio durante o desenvolvimento das atividades laboratoriais. Ao amigo Adriano Simões, um agradecimento especial pela amizade, desprendimento, ajuda e apoio. Sua contribuição foi fundamental durante o desenvolvimento do trabalho. Aos amigos que conheci durante o doutorado. Em especial à Fernanda Barbosa, Rafael Neto, Eduarda Costa e Maria Lúcia de Almeida. Obrigado a todos. Aos pacientes e familiares, por entenderem o quanto esse estudo era importante e assim permitir que eu levasse esse trabalho em frente. Por fim, expresso minha gratidão a todos que contribuíram, de forma direta ou indireta, e não foram mencionados. Serei eternamente grato.

“Demonstre gratidão a todos os que colaboram com suas vitórias”
(Carlos Hilsdorf)

ABSTRACT

SILVEIRA, Leonardo Lopes, D.Sc., Universidade Federal de Viçosa, April, 2025. **Oxinflammation affects transdifferentiation to myofibroblasts, and *Trichilia silvatica* extract controls the oxinflammatory response: A preclinical analysis**. Adviser: Reggiani Vilela Goncalves. Co-advisers: Mariáurea Matias Sarandy Souza and Manoela Maciel dos Santos Dias.

Controlling oxidative stress and inflammation (OxInflammation) is a major challenge during the tissue repair process, this may be due to a lack of understanding of the main mechanisms involved in this process or the absence of therapies that promote effective control of key molecules in tissue recovery. Added to this is the presence of chronic diseases that can further impair tissue repair, including diabetes mellitus. Thus, it is increasingly necessary to search for treatments that can control the OxInflammation process. Therefore, understanding these mechanisms is essential to develop effective therapeutic approaches for the treatment of various pathologies. In order to fill these gaps in knowledge, we produced three studies. The first aimed to understand the potential for modulation of inflammation and oxidative stress of plants such as Trichilia silvatica C.DC. In the second, the objective was to understand how the OxInflammation process can influence the transdifferentiation of fibroblasts into myofibroblasts and thus compromise the skin repair process. In the third, the aim was to verify the phytochemical characterization of T. silvatica extracts and how exposure to plant extracts of T. silvatica can control the oxinflammation process in macrophage cell cultures and thus control the harmful effects of oxidative stress and inflammation on cells. The systematic review followed the PRISMA guidelines, and searches were performed on Medline (PubMed), Scopus and Web of Science platforms. The included studies were limited to those that used diabetic murine models with excisional wounds. Bias analysis and methodological quality assessments were performed using the SYRCLE tool. The results confirm that oxinflammation generated by diabetes impairs the transformation of fibroblasts into myofibroblasts by affecting the expression of several growth factors, most notably transforming growth factor beta (TGF-ß) e o NOD-like receptor family, pyrin domain containing 3 (NLRP3). OxInflammation in diabetes also compromises pathways such as SMAD, c-Jun N-terminal kinase, protein kinase C and caspase activation pathways, nuclear factor kappa beta, leading to cell death. In the second study (experimental study), it was observed that hydroalcoholic extracts of the leaf and stem of the plant T. silvatica promoted an increase in free radical scavenging (DPPH and FRAP analysis); above 50%, both of the leaf and the

stem. In addition, exposure to the extract of T. silvatica increased cell viability and proliferation, in addition to protecting cells from oxidative stress caused by exposure to H2O2. Concentrations of 100 µg/ml and 250 µg/ml of the extracts increased catalase (CAT) activity, however reduced superoxide dismutase (SOD) activity and nitric oxide concentration. The leaf and stem extracts at concentrations of 100 µg/ml and 250 µg/ml reduced the expression of pro-inflammatory cytokines NFk-ß, TNF-a and COX-2, while the concentration of 250 µg/ml of leaf and 100 µg/ml of stem were able to increase the expression of IL-10 and H1F1 (anti-inflammatory). These results show that the T. silvatica extract revealed the presence of terpenes/steroids, coumarins, condensed tannins and phenolic acids, including chlorogenic and caffeic acids, in addition to having the potential to regulate the inflammatory response and oxidative stress in macrophages. These findings show that this therapy may represent a promising treatment to control the OxInflammation process and thus accelerate the healing process of various diseases.

Keywords: Meliaceae; inflammation; oxidative stress; healing; diabetes mellitus; antioxidant; activity.

RESUMO

SILVEIRA, Leonardo Lopes, D.Sc., Universidade Federal de Viçosa, abril de 2025. **A oxinflamação afeta a transdiferenciação em miofibroblastos, e o extrato de *Trichilia silvatica* controla a resposta oxinflamatória: Uma análise pré-clínica**. Orientadora: Reggiani Vilela Goncalves. Coorientadores: Mariáurea Matias Sarandy Souza e Manoela Maciel dos Santos Dias.

O controle do estresse oxidativo e da inflamação (OxInflammation) é um grande desafio durante o processo de reparo tecidual, seja pela falta de entendimento dos principais mecanismos envolvidos neste processo, ou pela ausência de terapias que promovam o controle eficaz de moléculas chave na recuperação tecidual. Soma-se a isto a presença de doenças crônicas que podem prejudicar ainda mais o reparo tecidual, entre elas podemos destacar o diabetes mellitus. Assim, é cada vez mais necessário a busca por tratamentos que consigam controlar o processo de OxInflamation. Portanto, entender esses mecanismos é fundamental para desenvolver abordagens terapêuticas eficazes para o tratamento de diversas patologias. Com o objetivo de preencher estas lacunas no conhecimento produzimos três estudos. O primeiro teve como objetivo entender o potencial para modulação da inflamação e do estresse oxidativo de plantas como a Trichilia silvatica C.DC. No segundo, o objetivo foi entender como o processo de OxInflammation pode influenciar a transdiferenciação de fibroblastos em miofibroblastos e assim comprometer o processo de reparo cutâneo. No terceiro, o objetivo foi verificar a caracterização fitoquímica dos extratos de T. silvatica e como a exposição a extratos vegetais de T. silvatica pode controlar o processo de OxInflammation em cultura de células de macrófagos e assim controlar os efeitos lesivos do estresse oxidativo e da inflamação sobre as células. A revisão sistemática seguiu as diretrizes PRISMA, e as buscas foram realizadas nas plataformas Medline (PubMed), Scopus e Web of Science. Os estudos incluídos foram limitados àqueles que usaram modelos murinos diabéticos com feridas excisionais. A análise de viés e as avaliações de qualidade metodológica foram realizadas usando a ferramenta SYRCLE. Os resultados confirmam que a OxInflamation gerada pelo diabetes prejudica a transformação de fibroblastos em miofibroblastos ao afetar a expressão de vários fatores de crescimento, mais notavelmente o fator de crescimento transformador beta (TGF-ß) e o NOD-like receptor family, pyrin domain containing 3 (NLRP3). A OxInflammation no diabetes também compromete vias como SMAD, c-Jun N-terminal kinase, proteína kinase C e vias de ativação da caspase, do fator nuclear kappa beta, levando à morte celular. No segundo estudo

(estudo experimental), observou-se que extratos hidroalcoólicos da folha e caule da planta T. silvatica promoveram um aumento no sequestro de radicais livres (análise de DPPH e FRAP); acima de 50%, tanto da folha como do caule. Além disso, a exposição ao extrato de T. silvatica aumentou a viabilidade e proliferação celular, além de proteger as células do estresse oxidativo causado pela exposição ao H 2 O 2 . As concentrações 100 µg/ml e 250 µg/ml dos extratos aumentaram a atividade da catalase (CAT), no entanto reduziram a atividade da superóxido dismutase (SOD) e a concentração de óxido nítrico. Os extratos da folha e caule nas concentrações 100 µg/ml e 250 µg/ml reduziram a expressão das citocinas pró-inflamatórias NFk-ß, TNF-a e COX-2, enquanto a concentração de 250 µg/ml da folha e 100 µg/ml de caule foram capazes de aumentar a expressão de IL-10 e H1F1 (anti-inflamatória). Estes resultados mostram que o extrato de T. silvatica revelou a presença de terpenos/esteroides, cumarinas, taninos condensados e ácidos fenólicos, incluindo os ácidos clorogênico e cafeico, além de ter potencial para regular a resposta inflamatória e o estresse oxidativo em macrófagos. Estes achados mostram que esta terapia pode representar um tratamento promissora para controlar o processo de OxInflammation e assim acelerar o processo de cura de várias doenças.

Palavras-chave: Meliaceae ; inflamação; estresse oxidativo; cicatrização; diabetes mellitus; atividade antioxidante

SUMÁRIO

CHAPTER I

PHYTOCHEMICAL PROFILE OF *TRICHILIA SILVATICA*: POTENTIAL FOR INFLAMMATION AND OXIDATIVE STRESS MODULATION

RESUMO

SILVEIRA, Leonardo Lopes, D.Sc., Universidade Federal de Viçosa, Maio de 2025. **Perfil fitoquímico de Trichilia silvatica: potencial para modulação da inflamação e do estresse oxidativo**. Orientadora: Reggiani Vilela Gonçalves. Coorientadoras: Mariáurea Matia Sarandy de Souza e Manoela Maciel dos Santos Dias.

Plantas nativas dos biomas brasileiros apresentam grande potencial biotecnológico devido à diversidade de compostos bioativos com propriedades terapêuticas. *Trichilia silvatica* é uma espécie amplamente distribuída em biomas tropicais e reconhecida por sua composição química rica em metabólitos secundários, como terpenoides, flavonoides, compostos fenólicos, saponinas e fitosteróis. Esses compostos exibem atividades antioxidantes, anti-inflamatórias e antimicrobianas, além de atuarem na modulação de mediadores inflamatórios e no controle do estresse oxidativo. Estudos indicam que extratos do gênero *Trichilia* demonstram efeitos promissores na redução da inflamação e na proteção celular contra espécies reativas de oxigênio (EROs), regulando a expressão de citocinas pró-inflamatórias e enzimas como a COX-2. Além disso, modelos experimentais sugerem que a planta pode ser eficaz no tratamento de distúrbios inflamatórios, como artrite e doenças associadas ao estresse oxidativo. A busca por alternativas naturais com menor toxicidade em comparação aos fármacos convencionais reforça o interesse científico e biotecnológico nessa espécie. Dessa forma, *Trichilia silvatica* pode apresentar um potencial terapêutico significativo e pode ser explorada para o desenvolvimento de novos produtos voltados à saúde humana, especialmente no tratamento de doenças inflamatórias e oxidativas. Estudos adicionais são necessários para melhor caracterizar seus mecanismos de ação e validar sua aplicação clínica.

Palavras-chave: Processo inflamatório; estresse oxidativo; *Oxinflammation*; espécies reativas de oxigênio.

ABSTRACT

SILVEIRA, Leonardo Lopes, D.Sc., Universidade Federal de Viçosa, May 2025. **Phytochemical profile of *Trichilia silvatica:* Potential for inflammation and Oxidative Stress Modulation.** Supervisor: Reggiani Vilela Gonçalves. Co-advisors: Mariáurea Matia Sarandy de Souza; Manoela Maciel dos Santos Dias.

Native plants from Brazilian biomes exhibit great biotechnological potential due to the diversity of bioactive compounds with therapeutic properties. *Trichilia silvatica* is a species widely distributed in tropical biomes and is recognized for its rich chemical composition of secondary metabolites, such as terpenoids, flavonoids, phenolic compounds, saponins, and phytosterols. These compounds exhibit antioxidant, anti-inflammatory, and antimicrobial activities, in addition to modulating inflammatory mediators and controlling oxidative stress. Studies indicate that from the genus *Trichilia* extracts demonstrated promising effects in reducing inflammation and protecting cells against reactive oxygen species (ROS), by regulating the expression of pro-inflammatory cytokines and enzymes such as COX-2. Furthermore, experimental models suggest that the plant may be effective in treating inflammatory disorders, such as arthritis and diseases associated with oxidative stress. The search for natural alternatives with lower toxicity compared to conventional drugs reinforces scientific and biotechnological interest in this species. Thus, *Trichilia silvatica* can present significant therapeutic potential and may be explored for the development of new health-related products, particularly for the treatment of inflammatory and oxidative stress-related diseases. However, further studies are required to elucidate its mechanisms of action and validate its clinical applications.

Keywords**:** Inflammatory process; oxidative stress; oxinflammation; reactive oxygen species.

1 STUDY ORGANISM

1.1 Meliaceae Family and *Trichilia* Genus

The use of plants with medicinal and therapeutic properties dates back to ancient times and remains a valuable resource in modern medicine, with approximately 70% of the population in developed countries utilizing them for healthcare (APPLEQUIST et al., 2020). The Meliaceae family is known for containing various bioactive compounds particularly tetranortriterpenoids, commonly known as limonoids or meliacins. Additionally, this family is also highly valued for its high quality wood, ease of cultivation, and diverse biological properties (ESTEVÃO, 2013).

The *Trichilia* genus, first described in 1956 by the researcher Browne, comprises approximately 70 species and is widely found in various tropical regions of Brazil and various African biomes. In Brazil, more than 15 species have already been cataloged. Beyond its ecological importance, *Trichilia* holds significant economic and medicinal value due to the presence of a wide variety of secondary metabolites. Its applications in the production of herbal medicines and cosmetics have contributed to a growing demand for these products, raising concerns about the potential depletion of natural resource (DA SILVA et al., 2021).

The increasing exploitation of natural resources in Brazil has driven deforestation in several biomes over the past decades, including those with a high concentration of Trichilia species, threatening their preservation. In this context, the conservation of these and other species found in Brazilian biomes is essential not only for maintaining biodiversity but also for ensuring the sustainable use of their bioactive compounds and promoting further research in this field (BATILLANI, SCREMIM-DIAS & SOUZA, 2005; BASTOS et al., 2018).

1.2 *Trichilia Silvatica* Species

The species *Trichilia silvatica*, commonly known as "catiguá," "catiguá-branco," or "cutia-vermelha" in the state of Santa Catarina, and as "rosa-branca" in Bahia (PENNINGTON, 1981), is a medium-sized tree that typically reaches a height of 5 to 10 meters (Figure 1). Its distribution spans various regions of Brazil, particularly in the Southeast, as well as in the states of Paraná, Santa Catarina, and southern Bahia. However, this species has been classified as vulnerable to extinction on the IUCN (International Union for Conservation of Nature) Red List

of Threatened Species (DE MORAES et al., 2011), highlighting the urgent need for conservation efforts.

Figure 1 - Representative photo of the *Trichilia silvatica* species. (A) and (B) Leaves. Trunk of the *Trichilia silvatica.*
Fonte: https://www.arvoresdobiomacerrado.com.br/site/2021/10/24/trichilia-silvatica-c-dc/.

Trichilia silvatica plays a crucial ecological role in its native biome, as environmental imbalances can impact both its survival and that of other plant species competing for resources or altering the environment in unfavorable ways (BERALDO, 2011). According to COUTRIM and SOUZA (2018), the conservation of native plants not only contributes to biodiversity maintenance but also strengthens sustainable practices, ensuring the well-being of local communities and promoting the development of safe and effective herbal medicines.

1.3 Therapeutic Applications

Trichilia silvatica has significant medicinal potential and has been the focus of numerous scientific studies exploring these properties. A study conducted by FIGUEIREDO (2010), which analyzed therapeutic compounds present in *T. silvatica* collected from different regions of Espírito Santo, identified the following compounds: the sesquiterpene ambrosanol-10,11-diol, the coumarin scopoletin, and the steroids β-sitosterol and stigmasterol. In the same

study, antimicrobial activity was tested, revealing positive effects against *Streptococcus salivarius* and *Streptococcus mutans*, as well as activity against *Aedes aegypti* larvae.

SILVA et al. (2018) observed that the administration of ethanolic extract from *T. silvatica* in rats with carrageenan-induced inflammation led to a significant reduction in edema (swelling) and inflammatory mediator production. In an advanced clinical study investigating the effects of a *T. silvatica*-based supplement in patients with rheumatoid arthritis, a significant reduction in pain, joint swelling, and morning stiffness was reported compared to the placebo group.

Although *T. silvatica* extracts show great therapeutic potential, it is essential to evaluate possible adverse effects and discomfort that the plant might cause. Scientific database studies have not indicated signs of toxicity or adverse effects at different concentrations, including clinical and experimental trials. However, like any bioactive substance, *T. silvatica* may present adverse effects, particularly when administered at high doses or in individuals with increased sensitivity (ALVARENGA & DE MELO, 2023).

2 DIVERSITY OF BIOACTIVE COMPOUNDS

Extracts from the *T. silvatica*¸ as well as other species of the *Trichilia* genus are rich in a diverse range of bioactive compounds, which are responsible for their therapeutic and medicinal properties. Research on these compounds has intensified in recent years, as new extraction techniques and molecular analysis methods have enabled the identification of chemical molecules with high pharmacological potential (PASSOS et al., 2021; SURILIGE et al., 2024; MAROYI, 2024).

Several studies exploring bioactive compounds associated with the *Trichilia* genus have identified, among others, terpenoids (triterpenes, sesquiterpenes, limonoids, and steroids), polyphenols (flavonoids and tannins), as well as saponins, alkaloids, lignans, and other less common compounds. These compounds exhibit a wide range of biological properties, including antimicrobial, anti-inflammatory, and antioxidant activities, which may be useful in the development of herbal medicines and cosmetics. Furthermore, the preservation of these species contributes to the conservation of native ecosystems and the continuity of scientific research (GARIMA, 2011; XU et al., 2013; DA SILVA, 2021).

2.1 Terpenoids

Secondary metabolites in plants are associated with a wide range of adaptive aspects related to their environment. Terpenes, or isoprenoids, are synthesized through two pathways from five-carbon precursor units derived from isoprene (C_5H_8), such as isopentenyl pyrophosphate (IPP) and its functional isomer, dimethylallyl pyrophosphate (DMAPP). Terpene synthases (TPSs) act on one or more of these universal precursors (including DMAPP) to produce a combinatorial diversity of terpenes. One pathway, predominantly in the cytoplasm, is the mevalonate (MVA) pathway, while the other, located in plastids, is the methylerythritol phosphate (MEP) pathway (BONCAN et al., 2020).

These terpenoid compounds play various roles in plant metabolism, including protection against environmental stress, attraction of pollinators, and defense against a wide range of pathogens. Additionally, they have pharmacological relevance due to their antioxidant, anti-inflammatory, antimicrobial, and anticancer properties (FAN et al., 2023).

The classification of this group of compounds is based on the number of isoprene units in their structure, starting with monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), triterpenes ($C_{30}H_{48}$), tetraterpenes ($C_{40}H_{64}$), and polyterpenes, as shown in figure 2. Monoterpenes, which have the lowest molecular weight, include compounds such as limonene and pinene, found in plant extracts, and are related to abiotic stress responses in plants due to their volatility, playing a role in signaling. In the medicinal field, they exhibit antimicrobial and sedative activities (BARRIENTOS, 2013). More complex terpenoids, such as sesquiterpenes (e.g., humulene and farnesene), display antioxidant and anti-inflammatory properties, while diterpenes, such as phytol, are notable for their immunomodulatory actions (TETALI et al., 2019).

The diversity of molecules derived from isoprenes, due to various cyclization processes, leads to the formation of triterpenes, including compounds such as beta-sitosterol and ursolic acid, which are studied for their anti-inflammatory and cholesterol-lowering properties. Tetraterpenes include carotenoids, such as β-carotene and lycopene, which are recognized for their antioxidant role and protection against cellular damage caused by reactive oxygen species. Polyterpenes, found in natural rubber, have both bioactive and industrial applications (FAIOLA et al., 2018; YANG, 2020).

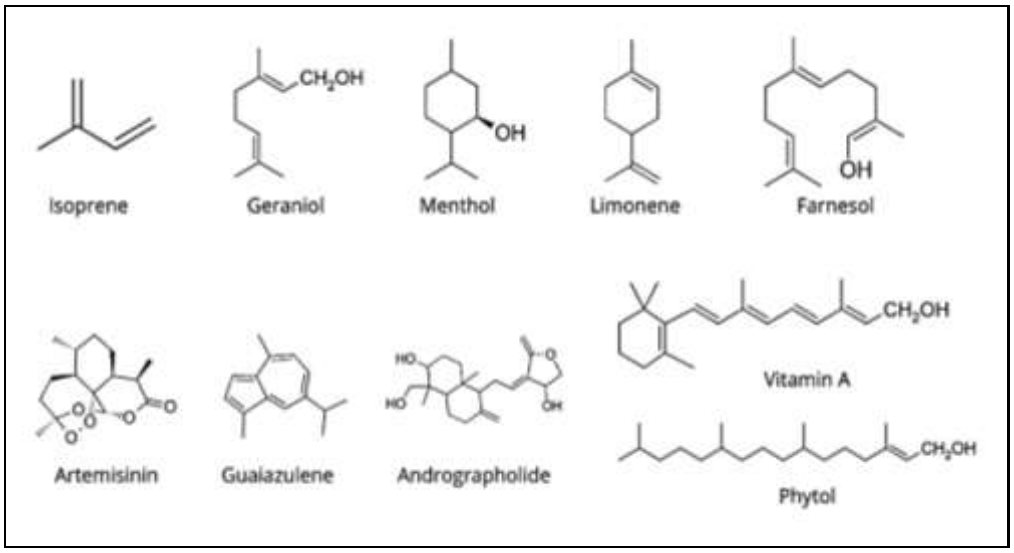

Figure 2 - Chemical structure of terpenoids. Chemical structure drawn using MarvinSketch (Chemaxon, Budapest, Hungary).

Studies related to the identification of terpenes through separation and quantification methods highlight the *Trichilia genus* as having a considerable concentration of terpenoids, with great biotechnological application potential in various fields. Research on *Trichilia martiana* has demonstrated that its triterpenes exhibit significant anti-inflammatory activity, inhibiting mediators such as TNF-α and IL-6, in addition to reducing the expression of pro-inflammatory enzymes like iNOS (PARK et al., 2020). Another study investigated limonene, a monoterpene with bactericidal, fungicidal, and insecticidal properties, extracted from *Trichilia* species and found. That seed extracts of *Trichilia havanensis* are effective in controlling agricultural pests. Research on *Trichilia rubescens* has also identified limonoids, a group of chemicals with antioxidant and cytoprotective effects (PASSOS et al., 2021).

2.2 Phenolic Compounds

Phenolic compounds are the most abundant secondary metabolites in plants, with an enormous diversity of structures. They are characterized by one or more aromatic rings with hydroxyl groups (-OH), bonded as glycosides or aglycones, as shown in figure 3, giving them a high electron-donating capacity and the ability to interact with reactive oxygen species (ROS) (DAI & MUMPER, 2010). This chemical characteristic forms the basis of their antioxidant, anti-inflammatory, and antimicrobial activities, which make these compounds crucial for cellular protection against oxidative stress and in the prevention of chronic diseases (ALARA, ABDURAHMAN & UKAEGBU, 2021).

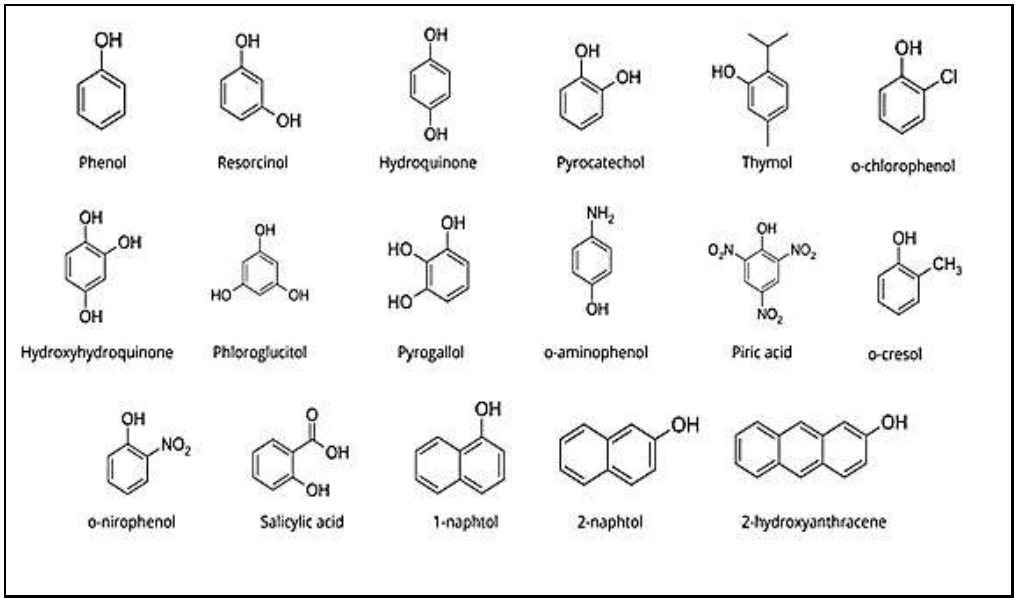

Figura 3 - Diversity of structures of phenolic compounds. Chemical structure drawn using MarvinSketch (Chemaxon, Budapest, Hungary).

Phenolic compounds, including flavonoids, a type of polyphenol, are found in a wide variety of plants and are characterized as secondary metabolites, which play protective roles in plant physiology, such as pigmentation, defense against biotic stresses (e.g., pathogen resistance), and abiotic stresses (e.g., protection against ultraviolet radiation). Additionally, numerous studies show that these compounds are capable of neutralizing free radicals, protecting cells from oxidative damage, and preventing cellular degeneration (KARAK, 2019).

The molecular structure of flavonoids consists of 15 carbon atoms organized in a diphenylpropane skeleton with two primary aromatic rings (A and B), connected by a three-carbon chain that may or may not form a heterocyclic ring (C), classified as aglycones, glycosides, and methyl derivatives (BRODOWSKA, 2017). Depending on the saturation and substitution pattern of ring C, which differentiates each flavonoid, they are divided into subgroups such as flavones, flavonols, flavanones, isoflavones, anthocyanins, and catechins, as shown in figure 4. These structural variations directly influence their mechanisms of action and consequently their antioxidant and biological properties (CHEN et al., 2005).

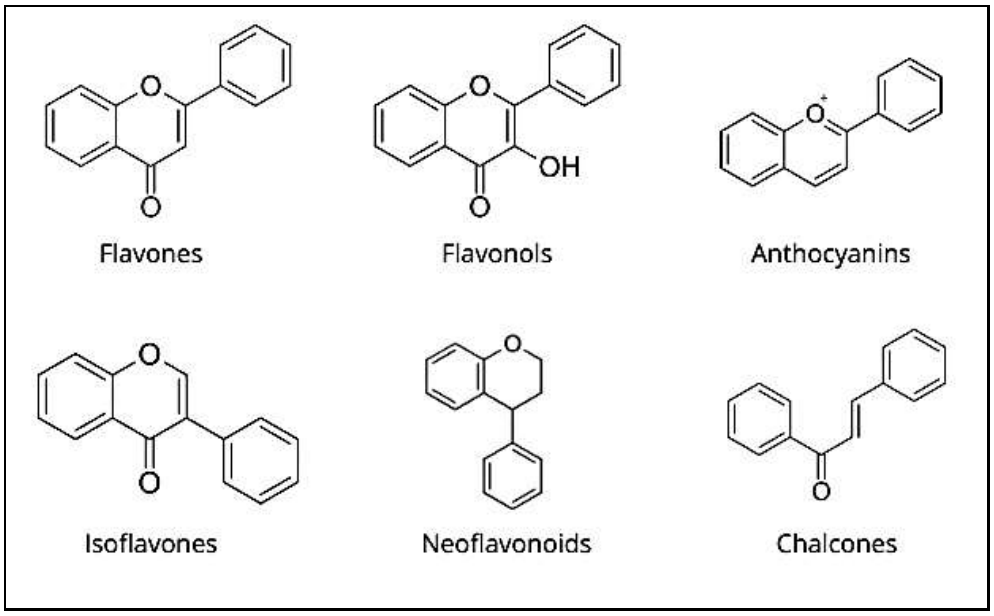

Figure 4 - Different types of flavonoids and their chemical structures. Chemical structure drawn using MarvinSketch (Chemaxon, Budapest, Hungary).

Both groups of phenolic derivatives, flavonoids and phenolic compounds, in addition to their contribution to the adaptation of plants in diverse environments, also possess bioactive properties with great potential in pharmacology and medicine, being used as phytotherapeutics. They are particularly noted for their antioxidant and anti-inflammatory properties, as the presence of hydroxyl groups (-OH) helps neutralize reactive oxygen species (ROS), preventing cellular damage caused by oxidative stress (HERNÁNDEZ-RODRÍGUEZ, BAQUERO & LARROTA, 2019). These compounds also modulate inflammatory pathways by inhibiting pro-inflammatory cytokines (such as TNF-α and IL-6) and the activity of inflammatory enzymes, such as cyclooxygenase-2 (COX-2), thereby helping to prevent various types of inflammation (PARK, 2020). Furthermore, they exhibit antimicrobial effects by inhibiting the growth of certain microorganisms, anticancer properties by affecting tumor cell proliferation, and cardio- and neuroprotective properties, improving markers of cardiovascular and neurological diseases (WEN et al., 2021).

Quercetin and kaempferol, two types of flavonoids present in plant extracts, have been associated with the reduction of inflammatory interleukins, such as IL-1β and TNF-α, and the inhibition of the cyclooxygenase enzyme (COX), a key factor in the production of mediators in oxidative inflammation processes (TIAN et al., 2021). Studies by Silva (2018), evaluating a range of plant extracts, indicated that these compounds play a significant role in reducing inflammatory processes and oxidative stress, by modulating the immune system. As a result,

they are associated with the prevention of chronic diseases related to oxidative stress, such as cardiovasculars and neurodegenerative diseases.

In the *Trichilia* genus, the phenolic compounds found play roles related to antioxidant, anti-inflammatory, and antimicrobial properties. Several species of this genus are rich in phenols and flavonoids, which contribute to their therapeutic activity. These properties make the phenolic compounds in *Trichilia* a subject of study for the development of products with medicinal applications (SILVA, 2018). Another study involving different species of the *Trichilia* genus, native plants from the Atlantic Forest, shows high concentrations of flavonoids and terpenes. These compounds have been widely investigated in modern medicine for their potential protective effects against chronic and degenerative diseases (RIBEIRO et al., 2013).

2.3 Saponins

Saponins are glycosidic compounds that are biologically active and can be found in a wide range of plants. They are characterized by a chemical structure consisting of a glycosidic (hydrophilic) portion attached to a triterpenoid or steroidal (hydrophobic) core, as shown in figure 5. This amphiphilic structure, capable of interacting with both polar and nonpolar compounds, gives saponins the ability to form foam in aqueous solutions. This characteristic makes them highly relevant and with great biotechnological potential, both in industrial applications and in the pharmaceutical field (EL AZIZ, ASHOUR & MELAD, 2019).

Saponins exhibit a wide range of biological activities, including anti-inflammatory and antioxidant effects, by reducing pro-inflammatory markers and decreasing reactive oxygen species (ROS) levels (YANG et al., 2022). These compounds demonstrate strong antimicrobial activity, as their amphiphilic nature allows them to interact with bacterial cell membranes, causing structural alterations and, in some cases, cell lysis. Additionally, they possess immunomodulatory properties, such as enhancing macrophage and natural killer (NK) cell activity, and they potentiate the adaptive immune response (DESAI, DESAI & KAUR, 2009; JI et al., 2015).

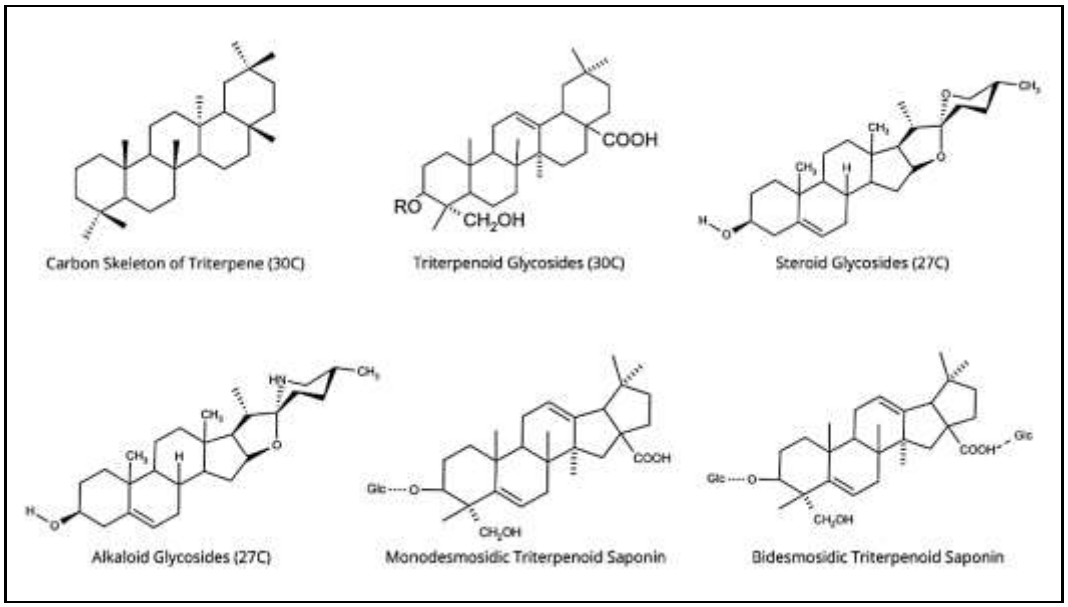

Figure 5 - Diversity of saponin structures. Chemical structure drawn using MarvinSketch (Chemaxon, Budapest, Hungary).

Given these properties, the application of these molecules in biotechnology enables the development of phytotherapeutics and functional foods, promoting cardiovascular health improvements, as well as immunological adjuvants that enhance vaccine efficacy (LIAO et al., 2021). Saponins are also used as surfactants to stabilize emulsions in various media (YATHAM et al., 2021).

Recent studies have demonstrated the ability of saponins to reduce cholesterol levels in both animals and humans, which can be attributed to their capacity to form insoluble complexes with bile acids in the digestive tract, forcing the body to utilize cholesterol for the synthesis of new bile acids. Additionally, they inhibit intestinal cholesterol absorption by interacting with micelles, thereby reducing its availability for transport by enterocytes (GONGARA-CHI et al., 2023; OAKENFULL & SHIDU, 2023). Waheed and colleagues (2012) isolated a novel steroidal glycoside saponin from *Fagonia indica*, capable of selectively inducing apoptosis or necrosis in cancer cells. Saponins are also used as precursors for the semisynthesis of steroidal drugs in the pharmaceutical industry. (SHENG & SUN, 2011). They have immense therapeutic potential such as hypolipidemic, hypoglycemic, antiasthmatic, antioxidant, antihypertensive and antimicrobial activity (SHARMA et al., 2023).

Studies conducted by PASSOS et al., (2021) have shown that extracts from *Trichilia* genus containing saponins exhibit antimicrobial activity against a variety of pathogens, including *Staphylococcus aureus* and *Escherichia coli*.

2.4 Phytosterols

Plant steroids, or phytosterols, constitute a class of bioactive compounds found in plants. Structurally, they are similar to animal cholesterol, featuring a tetracyclic steroid nucleus attached to different side chains, with more than 200 known compounds. The most common phytosterols include campesterol (24α-methylcholesterol), sitosterol (24α-ethylcholesterol), and stigmasterol (Δ22, 24α-ethylcholesterol), as illustrated in figure 6. These compounds are frequently found in vegetable oils, seeds, leaves, and medicinal plant extracts (MARAHATHA et al., 2021).

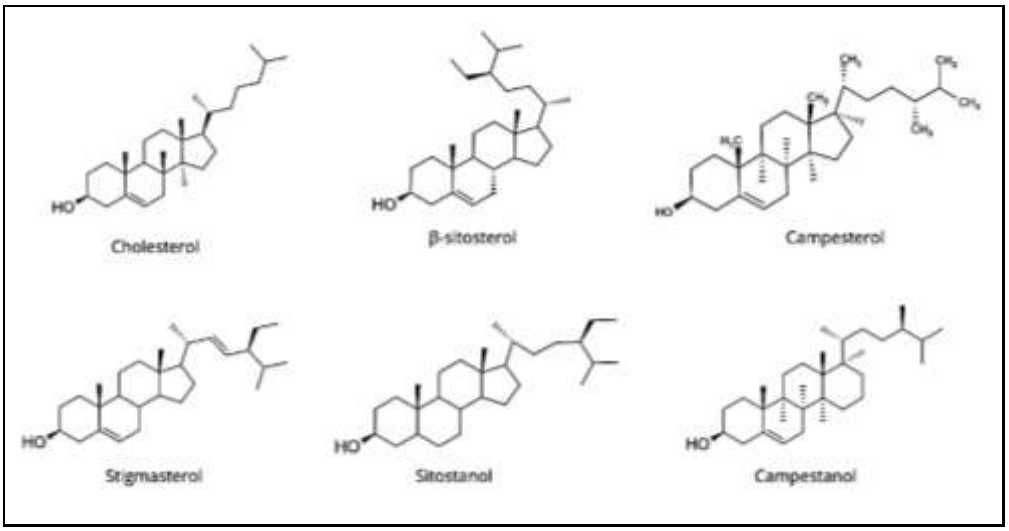

Figure 6 - Structural diversity of phytosterols. Chemical structure drawn using MarvinSketch (Chemaxon, Budapest, Hungary).

Phytosterols play essential roles in regulating plant metabolism, including modulating cell membrane fluidity and serving as precursors for the biosynthesis of hormones such as brassinosteroids, which influence plant growth and development. Additionally, they contribute to plant defense against pathogens and environmental stressors (NATTAGH-ESHTIVANI et al., 2022).

Phytosterols are associated with therapeutic benefits, such as cholesterol reduction, as they compete with cholesterol for intestinal absorption due to their structural similarity. By incorporating into micelles in the gastrointestinal tract, they reduce the absorption of exogenous cholesterol, leading to a decrease in serum LDL cholesterol levels (NATTAGH-ESHTIVANI et al., 2022). Another important property of phytosterols, such as β-sitosterol, is the modulation of inflammatory pathways by inhibiting the production of pro-inflammatory cytokines and

reducing the expression of enzymes such as COX-2 and iNOS (GOPALAKRISHNAN et al., 2016).

This group of steroid molecules i salso notable for its antimicrobial activity and is widely used in food preservation. For instance, β-sitosterol has demonstrated efficacy against *Bacillus subtilis*, among other tested microorganisms (BURČOVÁ et al., 2018). Other studies also highlight its role in lowering blood pressure, which is beneficial in combating cardiovascular diseases and renal dysfunction, allowing its use in combination with existing antihypertensive drugs (MORADI et al., 2018; STEPTOE et al., 2016). Additionally, antioxidant and anti-inflammatory effects have been observed, with modulation of inflammatory markers such as nuclear factor NF-κB, MAP kinase, cytokines, and interleukins, influencing the transcription of inflammatory proteins such as IL-6 and IL-8 (CHEN et al., 2017).

Various studies conducted in different regions of Brazil to isolate bioactive chemical compounds from *T. silvatica* have identified a diverse spectrum of secondary compounds. Among the most notable are pregnane-type and aromandrene-type steroids (2α,3β,4β-trihydroxypregnan-16-one, 2β,3β,4β-trihydroxypregnan-16-one, and cneorubin X), bicyclic clerodane-type diterpenes (Kolavelool and Kolavenol), as well as γ-tocopherol,3-O-β-D-glucopyranosyl-β-sitosterol,3-O-β-D-glucopyranosylstigmasterol, tocopherol, sesquiterpenes (2S,3S,6R,7R-humulene-2,3,6,7-diepoxide, 2R,3R,6R,7R-humulene-2,3;6,7-diepoxide), mustakone, and steroids such as β-sitosterol. Additionally, a mixture of triterpenes, including α-amyrin and β-amyrin, has been characterized, along with bioactive with anti-inflammatory potential, such as pseudotaraxasterol and lupeol (SOARES et al., 2014; FREITAS et al., 2014; FORMAGIO et al., 2012).

These compounds, identified and characterized in studies involving *T. silvatica,* are mainly classified as terpenes, phenolic compounds, flavonoids and coumarins found in extracts of both leaves and stems of this species. These bioactive compounds have possible antioxidant, antimicrobial and anti-inflammatory potential, among other applications (DA SILVA, 2018; DA SILVA, 2021).

3 INFLAMMATORY PROCESS AND ASSOCIATED NATURAL PRODUCTS

3.1 Inflammatory Process

The inflammatory process can be understood as the body's response mechanism to invading agents (pathogens), such as viruses, bacteria, and fungi. In addition, inflammation can be triggered by trauma or injury to enhance tissue repair, promoting increased blood flow to the affected area. This process can occur locally or systemically, affecting the entire body (MEDZHITOV, 2008). An injury caused by any agent results in the activation of a signaling cascade mediated by molecules such as cytokines and chemokines, which are released by damaged cells and adjacent tissues. This leads to the migration of immune system cells to the affected region, including leukocytes such as neutrophils and macrophages. These specialized cells are recruited and trigger the inflammatory process, followed by tissue repair (CRUVINEL et al., 2010).

The inflammatory mechanism involves a highly organized signaling cascade, including cellular and vascular responses. This process activates the production and secretion of humoral mediators specific to each type of inflammatory profile triggered. It includes the recruitment of white blood cells (monocytes, basophils, eosinophils, and neutrophils), as well as the recruitment of plasma and fluids to the inflamed area (ABDULKHALEQ et al., 2018). During the inflammatory process, signaling molecules such as histamine, prostaglandins, leukotrienes, thromboxanes, reactive oxygen and nitrogen species, and serotonin mediate the response generated (HUETHER & MCCANCE, 2015; ANWIKAR & BHITRE, 2010).

In the case of skin lesions, a series of events occur when the injury penetrates the first layer of epithelial tissue. Initially, there is a transient vasoconstriction, followed by vasodilation, allowing platelets to settle in the wounded area (REINKE & SORG, 2012). In response to the injury, various chemical mediators are released, promoting vasodilation and, consequently, increased blood flow, characteristic of an inflammatory event. This process is confirmed by the synthesis of cytokines and growth factors by platelets and leukocytes, as well as collagen synthesis, angiogenesis, and reepithelialization. Thus, inflammatory signaling plays a crucial role in promoting tissue repair in skin lesions (ROBSON, STEED & FRANZ, 2001; SILVA, FERNANDEZ & NEIVA, 2021).

The inflammatory process can be divided into two phases: acute and chronic. The acute phase is characterized by a primary and short-lived response of the immune system, mediated by specific cytokines with pro-inflammatory action (CHEN et al., 2017). The initial phase is

marked by transient vasoconstriction, followed by sustained vasodilation, mediated by molecules such as nitric oxide, histamine, and prostaglandins. These changes increase blood flow at the injury site, resulting in redness and heat, as illustrated in figure 7. Simultaneously, there is an increase in vascular permeability, allowing the recruitment of plasma proteins, such as fibrinogen, and leukocytes to the site of inflammation (HANNOODEE & NASURUDDIN, 2020).

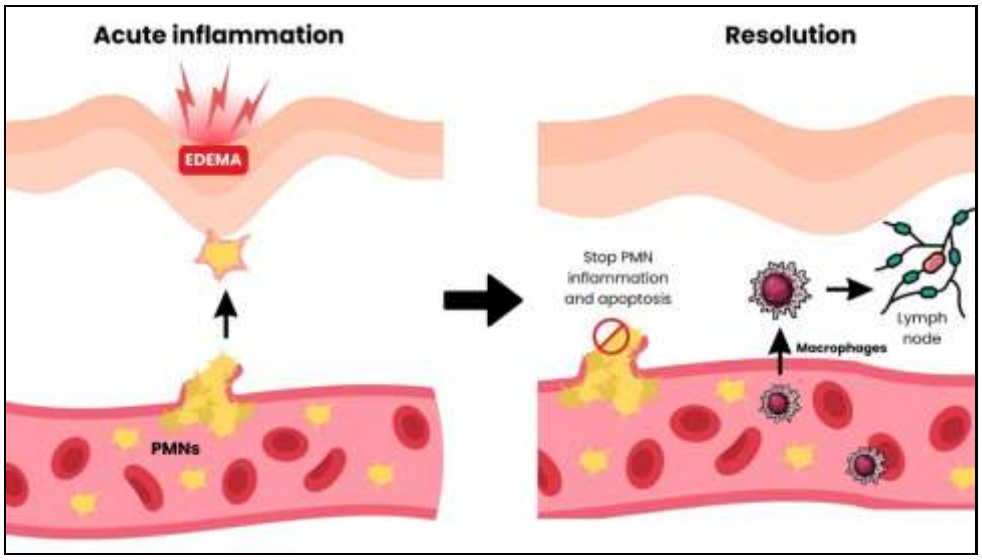

Figura 7 - The acute inflammatory process begins with a rapid influx of polymorphonuclear neutrophils (PMNs), which is soon followed by the recruitment of monocytes that differentiate into macrophages. This phase is also marked by the development of edema as a response to tissue injury. Image created using Canva (Canva Pty Ltd, Sydney, Australia).

The second phase of the acute inflammatory process, also known as the cellular phase, is characterized by the migration of neutrophils, the first leukocytes to arrive at the site of inflammation. This migration is stimulated by agents such as microbial endotoxins containing N-formyl-methionyl amino-terminal groups, the complement fragment C5a, interleukins, and basophil secretions, which attract chemokines such as IL-8 (MEDZHITOV, 2021). Neutrophils eliminate invading microorganisms and cellular debris through phagocytosis and the release of reactive oxygen species (ROS) and proteolytic enzymes. Subsequently, monocytes arrive at the inflamed area and differentiate into macrophages, which play a crucial role in removing cellular debris and releasing anti-inflammatory cytokines such as IL-10, initiating the resolution process (ABDULKHALEQ et al., 2018).

As a result of acute inflammation, three outcomes may occur: (1) Resolution, with the elimination of the causative agent of inflammation and subsequent repair of the injured tissue;

(2) Fibrosis, when the original tissue is replaced by connective tissue in cases of more extensive injuries; or (3) Development of a prolonged or chronic inflammatory response, when the causative agent is not completely eliminated, resulting in persistent inflammation (DEL CAMPO, GALLEGO & GRANDE, 2018).

The chronic inflammation profile occurs due to the persistence of the pathological stimulus, which culminates in the continuous activation of the immune system, as illustrated in figure 8. In this context, of mononuclear cell infiltration, such as monocytes and lymphocytes, occurs alongside fibroblast proliferation, collagen fiber formation, and connective tissue. These processes contribute to the development of granulomatous structures, which can reach up to 2 mm in diameter (GLESSOM et al., 2011). Additionally, the continuous recruitment of immune cells, including macrophages and lymphocytes, may exacerbate infection severity and contribute to irreversible tissue damage. In this scenario, characteristic symptoms such as pain, redness, swelling, heat, and eventually loss of function are observed (MURAKAMI, 2012). Tissue degeneration is often mediated by nitrogen species, proteases, and other reactive oxygen species released by infiltrated inflammatory cells, as a result of the release of the previously mentioned chemical mediators (MEDZHITOV, 2008; GILROY et al., 2004).

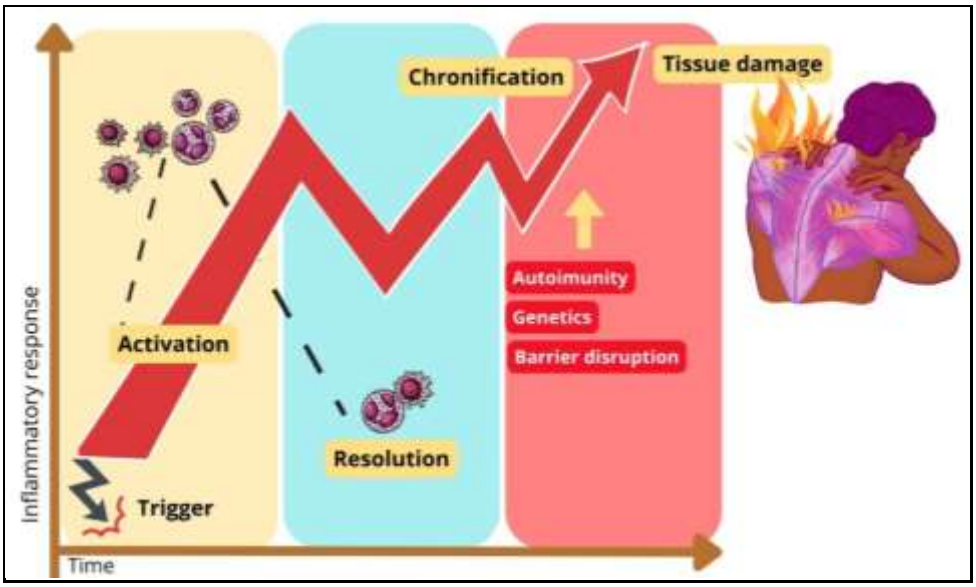

Figure 8 - Activation, Resolution, and Progression of the Chronic Inflammatory Process (SCHETT & NEURATH, 2018). Image created using Canva (Canva Pty Ltd, Sydney, Australia).

Several factors can interfere with the inflammatory response of an organism, including genetic characteristics, environmental factors, and phenotypic aspects, all of which modulate the intensity of inflammation. Proper regulation of this process is crucial for effective tissue

repair (MARUYMA et al., 2020). Inflammation can be managed through pharmacological interventions, with widely available anti-inflammatory angents playing a key role. Notably, synthetic drugs, such as analgesics, corticosteroids, and non-steroidal anti-inflammatory drugs (NSAIDs), are commonly used for this purpose (FORRESTER et al., 2018).

Another approach involves the use of natural products, which exhibit anti-inflammatory properties due to the presence of bioactive compounds, such as polyphenols. These compounds modulate cellular signaling pathways, reducing the inflammatory response and contributing to the control of oxidative stress by acting on the neutralization of free radicals (SCALBERT et al., 2005).

3.2 Anti-inflammatory Drugs and Associated Natural Products

Anti-inflammatory drugs exert their effect primarily inhibiting the cyclooxygenase (COX) enzyme, with selectively varying depending on the specific drug (SOUZA, 2013). Although these drugs exhibit excellent anti-inflammatory properties and are widely used in clinical practice, their recurrent use may result in adverse effects, including mild to severe gastric irritation, such as ulcers, as well as skin reactions, impaired renal function, and analgesic nephropathy due to chronic use. Additionally, less common but significant adverse effects include central nervous system (CNS) disturbances, bone marrow changes, and liver dysfunction (CECIL, 2005).

Given the complexities associated with conventional anti-inflammatory therapies, the pharmaceutical industry and the scientific community have intensified efforts to develop formulations derived from natural products, aiming to provide alternatives with comparable efficacy to conventional drugs but with a reduced risk of adverse effects. One example is Acheflan, an anti-inflammatory derived from *Cordia verbenacea*, a plant native to the Brazilian Atlantic Forest. Its mechanism of action involves selective inhibition of the COX-2 enzyme, leading to reduced prostaglandins synthesis and, consequent attenuation of inflammation (PASSOS et al., 2021). Another example is Védica, a a phytotherapeutic formulation, extracted from *Boswellia serrata*, which has been employed in the treatment of intestinal inflammation (SADIQ et al., 2020).

Natural products have become increasingly recognized in the therapeutic applications due to their anti-inflammatory properties, low cost, and lower toxicity. Their bioactive compounds modulate the immune response by reducing the production of inflammatory cytokines, reactive oxygen species (ROS), and pro-inflammatory enzymes. Among the most

extensively studied are polyphenols, that are structurally defined in advance by an aromatic ring associated with hydroxyl groups. This diverse class includes flavonoids, curcuminoids, phenolic acids, tannins, and tocopherols - molecules that exhibit antioxidant and anti-inflammatory properties. These compounds contribute to downregulation of plasma prostaglandins, leukotrienes, and transcription factors, such as NF-κB. Moreover, they neutralize reactive species, including singlet oxygen and hydroxyl radicals, and can either interact with other phenolic acids or persist in their free form (CHEN et al., 2018).

In addition to polyphenols, other naturally occurring compounds with anti-inflammatory activity include terpenes and their volatile derivatives, often available as essential oils. These compounds are responsible for reducing the production of prostaglandins and inflammatory cytokines, as well as inhibiting COX-2 (EDRIS, 2007). Alkaloids, typically found in medicinal plants, also play a significant role in suppressing nitric oxide production, thereby contributing to the reduction of inflammatory symptoms (HOSSEINZEDAH, 2016).

Various natural products have been widely used in traditional medicine due to their anti-inflammatory properties, attributed to the presence of bioactive molecules that modulate metabolic pathways and reduce the inflammatory cascade. A notable example is propolis, a byproduct produced by bees, which possesses multiple pharmacological properties, including anticancer, antioxidant, antifungal, antibacterial, antiviral, and anti-inflammatory activities. Its chemical composition is rich in phenolic compounds, flavonoids, and other bioactive secondary metabolites (BARRIENTOS et al., 2013).

Other natural sources of anti-inflammatory compounds include extracts from leaves, stems, and roots of medicinal plants, such as Aloe vera, chamomile, guaco, quebra-pedra, boldo, and ginger, which are widely distributed throughout Brazil and frequently cultivated in households (GUPTA et al., 2003) These extracts have been associated with the increased production of the cytokines interleukin-4 (IL-4) and interleukin-10 (IL-10), signaling molecules of the immune system that play a key role in regulating inflammation (LIBÉRIO et al., 2011). Activation of these cytokines leads to COX inhibition, reducing the synthesis of prostaglandins and alleviating inflammatory symptoms (VIEIRA et al., 2014).

Among the various medicinal plants studied, *Trichilia* genus has gained increasing interest due to its anti-inflammatory properties. Scientific studies show that extracts from its leaves and bark are rich in phenolic compounds and flavonoids, which modulate pro-inflammatory pathways, such as NF-κB, as well as reduce the expression of inflammatory mediators like interleukins and prostaglandins. Furthermore, recent research indicates its

potential in the treatment of joint inflammation and dermatological diseases, with a favorable safety profile and low risk of adverse effects (CARVALHO et al., 2022).

4 OXIDATIVE STRESS AND NATURAL ANTIOXIDANT PRODUCTS

4.1 Oxidative Stress

Redox reactions are fundamental for homeostasis, physiology, and cellular metabolism, maintaining the balance of ions within the cellular environment. Oxidative stress, on the other hand, is characterized by an imbalance in redox homeostasis and pH in the cellular environment, leading to the excessive production of reactive oxygen species (ROS). This phenomenon directly affects bioenergetics, metabolism, and vital cellular functions, reducing the ability of cells to neutralize these ROS through antioxidant systems (SIES, BERNDT & JONES, 2017).

Molecular oxygen, in its ground state, has two unpaired electrons with parallel spins in its two separate anti-bonding orbitals. This spin restriction limits its reactivity, requiring a stepwise reduction to accomodate eléctron pairs from donor melucules. As a result, molecular oxygen functions as the as the terminal electron acceptor in various biochemical and oxidative process (GULCIN, 2020).

ROS can be defined as highly reactive molecules that have one or more unpaired electrons in their outer shell, classifying them as free radicals. These species are generated through the interaction of oxygen with specific molecules (LIGUORI et al., 2018). ROS can exist as charges free radicals, such as the superoxide anion ($O_2 \cdot^-$) and the hydroxyl radical ($OH\cdot$), as non-radical species, such as hydrogen peroxide (H_2O_2). Additionally, other molecules including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase (MPO), lipoxygenase, and angiotensin II contribute to ROS production through their degradation, leading to the formation of ionic species (ALKADI, 2020).

The production of these reactive species occurs naturally as a byproduct of cellular metabolic reactions and can be harmful to the intracellular environment. In addition to the compartmentalized metabolic reactions in the mitochondria during oxidative phosphorylation, several exogenous sources also contribute to ROS generation, including exposure to ultraviolet (UV) rays and chemicals (DI MEO & VENDITTI, 2020).

The imbalance of the redox system, known as oxidative stress, can be exacerbated by exogenous exposures that accelerate the process of cellular damage. Among these factors are air and water pollution, smoking, alcohol consumption, exposure to heavy or transition metals,

the use of certain drugs (such as cyclosporine, tacrolimus, gentamicin, and bleomycin), industrial solvents, processed foods (such as smoked meat, residual oil, and fats), and radiation. Within the body, these substances are metabolized, generating reactive oxygen species (ROS), which are associated with various diseases, including cancer, diabetes mellitus, cardiovascular and neurodegenerative disorders, as well as aging (PHANIENDRA, JESTADI & PERIYASAMY, 2015; LIGUORI et al., 2018).

Excessive ROS production can cause significant and irreversible damage to biomolecules. In lipids, peroxidation occurs, leading to the formation of toxic compounds such as malondialdehyde (MDA), which compromises the integrity of cell membranes. In proteins, oxidation of amino acid residues can alter their structure and function, resulting in loss of enzymatic activity and protein aggregation. In DNA, oxidative stress can induce single-strand breaks and modifications in nitrogenous bases, promoting mutations that contribute to carcinogenesis (SIES et al., 2017).

To mitigate the harmful effects of ROS and repair cellular damage, cells have evolved sophisticated antioxidant defense mechanisms. Among endogenous enzymatic antioxidants, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) play key roles in catalyzing the convertion of reactive species into non-toxic byproducts, thereby minimizing oxidative damage (BIRBEN et al., 2012). In addition to enzymatic defenses, non-enzymatic antioxidants, such as reduced glutathione, vitamins C and E (α-tocopherol), bilirubin, β-carotene, and phenolic compounds (such as resveratrol, phenolic acids, and flavonoids), also contribute significatly to cellular protection. In plasma, albumin and uric acid together account for approximately 85% of the total antioxidant capacity (WU, KOSTEN & ZHANG, 2013). These exogenous antioxidants not only reduce the formation of free radicals, such as hydroxyl and superoxide radicals, but also inhibit lipid peroxidation, further preventing oxidative damage (PISOSCHI & POP, 2015).

4.2 Antioxidants and Associated Natural Products

Antioxidants play a fundamental role in the homeostasis of organisms, as they are responsible for inhibiting the oxidation of other substances, thereby reducing lipid peroxidation and the formation of its secondary products. These compounds have been extensively studied due to their association with the delay and prevention of various chronic diseases, such as diabetes mellitus, cancer, cardiovascular diseases, and neurodegenerative disorders (GULCIN, 2020).

Antioxidants can be categorized into two main groups. The first group consists of endogenous, or enzymatic, antioxidants, which are synthesized by the organism and play a crucial role in mitigating oxidative stress through enzymatic reactions. Key enzymes in this group include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which catalyze the conversion of reactive oxygen species (ROS) into less harmful molecules, thereby maintaining cellular redox balance (DI MEO & VENDITTI, 2020). The second group consists of exogenous, or non-enzymatic, antioxidants, which are acquired through dietary intake. This group includes polyphenols, carotenoids, vitamins C and E, as well as minerals such as selenium and zinc, which contribute to oxidative stress reduction through diverse pathways (BUNACIU et al., 2016), as illustrated in figure 9.

The effectiveness of dietary antioxidants is largely influenced by their bioavailability, which defined the proportion of a given nutrient that is digested, absorbed, and metabolized within the body. In the case of polyphenols, their intestinal absorption is highly dependent on their chemical structure and physicochemical Properties including molecular weight, degree of polymerization, solubility, and interactions with other dietary componentes. These factors influence their stability, transport across the intestinal epithelium, and subsequent metabolic transformation, ultimately determining their biological activity and antioxidant potential (JAKOBEK, 2015).

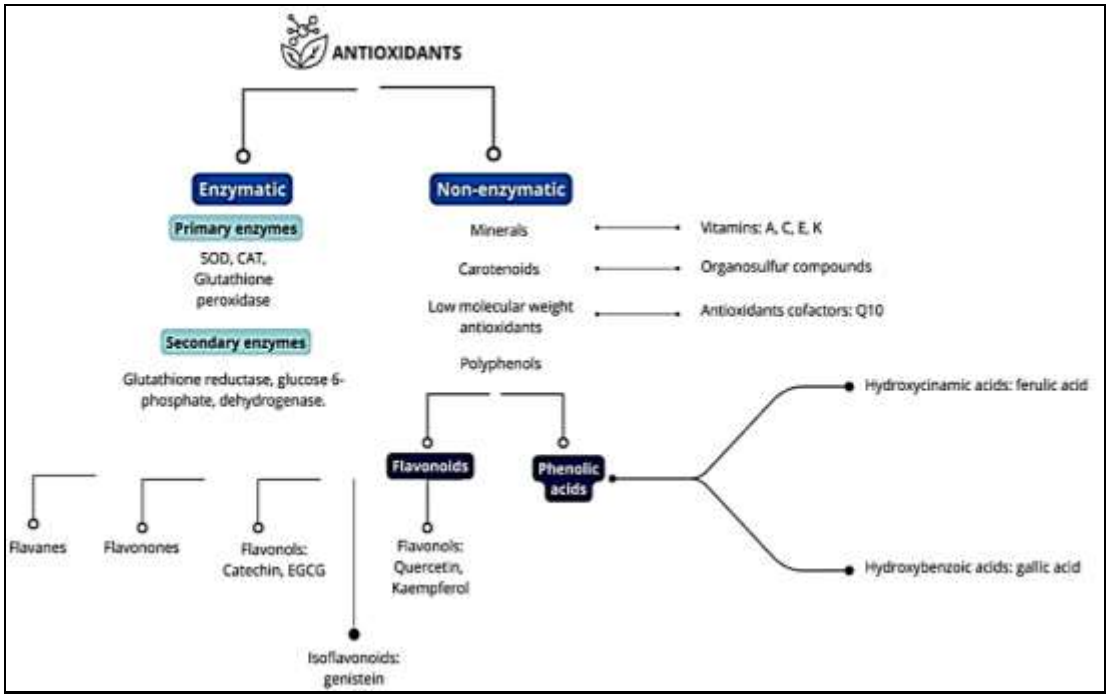

Figure 9 - Types of Antioxidants and Classification. Image created using Canva (Canva Pty Ltd, Sydney, Australia).

These compounds mitigate free radical activity through three main mechanisms. The first mechanism involves direct electron donation by the antioxidant compound to neutralize free radicals, thereby stabilizing them and preventing oxidative damage. An example of this process occurs with phenolic acids, whose antioxidant activity is influenced by the number and position of hydroxyl groups attached to the aromatic ring. Additionally, the binding site location and the mutual arrangement of hydroxyl groups within the ring play a crucial role in determining their reactivity and effectiveness in scavenging free radicals (GULCIN, 2012).

The second mechanism involves enzymatic activation, in which compounds such as polyphenols stimulate cellular signaling pathways that enhance the expression of antioxidant enzymes, such as CAT and SOD. This enzymatic modulation contributes to the neutralization of reactive oxygen species (ROS) (CHEN et al., 2018).

The third mechanism refers to the regulation of the inflammatory process. Compounds such as carotenoids and flavonoids play an essential role in modulating the immune response by inhibiting the production of pro-inflammatory cytokines and activating transcription factors, such as NF-κB, which are directly involved in chronic inflammatory responses.

A well-documented example of an antioxidant mechanism is that of tocopherol (vitamin E), a highly lipophilic compound that plays a critical role in protecting cell membranes and lipoproteins from oxidative damage. Its antioxidant activity is primarily associated with the inhibition of lipid peroxidation, a process in which tocopherol scavenges lipid peroxyl radicals, converting them into less reactove lipid hydroperoxides. This reaction results in the formation of a tocopheroxyl radical, which can subsequently be regenerated into its active form through interations with other antioxidants within the biological system (GULCIN, 2020). A visual representation of this mechanism is provided in figure 10.

Among the primary natural antioxidant compounds, polyphenols, carotenoids, alkaloids, and essential oil constituints are recognized for their significant biological activity. Polyphenols, widely distributed in fruits, vegetables, teas, and wines, are known for their ability to inhibit free radical formation and chelate transition metals. Notable examples include resveratrol, found in grapes and red wine, which protects against oxidative damage associated with cardiovascular and neurodegenerative diseases. Studies have demonstrated its efficacy in reducing oxidative stress associated with atherosclerosis (BRITO et al., 2019). Additionally, green tea catechins have shown beneficial effects in mitigating oxidative stress in metabolic disorders (SINGH et al., 2021).

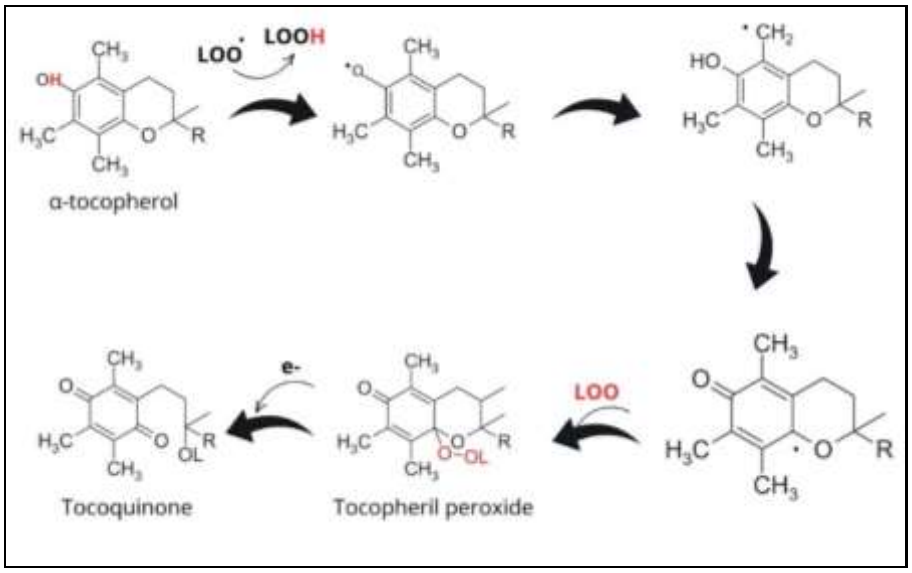

Figure 10 - Antioxidant Mechanism of Action of Tocopherol. Image created using Canva (Canva Pty Ltd, Sydney, Australia).

Carotenoids, such as β-carotene, lutein, and lycopene, are lipophilic compounds present in colorful fruits and vegetables. They play an essential role in oxidative damage protection by neutralizing ROS and stabilizing cell membranes (LIU et al., 2020). Studies indicate that lycopene supplementation can significantly reduce oxidative stress markers in patients with metabolic syndrome (AHN & KIM, 2021). Lutein, found in foods such as spinach and corn, has been associated with the prevention of oxidative stress-related eye diseases, such as macular degeneration (BONE, LANDRUM & MUKHERJEE, 2018). Meanwhile, alkaloids such as berberine exhibit both anti-inflammatory and antioxidant properties, proving particularly effective in modulating oxidative stress in metabolic diseases (CHANG et al., 2020).

Essential oils, extracted from aromatic plants such as rosemary (*Rosmarinus officinalis*) and lavender (*Lavandula angustifolia*), are rich in volatile compounds like terpenes and phenylpropanoids, which stabilize cell membranes and exhibit antioxidant properties (OLIVEIRA et al., 2021). Additionally, certain fatty acids found in vegetable oils, such as omega-3 fatty acids, have been associated with reduced oxidative stress biomarkers, such as malondialdehyde (MDA), contributing to cardiovascular protection (CALDER, 2020).

In neurodegenerative health, natural compounds such as curcumin and green tea polyphenols have demonstrated potential in reducing the accumulation of oxidized proteins, thereby slowing the progression of diseases like Alzheimer's and Parkinson's (FERREIRA et al., 2020). Furthermore, cinnamon and blueberry extracts have shown benefits in diabetic patients by improving insulin sensitivity and reducing oxidative stress (ZHU et al., 2021).

Natural products also have broad applications in the cosmetic field, particularly vitamin E, found in vegetable oils and seeds, and ascorbic acid (vitamin C), present in citrus fruits. Both are widely used in formulations designed to protect the skin from ultraviolet radiation-induced damage (JUNG et al., 2020). However, their application is often limited by challenges such as variability in chemical composition, difficulties in standardization, and the low bioavailability of certain compounds still. To overcome these limitations, innovative approaches are being explored, including the developmente of advanced formulations incorporating nanocarriers to enhance the absorption and efficacy of these bioactive compounds (TAN et al., 2022).

5 CONCLUSION

Several plants contain bioactive compounds capable of modulating inflammation and oxidative stress, thereby offering potential health benefits. Among them, *Trichilia silvatica* is particularly notable for its high content of phenolic compounds and flavonoids, which are essential for maintaining redox homeostasis. However, to maximize its therapeutic efficacy, the development of advanced delivery systems that enhance the bioavailability and stability of these bioactive compounds is essential. Thus, the exploration of innovative formulation strategies may contribute to the management of treatment of pathologies associated with inflammation and oxidative stress, facilitating cellular repair and mitigating the process known as OxInflammation.

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CHAPTER II

OXINFLAMMATION AFFECTS TRANSDIFFERENTIATION TO MYOFIBROBLASTS, PROLONGING WOUND HEALING IN DIABETES: A SYSTEMATIC REVIEW[1]

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ABSTRACT

Skin wounds, primarily in association with type I diabetes mellitus, are a public health problem generating significant health impacts. Therefore, identifying the main pathways/mechanisms involved in differentiating fibroblasts into myofibroblasts is fundamental to guide research into effective treatments. Adopting the PRISMA guidelines, this study aimed to verify the main pathways/mechanisms using diabetic murine models and analyze the advances and limitations of this area. The Medline (PubMed), Scopus, and Web of Science platforms were used for the search. The studies included were limited to those that used diabetic murine models with excisional wounds. Bias analysis and methodological quality assessments were undertaken using the SYRCLE bias risk tool. Eighteen studies were selected. The systematic review results confirm that diabetes impairs the transformation of fibroblasts into myofibroblasts by affecting the expression of several growth factors, most notably transforming growth factor beta (TGF-beta) and NLRP3. Diabetes also compromises pathways such as the SMAD, c-Jun N-terminal kinase, protein kinase C, and nuclear factor kappa beta activating caspase pathways, leading to cell death. Furthermore, diabetes renders the wound environment highly pro-oxidant and inflammatory, which is known as OxInflammation. As a consequence of this OxInflammation, delays in the collagenization process occur. The protocol details for this systematic review were registered with PROSPERO: CRD42021267776.

Keywords: *OxInflammation process; wound healing; myofibroblasts; diabetes mellitus; NLRP3; TGF-beta; inflammasome; cytokines.*

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1 Published in the *International Journal of Molecular Science*, date 19 August 2024. https://doi.org/10.3390/ijms25168992.

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1 INTRODUCTION

Skin wounds affect a large proportion of the global population and represent an expensive public health issue that affects the entire health system, generating billions of dollars of preventive and treatment expenses, according to the World Health Organization (WHO) [1]. In the wound healing process, there are four steps or events that overlap, which are hemostasis, inflammation, proliferation, and remodeling. Among the most common complications in wound healing are those related to diabetes mellitus (such as vasculopathy and neuropathies), which are especially prevalent and contribute to diabetic ulcer formation [2]. Diabetic ulcers are characterized by a prolonged inflammatory response due to a significant imbalance in cytokine and inflammatory marker expression and the compromised formation and maturation of granulation tissue [3]. Diabetic individuals represent an example of impaired healing, with several impairment factors, including peripheral vascular disease and reduced blood pressure. Uncontrolled and excessive inflammation and, consequently, oxidative stress in the tissue delay the wound healing process [4].

Persistent inflammation has been associated with high levels of IL-1, IL-2, IL-6, IL-17, and tumor necrosis factor (TNF) cytokines, which can result in fibroblast apoptosis and extracellular matrix (ECM) degradation [5–7]. During the end of the inflammatory phase, fibroblasts and endothelial cells move into the wound, producing an extracellular matrix and angiogenesis, thereby forming granulation tissue [8]. Many of these fibroblasts acquire the morphological and biochemical appearance of smooth muscle cells, called myofibroblasts [9]. Myofibroblasts, expressing alpha-smooth muscle actin (alpha-SMA), play an essential role in wound healing, mediating growth factor secretion, ECM synthesis, and angiogenesis [10]. In this context, the formation of myofibroblasts occurs when mediators such as platelet-derived growth factor (PDGF), chemokines, and transforming growth factor beta (TGF-beta) bind to their membranes, activating intracellular pathways such as SMAD [11]. Activated receptor complexes mediate canonical TGF-beta signaling by phosphorylating the receptor-regulated effector proteins (R-SMADs) at their carboxy terminals, via the TGF-beta/SMAD signaling pathway. Among the R-SMADs, SMAD2 and SMAD3 mediate the TGF-beta signaling pathway [12] and are responsible for the collagen type I transcription increase in fibroblast cell lines. Therefore, a method to positively target the TGF-beta pathway would be extremely useful in treating diabetic wounds, mainly because it is already known that TGF-beta/SMAD signaling is downregulated in diabetes, leading to the decreased expression and deposition of collagen type I, as well as increased matrix metalloproteinase (MMP) expression [13].

Moreover, TGF-beta can use non-SMAD effectors to mediate some of its biological responses, including non-receptor tyrosine kinase proteins such as Src and FAK, mediators of cell survival (e.g., NF-kB, PI3K/Akt pathways), MAPK (ERK1/2, p38 MAPK, and JNK, among others), and Rho GTPases like Ras, RhoA, Cdc42, and Rac1. Notably, these pathways can also regulate the canonical SMAD pathway and are involved in TGF- beta-mediated biological responses [14]. Some of these pathways are involved in the wound remodeling and closure phase and the release of mediators such as TGF-beta, which stimulate myofibroblast differentiation, which in turn promotes wound contraction and closure [15]. Myofibroblasts expressing alpha-SMA promote contraction and synthesize high protease levels, degrading the extracellular matrix. Thus, beyond their critical role in wound contraction via alpha-SMA expression, myofibroblasts participate in synthesizing extracellular matrix components, influencing wound closure and scars' mechanical strength [16]. Importantly, during the healing process, the ability of fibroblasts to transform into myofibroblasts is altered in people with diabetes [17]. The versatility of TGF-beta shows that these molecules have a central role in wound healing due to their influence on the inflammatory response and, consequently, the oxidative stress in the tissue. TGF-beta expression can also control angiogenesis, granulation tissue formation, reepithelization, extracellular matrix deposition, and remodeling [18].

The intensified inflammation process is usually associated with increased free radicals and reactive oxygen species (ROS) generation. This process is known as OxInflammation, in which ROS work as secondary messengers, activating inflammation and oxidative stress in positive damage feedback. The lengthy inflammatory phase leads to excessive ROS accumulation, thereby impairing and compromising the entire wound healing process. Excessive ROS generation in diabetes is mainly due to acute increases in serum glucose and oxidative stress generation. Oxidative balance is critical in healing, and the role of ROS can be favorable or deleterious [19]. It is well known that inflammatory and oxidative markers play an essential role in skin wound closure, but little is known about the relationship between inflammatory and oxidative effectors during the transformation of fibroblasts into myofibroblasts in the wound healing process. A comprehensive analysis of the signaling pathways and the relation of the mechanisms involved in oxidative damage and its physiological response has not been systematically undertaken. Furthermore, an overview of the current evidence regarding the advances and limitations of the studies in this field has never been carried out. The development of new therapies, as well as the improvement of gold-standard procedures, is a field that has been heavily explored and described in the literature for the treatment of diabetic wounds. Among the new treatments are negative pressure suction,

autologous skin transplantation, and stem cell therapy, as well as advanced drug delivery systems using nanoparticles, hydrogels, liposomes, and others [20,21]. In this context, in this systematic review, we investigated how the excessive presence of reactive oxygen species can affect transdifferentiation to myofibroblasts and compromise the wound healing process in diabetic murine models. In this sense, the data of this study could help to understand the main mechanisms involved in the delay of the wound healing process in diabetic ulcers and provide a guideline for decision-makers or even researchers in developing new products and treatments that can accelerate skin wound closure associated with this comorbidity.

2 METHODS

2.1 Search Strategy

This systematic review was carried out using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). Its main question was the following: How does excessive ROS' presence affect transdifferentiation to myofibroblasts and compromise the wound healing process in diabetic murine models? The studies were selected through an advanced search on PubMed–Medline (https://www.ncbi.nlm. nih.gov/pubmed), Scopus (https://www.scopus.com/home.uri), and Web of Science (https://www.webofknowledge.com); both platforms were accessed on 10 April 2021. The protocol details for this systematic review were registered in the Prospective International Registry of Systematic Reviews (PROSPERO: CRD42021267776). For a comprehensive search of relevant papers, the strategy employed was to firstly search the databases for appropriate studies and then search the bibliographic references. PubMed–Medline platform filters were created using the hierarchical distribution of the Medical Subject Headings (MeSH) terms to retrieve the indexed studies. Non-MeSH descriptors were characterized using the Title and Abstract (TIAB) algorithm. A standardized experimental animal filter was applied [22]. The search filters used for the PubMed–Medline search platform were adapted to the Scopus and Web of Science databases, except for the experimental animal filter used in Scopus, which was made available by the website. The complete search strategy is shown in the Supplementary File (Table S1).

2.2 Eligibility Criteria

Three independent researchers (LLS, MMS, and MMS) performed the selection of potentially relevant studies. Initially, the abstracts of all papers recovered in the electronic databases were screened. Duplicate studies were excluded (433 articles), and 826 articles were excluded because the studies were conducted using clinical, ex vivo, and in vitro models. Only in vivo preclinical studies investigating the main mechanisms involved in differentiating fibroblasts into myofibroblasts in excisional wound healing in diabetic models were subjected to the eligibility analysis and considered for inclusion in the systematic review. After initial screening, the full texts of all potentially relevant studies were recovered and evaluated for eligibility. Studies were excluded based on the following criteria: (i) no full text available, (ii) secondary studies (i.e., editorials, commentaries, letters to the editor, and literature reviews without original data). The researchers (RDN, MMS, and RVG) independently analyzed the eligibility criteria, and doubts were resolved by consensus reached through discussion. The interrater agreement obtained via our search strategy was evaluated using the kappa coefficient (0.899). Finally, studies were selected that used diabetic murine models with excisional wounds, some of which implemented interventions and some did not, and which reported the performance of myofibroblasts in the healing process. After selecting eligible studies, the indirect screening of the reference lists of all selected studies was performed.

2.3 Data Extraction and Management

Considering the detailed characterization of all studies in the systematic review, qualitative and quantitative data were extracted using structured tables. Each table was formulated according to basic methodological requirements used to characterize the studies by descriptive level as follows:

Publication characteristics (author, year of publication, and country of origin); Experimental model characteristics (animal strain, sex, age, and weight);

Skin wound (type of lesion, site, initial area, number, anesthesia, asepsis, and injury time); Primary outcome (labeling for myofibroblasts and main cellular pathways activated); Secondary results (cellularity, synthesis of extracellular matrix components, neo angiogenesis, cell death, and immunological effectors).

2.4 Bias Analysis

The risk of bias was analyzed using the bias risk tool (RoB) from the Center for Systematic Review for Experimentation with Laboratory Animals (SYRCLE, Nijmegen, The Netherlands) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments, London, UK) guidelines. The SYRCLE instrument is based on the Cochrane Collaboration's RoB Tool, which is adjusted for bias factors that play a specific role in animal intervention studies. The objective was to avoid discrepancies in evaluating the methodological quality in animal experimentation. Signaling questions were answered to facilitate judgment based on the following domains and to increase the transparency and applicability: (i) the sequence generated was randomized; (ii) the blind selection bias of the animals was inadequate; (iii) the blinding of participants and personnel; (iv) the blinding of the outcome assessment; (v) incomplete outcome data; (vi) the data evaluation was complete; (vii) complete results data; (viii) allocation conditions; (ix) intervention data; (x) allocated in groups or individually; (xi) ethics committee data; (xii) data on participant exclusion; (xiii) data on the applied methodology; (xiv) statistical data; (xv) addressed the issue of revision; (xvi) bias due to problems not covered elsewhere in the table. The ARRIVE strategy requires the complete screening of all manuscript sections (abstract to acknowledgments and funding) to evaluate the completeness of scientific reports on animal studies. The screening strategy was based on short descriptions of essential characteristics such as baseline measurements, the sample size, animal allocation, randomization, experimental concealment, the statistical methods, ethical statements, and generalizability. A table summarizing all relevant and applicable aspects was constructed considering the specificity and aims of the systematic review. Individual adherence to bias criteria and the overall mean adherence were expressed as absolute and relative values [23] (Table S4).

3 RESULTS

3.1 Publication Characteristics

The initial search obtained 1405 studies, of which 706 were from PubMed–Medline, 260 from Scopus, and 439 from the Web of Science database. Initially, 433 were excluded as duplicates. After reading the titles and abstracts, 829 were excluded, with 143 studies being selected for full reading. Following the reading, 18 studies met the inclusion criteria and were

included in the final systematic review. No additional articles were found after reading the bibliographic references of the selected studies, as shown in figure 1.

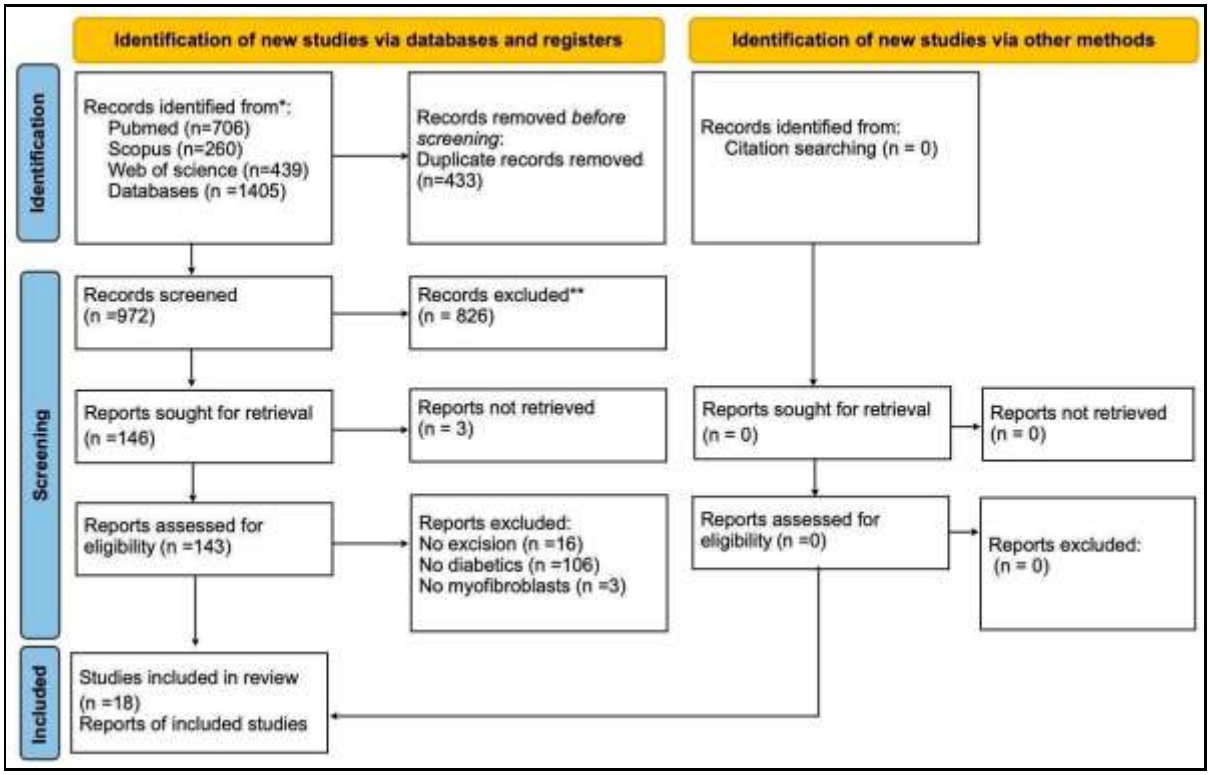

Figure 1 - PRISMA diagram. * Consider, when feasible, reporting the number of records identified from each database or register searched (rather than the total number across all databases/registers). ** If automation tools were used, indicate how many records were excluded by a human and how many were excluded by automation tools [24]. For more information, visit http://www.prisma-statement.org, accessed on 17 July 2021. Different phases of the selection of studies to conduct qualitative and quantitative analyses. Flow diagram of the systematic review literature search results. Based on "Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement", http://www.prisma-statement.org.

The 18 studies selected were from different countries, with China presenting the largest number at six in total (33.33%), followed by the US with three (16.6%); Japan with two (11.1%); and Malaysia, Russia, Korea, Spain, Iran, India, and Germany with one study each, representing 5.55%, respectively (Table S2). According to the number of articles by year, from 2012 onwards, more studies were described and selected for this research, with a more significant number of studies identified in 2020 (Figure S1). This indicates that, over the last few years, more researchers have investigated closure mechanisms for wounds associated with diabetes, especially regarding the main pathways involved. This increase has led to significant advances and/or improvements in treatments in this field.

3.2 Characteristics of Experimental Murine Models

The animals chosen were murine models, including both rats and mice. The most used strains of mice were C57BL/6 in 50% of studies (n = 9), while two studies (11.1%) used BKS mice, followed by Swiss albino (5.55%, n = 1) and ICR (5.55%, n = 1). The rats used included Sprague Dawley in 16.6% of studies (n = 3), followed by Wistar (11.1%, n = 2). Most studies used males (72.2%, n = 13), 22.2% used females (n = 4), and one (5.55%) used females and males. All animals used were diabetic; for the majority, this was primarily streptozotocin-induced, namely in 55.5% (n = 10), while 38.8% (n = 7) were genetically modified. Nine studies (50%) performed further tests to assess glucose levels. Animal ages were reported in 14 studies (77.7%). The ages ranged between 6 and 12 weeks in mice, while, in rats, only one study (5.55%) reported an age of between 8 and 10 weeks. Two studies (11.1%) reported using adult animals, while two others (11.1%) provided no details regarding the animals' age (Table S2).

3.3 Excisional Diabetic Wound Characteristics

All animals (100%) received dorsal excisional wounds. Asepsis was described in nine of the 18 studies (50%) selected. Sterilization with 70% ethanol was used in almost all studies (n = 8, 44.4%) that reported asepsis. All studies reported the excision wound size, ranging from 4 to 20 mm, with 10 mm being the most common size, described in four studies (22.2%), followed by 8 mm, described in three studies (16.6%). Incisional wounds of 4, 5, 6, 7, and 9 mm were performed in two studies (11.1%), and 9, 15, and 20mm wounds were created in one study each (5.5%). The number of wounds per animal was described in 13 studies (72.2%), ranging from one single wound to six wounds performed in mice and from one to four wounds in rats. Most studies (8–44.4%) reported the use of bandages to prevent catching, biting, and infection, followed by the individual conditioning of the animals (7–38.8%). Three studies (16.6%) reported no care following wounding, and one study (5.5%) only mentioned that no dressing was used.

The time spent observing the wound for further analysis was described in all studies (100%), with seven studies (38.8%) reporting a time period of up to 10 days. In six studies (33.3%), the time was between 11 and 14 days, while five studies (27.7%) adopted a time period of between 15 and 28 days. Twelve studies (66.6%) indicated the anesthetic used for the procedures, with pentobarbital being used in four studies (22.2%) and ketamine and xylazine used in different proportions in mg/kg in three studies (16.6%). The method used for animal

euthanasia was described in only two studies (11.1%): one by cervical dislocation and the other by pentobarbital overdose (Table S3).

3.4 Primary Outcomes

3.4.1 Updates and main results for TGF-Beta/SMAD signaling pathway

In most studies, n = 16 (88.8%), skin repair was impaired in diabetic animals, with a concomitant decrease in wound contraction and the reepithelialization rate for healing. TGF-beta played a central role in controlling these pathways, and the most instigated pathway described in this review was the TGF-beta/SMAD (n = 14; 77.7%). The results verified in this revision showed that the downregulation of TGF-beta1/SMAD signaling was an essential pathogenic mechanism in wound healing. This mechanism occurs after the phosphorylation of the type I receptor. It specifically recognizes and phosphorylates R-SMADs, including SMAD2 and SMAD3, and the phosphorylation of both destabilizes the interaction with the SMAD anchor for receptor activation (SARA) and increases the affinity for SMAD4 (also called Co-SMAD). This transcriptional complex (R-SMAD/Co-SMAD) is then translocated into the nucleus, where it regulates the transcription of TGF-beta target genes, such as the production of the alpha-SMA protein in the cytoskeleton, responsible for the differentiation of fibroblasts into myofibroblasts [25].

We observed that, in chronic diabetic disease, the TGF-beta I-II receptors are downregulated, with the subsequent absence of the phosphorylation of R-SMADs, which compromises the entire TNF-beta signaling pathway. These alterations were followed by TNF-alpha and MMP-9 upregulation and, consequently, an increase in the inflammatory phase.

3.4.2 Pathways related to oxidative stress and myofibroblast differentiation

Most of the studies included in this review also observed increased free radicals and reactive oxygen species (ROS) generation. Some studies named this process OxInflammation, in which the ROS work as secondary messengers, activating inflammation and oxidative stress in positive damage feedback. Other vital pathways related to myofibroblast differentiation were NFK-b, PKC, ROS/Bax-2/Bcl-2/Caspases, and JNK (n = 4, 22.2%); each pathway was mentioned separately in one of the studies. Six studies (33.3%) reported the activation of some pathways through ROS' presence. Increased oxidative stress in diabetic disease resulted in

decreased TGF-beta expression and, consequently, an inactive TAK/JNK or PKC/ERK pathway. This reduction inhibited the differentiation and proliferation of genes responsible for fibroblast differentiation into myofibroblasts, delaying the wound healing process.

Five studies (27.7%) reported that excess free radicals and ROS stimulated the expression of pro-inflammatory cytokines such as IL-1 beta, IL-6, and IL-18. The release of these cytokines intensifies the inflammatory process, compromising vascularization at the wound site and decreasing TGF-beta and alpha-SMA factors, which further promotes negative feedback between pro-inflammatory cytokine expression and TGF-beta/SMAD expression. Some studies also described the nuclear factor kappa beta (NF-κB) pathway as critical in the OxInflammation process, increasing in the wounds in diabetic models. The studies included in this review specifically described the role of NF-κB in regulating the survival, activation, and differentiation of innate immune cells and inflammatory T cells [26]. Moreover, in our review, we observed that the activation of the STAT1, IP3, and NF-κB pathways and membrane receptors like IFN-γR, TNFR, and toll-like in phagocytes, especially macrophages, occurred at the beginning of the wound healing process.

Some studies have also described ROS/Blc-2/Bax-2/Caspase pathway expression. These pathways are involved in the release of calcium into cell cytosol, activating pro- caspases and promoting apoptosis and necrosis in cells. The consequences of these path ways are the decreased expression of genes involved in myofibroblast differentiation and diminished collagen secretion and wound healing in diabetes. In addition, Ca+2 mobilization occurs, producing ROS and, consequently, NLRP3 inflammasome activation, resulting in the proteolytic cleavage of Caspase-1, which stimulates IL-1beta and IL-18 maturation, which is responsible for the infiltration of immune cells, vasodilatation, and an endothelial cell response, compromising the performance of TGF-beta and SMAD expression in myofibroblasts.

Therefore, this systematic review revealed how the different pathways are compromised in diabetic models, especially given that the TGF-beta, NF-κB, and NLRP3 molecules are key aspects of the wound healing process, as shown in figure 2. By deregulating these pathways, the OxInflammation process compromises the entire cascade of events that lead to healing.

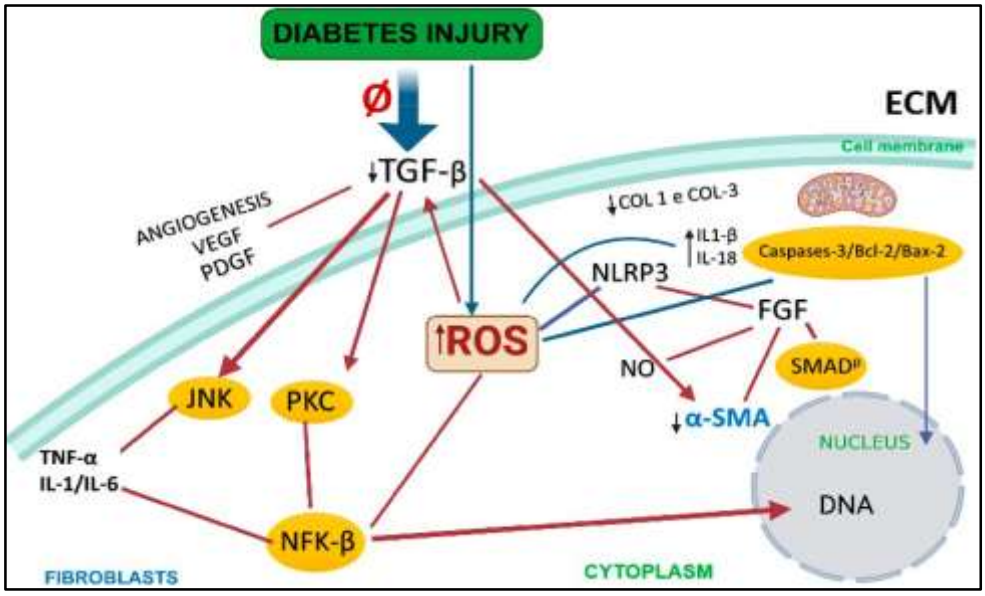

Figure 2 - In diabetics, the TGF-β molecule is downregulated, affecting different pathways that promote fibroblast differentiation into myofibroblasts, compromising wound healing. Growth factors VEGF and PDGF, as well as the angiogenesis process, are also severely reduced in diabetics. FGF suppresses α-SMA expression, which, under normal conditions, is activated by the TGF-β molecule. The pathways reported as affected and found in the examined studies are SMAD, JNK, NFK-β, and PKC. During the healing process, when the inflammatory response is accentuated and the ROS levels increase, pathways such as Bcl-2 and Bax-2 are activated, leading to increased Caspase levels and cell death. Therefore, an excessive ROS presence directly affects the pathways studied and compromises healing. Red arrows: inactive myofibroblast pathways. Blue arrow: active. Yellow circles: activated route. ECM = extracellular matrix; TGF-β = transforming growth factor beta; VEGF = vascular endothelial growth factor; PDGF = platelet-derived growth factor; ROS = reactive oxygen species; JNK = c-Jun N-terminal kinase; PKC = protein kinase c; NFK-β = factor nuclear kappa beta; TNF-α = tumor necrosis factor-alpha; ILs = interleukins; COL 1 and COL 3 = collagens 1 and 3, respectively; NLRP3 = NOD, LRR, and pyrin domain-containing protein 3; FGF = fibroblast growth factor; NO = nitric oxide; α-SMA = alpha-smooth muscle actin. Created with BioRender.com.

3.5 Risk of Bias and Methodological Quality Assessments

The bias of reports based on the SYRCLE analysis is detailed in Figures 3 and 4. The original studies adhered to a mean of 50 bias items (Figure 5). According to the methodological analysis criterion, none of the studies met all of these criteria (100%). We can highlight the low risk of bias for incomplete outcome data (attrition bias), selective reporting (reporting bias), and ethical approval. Most studies did not report the sequence generation process. The inadequate concealment of allocation before assignment was not reported in 12 studies (66.6%). No study reported information regarding participants' knowledge about the interventions (100%) or reported on the understanding of the interventions among the evaluators of the results (100%). The amount, nature, or handling of incomplete results and data reports due to the selective reporting of results and data on wound closure showed no sign of bias (100%). The conditions in which animals were kept were described in 61.1% of studies. Data on any intervention were reported in almost all studies (94.5%). Seven studies (38.8%) did not specify

whether the animals were kept in groups or cages. All studies (100%) considered relevant ethical aspects. The methodology used to obtain the results was reported in all cases (100%). Concerning the statistical methods used and the adequacy of these methods, only one study required clarification (5.55%). There was no sign of bias regarding significant populations that were excluded from the studies (100%). Finally, only two studies (11.1%) needed to clarify problems not covered in other parts of the work. All highlighted information is shown in figures 3, 4 and 5. Therefore, we can conclude that the results of the individual studies show that the current evidence is reliable, due to low levels of bias.

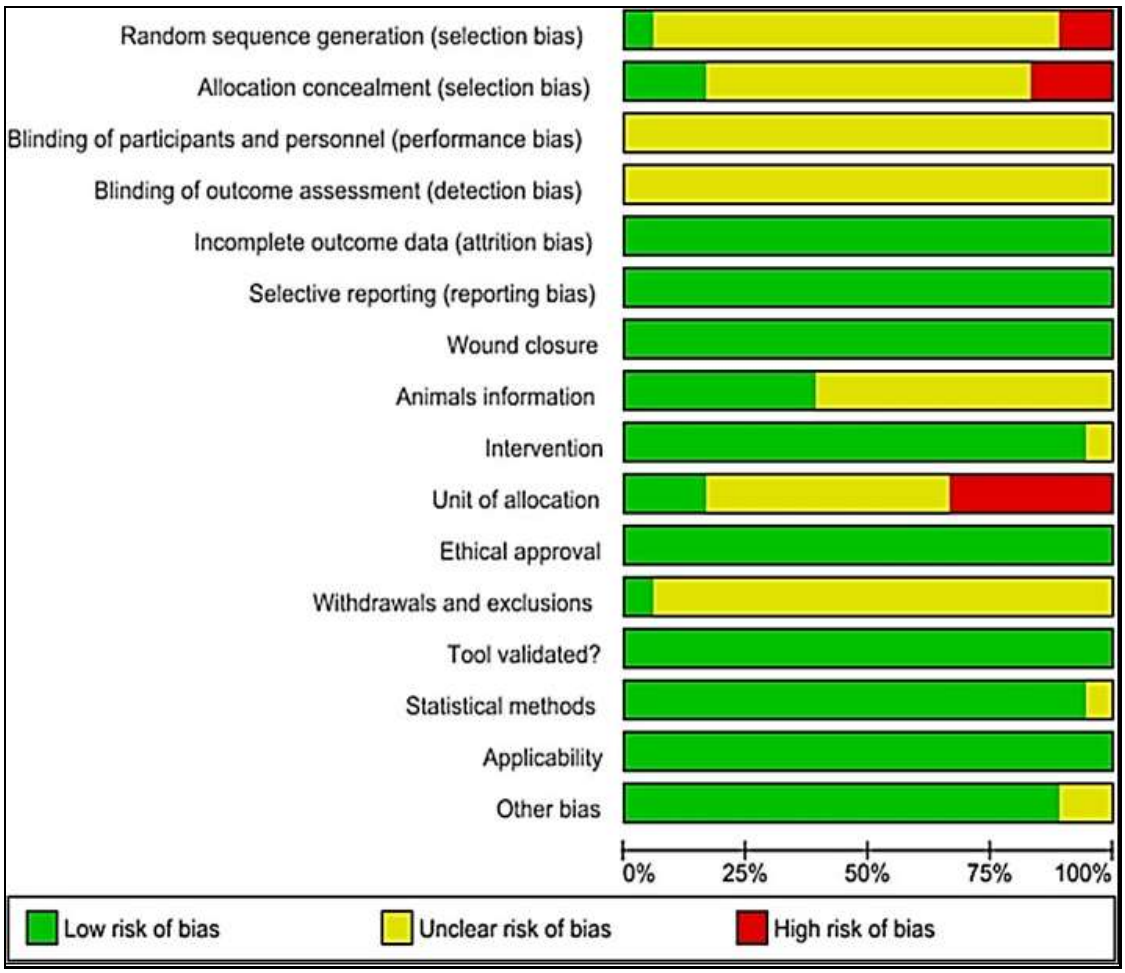

Figure 3 - Risk of bias and methodological quality indicators for all studies included in the systematic review using the Systematic Review Center for Laboratory Animal Experimentation (SYRCLE) bias risk assessment. Green: indicating low risk of bias (green); red: indicating high risk of bias; yellow: indicating that the item was not reported, resulting in an unknown risk of bias.

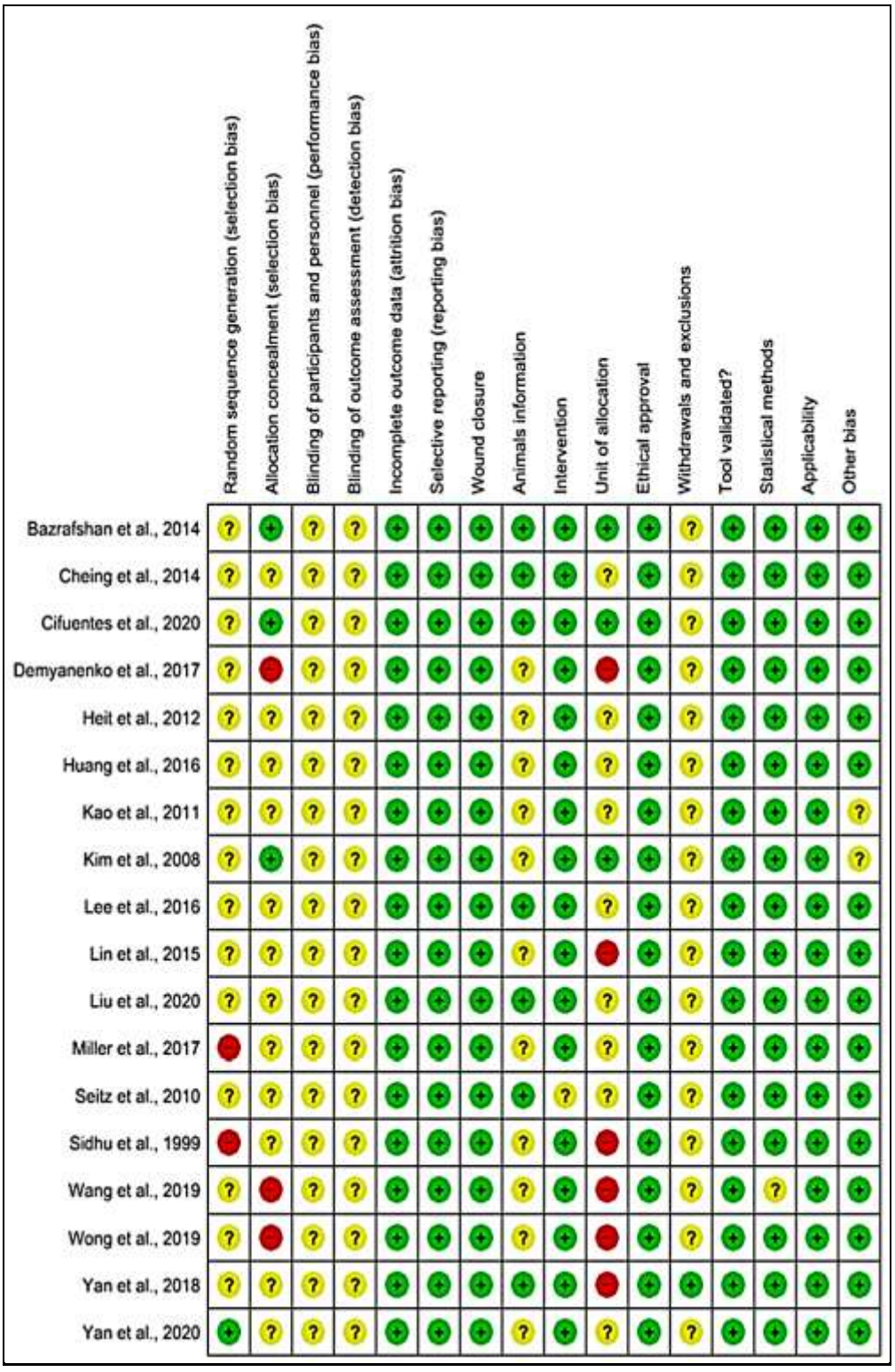

Figure 4 - Risk of bias summary: review authors' judgments about the risk of bias items for each included study. Green: low risk of bias; yellow: unclear risk of bias; and red: high risk of bias. References of the articles in the figure: Bazrafshan et al., 2014 [27]; Cheing et al., 2014 [28]; Cifuentes et al., 2020 [29]; Demyanenko et al., 2017 [30]; Heit et al., 2012 [31]; Huang et al., 2016 [32]; Kao et al., 2011 [33]; Kim et al., 2008 [34]; Lee et al., 2016 [35]; Lin et al., 2015 [36]; Liu et al., 2020 [37]; Miller et al., 2017 [38]; Seitz et al., 2010 [39]; Sidhu et al., 1999 [40]; Wang et al., 2019 [41]; Wong et al., 2019 [42]; Yan et al., 2018 [43]; Yan et al., 2020 [44].

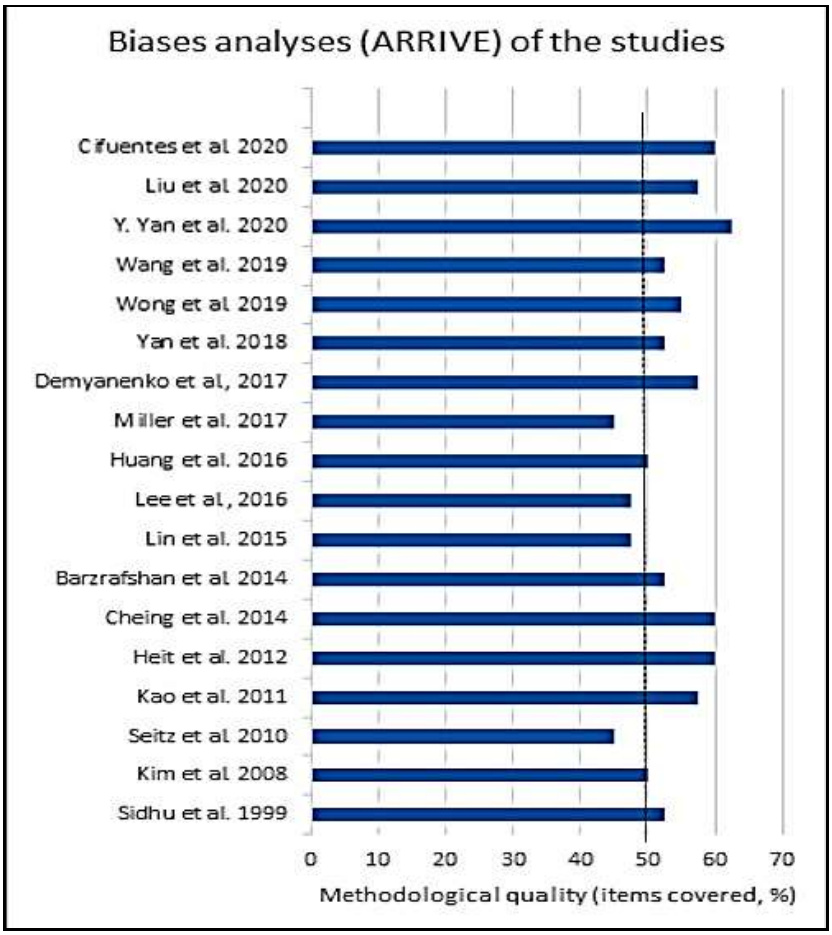

Figure 5 **-** Analysis of methodological bias (reporting quality) for each study included in the review. Based on Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (http://www. nc3rs.org.uk/arriveguidelines), accessed on 17 July 2021. The dotted line indicates the mean quality score (%). Detailed bias analysis stratified by domains and items evaluated is presented in Supplementary Materials. References of the articles in the figure: Cifuentes et al., 2020 [29]; Liu et al., 2020 [37]; Yan et al., 2020 [44]; Wang et al., 2019 [41]; Wong et al., 2019 [42]; Yan et al., 2018 [43]; Demyanenko et al., 2017 [30]; Miller et al., 2017 [38]; Huang et al., 2016 [32]; Lee et al., 2016 [35]; Lin et al., 2015 [36]; Bazrafshan et al., 2014 [27]; Cheing et al., 2014 [28]; Heit et al., 2012 [31]; Kao et al., 2011 [33]; Seitz et al., 2010 [39]; Kim et al., 2008 [34]; Sidhu et al., 1999 [40].

4. DISCUSSION

4.1 Studies' Characteristics

The search for therapies that help to heal skin wounds in diabetic models has grown significantly in recent years [45–47]. Studies show that skin wound healing is impaired in chronic diseases such as diabetes, showing decreased wound contraction and reepithelialization [48]. However, there is a lack of knowledge of the main mechanisms and pathways involved in

the contraction process of myofibroblasts in diabetic wounds [49]. Our study conducted a careful systematic review to verify and investigate the main mechanisms involved in differentiating fibroblasts into myofibroblasts in the contraction of cutaneous wounds in diabetic murine models. In this regard, we assembled research in this area and critically assessed the quality of the selected studies. Our results provide strong evidence that oxidative stress affects transdifferentiation to myofibroblasts, compromising the wound healing process in diabetes. In addition, it was possible to observe that oxidative stress coexists with inflammation, with a clear overlap in pathways and mechanisms during diabetes. The consequence of this crosstalk is a prolonged wound healing process due to increases in NLRP3 and consequently in IL-1b and IL-18, leading to the intensification of the downregulation of TGF-beta, a critical wound healing pathway. The OxInflammation environment also compromises the SMAD, c-Jun N-terminal kinase, protein kinase C, and nuclear factor kappa beta activating caspase pathways, leading to cell death.

Animal models are an important tool in recreating conditions that allow the investigation of clinical conditions to understand the wound healing process better and test new interventions. The species currently used in in vivo chronic wound models present advantages and limitations. It is known that the natural physiological mechanisms of wound healing in rodents differ from those of humans. In rodents, the Panniculus carnosus participates in wound contraction, resulting in its closure, while, in humans, the healing process occurs through reepithelialization [50]. Therefore, rodent wound models contract and heal at a rate that human skin does not achieve [51]. On the other hand, the porcine model has advantages in terms of its anatomical and physiological similarity to human skin. However, the number of studies that use this animal model is relatively small and variable, making the development of critical studies for systematic reviews difficult. Its more limited use is probably due to it being more costly to purchase, maintain, and keep, as well as requiring specialized handling for anesthetic and surgical interventions [52]. Given this, experimental murine models are widely used to investigate the action mechanisms and pathways related to wound healing, mainly given their low cost, availability, and ease of care and handling, allowing researchers to use a considerable number of animals in experiments, thus producing more reliable results [53]. Additionally, these experimental models are extensively used due to their more similar physiology to that of humans, the possibility of the histological monitoring of the healing process, and the ability to perform macroscopic, biochemical, and biomechanical measurements [54]. Although there are negative aspects to using murine models, the advantages generally outweigh the disadvantages [55].

An important parameter that should be analyzed for wound studies is the wound size and position. Choosing an appropriate location minimizes differences and interference, especially regarding the tensile strength and resistance of the cutaneous tissue [56]. All studies selected in this review performed excisional wounds on the dorsal areas of the animals, being an easily manipulable area that facilitates tissue collection to analyze factors such as wound contraction. Furthermore, it is a place that is difficult for animals to lick when they are properly and individually caged, which is advantageous, as the salivary glands are reservoirs of many rodent growth factors [57].

4.2 Transdifferentiation Pathways

According to our review, TGF-beta/SMAD signaling was the most cited pathway, followed by the NLRP3 pathway, being compromised in the diabetic models in the selected studies. TGF-beta is directly linked to the phosphorylation process of the SMAD complex, which, when phosphorylated, is activated and translocated into the nucleus, where it regulates TGF-beta target gene transcription, and ACTA2 gene transcription is activated to increase alpha-SMA protein production in the cytoskeleton. Once the TGF-beta-induced SMAD pathway is activated, numerous feedback mechanisms are activated to modulate the signaling duration and thus stimulate healing [25]. In diabetes, there is the negative regulation of TGF-beta/SMAD and a decrease in type I collagen transcription in fibroblast cell lines and, consequently, a reduction in tissue tensile strength [28]. Moreover, patients with chronic diabetic ulcers were characterized by the downregulation of the TGF-beta I-II receptors and the subsequent absence of the phosphorylation of R-SMAD [58], which compromises the entire TNF-beta signaling pathway. The long-lasting inflammatory phase leads to excessive ROS accumulation, which compromises the overall wound healing process. The TGF-beta pathway regulates the proliferation, survival, apoptosis, and cellular differentiation of many cells. It plays a role in all repair phases and is critical in regulating collagen deposition [23]. In this case, TGF-beta is a candidate mediator of ECM production and remodeling and plays a key role in the deposition and reorganization of the impaired ECM [59]. In this context, a reduction in TGF-beta was observed in diabetic murine models, the most important regulator of alpha-smooth muscle actin expression (alpha-SMA) in myofibroblasts, which impaired the contractile capacity of this cell type [13]. In diabetes, fibroblasts lose the ability to transform into myofibroblasts and differentiation depends on different pathways and mechanisms underlying the role of TGF-beta [17].

Among the pathways involved in this process, we can highlight SMAD, PKC, JNK, and NFK-beta, which are linked to TGF-beta receptor activation. According to Yan et al. [43], an inhibited PKC pathway in diabetics reduced the TGF-beta and alpha-SMA levels and, consequently, fibroblasts' differentiation into myofibroblasts. Similarly, the JNK pathway is con- trolled by TGF-beta receptor activation. It controls pro-apoptotic genes that lead fibroblast cells to cell death by apoptosis and necrosis, especially in diabetic models [60]. Addition- ally, JNK cellular apoptosis pathways can overlap with the ROS/BCL2/Bax 2/Caspase-3 pathways in diabetic models, which can be overexpressed. This promotes cytochrome release, apoptosis pathway activation, and consequently decreased collagen secretion and proliferation capacities, thereby compromising the scar density [44]. On the other hand, the NFK-beta pathway also regulates nitric dioxide (NO2), which macrophages produce during respiratory bursts. Together with IL-1 beta and TNF-alpha, it is involved in inflammatory skin reactions. Importantly, IL-1 beta and TNF-alpha expression are positive in diabetics, which might explain the increased inflammatory process in diabetes [37]. Consequently, there is excessive ROS generation in diabetes, mainly due to acute increases in serum glucose and oxidative stress generation. As such, we can conclude that NF-κB is a transcription factor involved in controlling the expression of several genes linked to the inflammatory and oxidative stress responses and plays a critical role in regulating the survival, activation, and differentiation of innate immune cells and inflammatory T cells [26].

Oxidative balance is critical in healing, and the role of ROS can be favorable or deleterious [19]. As reported by Lin et al. [36], NADPH oxidase 4 (NOX 4) produces free radicals and ROS, especially superoxide and H2O2, inside phagocytes. The ROS generated from the NOX system mediate pro-inflammatory effects in vascular tissue, and, in phagocytic cells, electrons are transported across the membrane to extracellular oxygen. These superoxides (oxygen) may interact with intracellular messengers, activating redox-sensitive transcription factors such as kappa B and expressing a wide range of adhesion molecules involved in inflammation processes [7]. Different isoforms have been linked to the development of various diseases and disorders, such as cancer, hypertension, stroke, heart failure, neurodegenerative diseases, and diabetes [61]. However, their role is still controversial, and research is ongoing. However, we know that, in diabetes, there is an increase in NOX 4 and ROS. Furthermore, the SMAD pathway, which TGF-beta induces, is inhibited and decreases the activation of myofibroblasts. The excessive ROS generation in diabetes is mainly due to significant increases in serum glucose and oxidative stress. In addition, increased levels of ROS also increase the NLRP3 inflammasome pathways, promoting an OxInflammation environment. Therefore, by

decreasing oxidative stress, the inflammatory phase is ameliorated, but this cannot be long-lasting given the decreased levels of IL-1 beta and IL-18 cytokines, increased vascularization due to the release of VEGF, increased TGF-beta presence, and consequently increased alpha-SMA and myofibroblast expression [30].

Considering the OxInflammation environment, different proteins, essential in myofibroblast differentiation, are suppressed in the diabetic model, negatively interfering with wound healing [37]. We can highlight the protein complex NLRP3, which is vital for the inflammatory process. The excessive or altered regulation of NLRP3 inflammasome activity is related to the pathogenesis of a wide variety of inflammatory, autoimmune, and degenerative diseases. There are two signals for NLRP3 inflammasome activation. Signal 1 is triggered by pattern recognition receptor signaling or cytokines, leading to the transcriptional activation of NLRP3 inflammasome components [62], and signal 2 occurs when numerous molecular and cellular events, including excessive mitochondrial ROS pro- duction (mtROS), ion flux, and lysosomal damage, are involved in NLRP3 inflammasome activation and enhancement [30]. A better understanding of the molecular mechanisms underlying NLRP3 inflammasome activation will help us to develop prevention and treatment methods for NLRP3 inflammasome-related diseases [62].

Another protein that is compromised in diabetes patients is the proliferating cell nuclear antigen (PCNA) protein, which is involved in DNA replication, repair, and cell cycle regulation. The reduction of this protein negatively affects cell proliferation and decreases angiogenesis and fibroblast growth factor (FGF), known as an essential mitotic factor [27], thereby impairing healing due to the reduced vascularization arising in diabetics [63]. The high metabolic rate in injured tissue requires a good supply of oxygen and nutrients, such that forming new vessels and restoring blood flow is essential for tissue repair [64]. Growth factors were also reported to be inhibited or reduced in diabetics. Fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) act in conjunction with TGF-beta to regulate the migration, proliferation, angiogenesis, and production of the fibroblast ECM [33]. As such, this study focused on mechanisms that influence the differentiation of fibroblasts into myofibroblasts. Here, we observed that the OxInflammation environment plays a crucial role, affecting wound healing in different ways, regardless of the wound size or delays in healing onset. Understanding these pathways is essential to help identify therapeutic targets and the resolution levels involved in regulating the inflammatory response at a clinical level, improving the quality of skin regeneration. It would be appropriate to conduct experimental studies in future research to understand the role of the

most important markers of the OxInflammation process, such as 4-hydroxy-2-nonenal (4-HNE) and 8-hydroxy-2′-deoxyguanosine (8-OHdG), and antioxidant enzymes such as catalase, superoxide dismutase, and glutathione, and to understand the whole oxidative cellular chain during the inflammatory and oxidative stress process.

4.3 Future Perspectives

Prolonged wound healing can result in increased damage to cells, chronic inflammation, and high levels of ROS, compromising the repair process. Therefore, it is essential to promote effective and rapid healing whenever possible to minimize these adverse effects. Vascular diseases, obesity, burns, and diabetes are the main factors contributing to chronic wounds. The hypoxia response pathway is one of the most important activated pathways, particularly in hyperglycemic patients, resulting in impaired cell migration and vascularization. In this context, understanding the mechanisms activated during wound healing is crucial in designing appropriate treatment strategies to minimize the risk of infection. De- spite the availability of several treatment methods, progress in this field is hampered by the limited understanding of the primary mechanisms activated during tissue recovery. These complex mechanisms involve biological processes that must be thoroughly understood to enhance the treatment efficacy. In this context, this review allows an understanding of the ways in which oxidative stress affects transdifferentiation to myofibroblasts and compromises the wound healing process in diabetes. It was found that it is necessary to obtain more information about the mechanisms activated using a preclinical model before the results are translated to the human context. In addition, this systematic review indicates that investing in therapies with high antioxidant potential is necessary to promote the good recovery of skin lesions in diabetes.

4.4 Limitations

Although the systematic review has been listed as a high-level method for the blind evaluation of studies using specific tools, there are some limitations. After reviewing all possible pathways outlined in this review, it is clear that the relationship between inflammation and oxidative stress mechanisms is not well understood. It has been supposed that the process could occur through multiple pathways, but the bias analysis of the in vivo studies also uncovered some underreported information. The primary information not re- ported included random sequence generation, blinding, and personal and random outcome assessment. The

significant heterogeneity, primarily associated with in vivo experimental results, makes it difficult to replicate the work and diminishes the reliability of the research. For example, a lack of information regarding the animal age was identified in most studies, which may reflect a reporting bias as it compromises the report's quality. Although the individual bias scores were variable, they did not present a temporal influence (year of publication). This finding indicates that the reporting bias was systematically reproduced through the research process, independent of well-known advances in analytical and statistical methods and the increasing availability of guidelines and regulatory strategies adopted to ensure the completeness of the scientific reports in preclinical studies. In all studies included, simple constructs such as experimental blindness, animal allocation and age, sample size calculation, and the rational choice of the administration route were the primary sources of bias. Although these elements are essential sources of intrinsic bias, they also are easily adjustable. The construction of more rigorous experimental designs with acceptable construct validity can be achieved in future studies, especially for translation to the clinical context.

5 CONCLUSIONS

Based on the data collected from this systematic review, we can conclude that diabetes impairs the transformation of fibroblasts into myofibroblasts, primarily by affecting the expression of growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-beta), compromising fibroblast migration and proliferation, angiogenesis, and the production of the extracellular matrix (ECM), especially collagens I and III. The authors observed that the excessive ROS presence in diabetes promoted an OxInflammation environment, intensifying TGF-beta's downregulation and NLRP3's upregulation, which are critical wound healing pathways. The OxInflammation environment also compromised the SMAD, c-Jun N-terminal kinase, protein kinase C, and nuclear factor kappa beta activating caspase pathways, leading to cell death. Therefore, our results provide new insights into how the OxInflammation environment can affect myofibroblasts' transdifferentiation, delaying wound healing. The findings indicated that the main methodological limitations of the identified studies were based on the recurrent underreporting of the experimental designs, but the development of more comprehensive and controlled studies seems feasible. However, more randomized and controlled studies are required to determine whether and to what extent additional OxInflammation mechanisms can affect transdifferentiation to myofibroblasts,

prolonging the wound healing process. In addition, further studies are needed to analyze the role of the critical markers of OxInflammation and better understand the oxidative cellular chain in diabetes, especially in the clinical context.

Supplementary Materials
The following supporting information can be downloaded at: https:// www.mdpi.com/article/10.3390/ijms25168992/s1.

Author Contributions
Conceptualization, L.L.S. and M.M.S.; methodology, L.L.S. and M.M.S.; validation, R.D.N., M.M.-S. and R.V.G.; formal analysis, L.L.S., M.M.-S. and R.V.G.; investigation, L.L.S. and M.M.-S.; resources, L.L.S.; data curation, L.L.S., M.M.-S. and R.D.N.; writing-original draft preparation, L.L.S. and M.M.-S.; writing—review and editing, L.L.S., M.M.-S., R.D.N., M.M.-S. and R.V.G.; visualization, M.M.-S. and M.M.-S.; supervision, M.M.-S. and R.V.G.; funding acquisition, M.M.-S. and R.V.G. All authors have read and agreed to the published version of the manuscript.

Funding
This work was supported by the Brazilian agencies Fundação do Amparo à Pesquisa do Estado de Minas Gerais—FAPEMIG (processes APQ-01325-21, AQP-03519-22, and AQP-04164-22) and Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (310413/2023- 0; 403194/2023-7; and 306733/2023-4). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil CAPES (finance code 001).

Institutional Review Board Statement: Not applicable.
Informed Consent Statement: Not applicable.

Data Availability Statement
The original contributions presented in the study are included in the article/Supplementary Materials; further inquiries can be directed to the corresponding author/s.

Acknowledgments
The authors gratefully acknowledge the Fundação do Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES), which have consented to the acknowledgement.

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

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SUPPLEMENTARY MATERIAL

Table S1 - Supporting Information

S1 Table Complete search strategy filters and number of studies recovery and data bases is PubMed and SCOPUS and Web of Science.

Data base	Descriptors	Items Found	Time	Date
PUBMED	#1 Wound Healing" [MeSH terms] OR " Wound Healing "[TIAB]	169 961	13:41:45	04/10/2021
	#2 "myofibroblasts"[MeSH Terms] OR "myofibroblasts"[TIAB]	11 312	13:42:13	04/10/2021
	# 3 ("Skin" [MeSH terms] OR "Dermis" [MeSH terms] OR "Granulation Tissue"[MeSH terms] OR "Epidermis"[MeSH terms] OR "Keratinocytes"[MeSH terms] OR "Integumentary System"[MeSH terms] OR "Dermatology"[MeSH terms] OR "Dermoscopy"[MeSH terms] OR "Wounds and Injuries"[MeSH terms] OR "Fibrosis"[MeSH terms] OR "Skin injuries"[TIAB] OR "Skin fibrosis"[TIAB] OR "Skin scars"[TIAB] OR "Cicatrix"[MeSH terms])	1 364 342	13:42:35	04/10/2021
	#4 Animal ("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSH Terms:noexp] OR "chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSH Terms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSH Terms:noexp] OR "primates"[MeSH Terms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSH Terms:noexp] OR	7 273 832	13:43:13	04/10/2021

	"strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR "catarrhini"[MeSH Terms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "hominidae"[MeSH Terms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongo pygmaeus"[MeSH Terms]) OR ((animals[TIAB] OR animal[TIAB] OR mice[TIAB] OR mus[TIAB] OR mouse[TIAB] OR murine[TIAB] OR woodmouse[TIAB] OR rats[TIAB] OR rat[TIAB] OR murinae[TIAB] OR muridae[TIAB] OR cottonrat[TIAB] OR cottonrats[TIAB] OR hamster[TIAB] OR hamsters[TIAB] OR cricetinae[TIAB] OR rodentia[TIAB] OR rodent[TIAB] OR rodents[TIAB] OR pigs[TIAB] OR pig[TIAB] OR swine[TIAB] OR swines[TIAB] OR piglets[TIAB] OR piglet[TIAB] OR boar[TIAB] OR boars[TIAB] OR "sus scrofa"[TIAB] OR ferrets[TIAB] OR ferret[TIAB] OR polecat[TIAB] OR polecats[TIAB] OR "mustela putorius"[TIAB] OR "guinea pigs"[TIAB] OR "guinea pig"[TIAB] OR cavia[TIAB] OR callithrix[TIAB] OR marmoset[TIAB] OR marmosets[TIAB] OR cebuella[TIAB] OR hapale[TIAB] OR octodon[TIAB] OR chinchilla[TIAB] OR chinchillas[TIAB] OR gerbillinae[TIAB] OR gerbil[TIAB] OR gerbils[TIAB] OR jird[TIAB] OR jirds[TIAB] OR merione[TIAB] OR meriones[TIAB] OR rabbits[TIAB] OR rabbit[TIAB] OR hares[TIAB] OR hare[TIAB] OR diptera[TIAB] OR flies[TIAB] OR fly[TIAB] OR dipteral[TIAB] OR drosphila[TIAB] OR drosophilidae[TIAB] OR cats[TIAB] OR cat[TIAB] OR carus[TIAB] OR felis[TIAB] OR nematoda[TIAB] OR nematode[TIAB] OR nematoda[TIAB] OR nematode[TIAB] OR nematodes[TIAB] OR sipunculida[TIAB] OR dogs[TIAB] OR dog[TIAB] OR canine[TIAB] OR canines[TIAB] OR canis[TIAB] OR sheep[TIAB] OR sheeps[TIAB] OR mouflon[TIAB] OR mouflons[TIAB] OR ovis[TIAB] OR goats[TIAB] OR goat[TIAB] OR capra[TIAB] OR capras[TIAB] OR rupicapra[TIAB] OR chamois[TIAB] OR haplorhini[TIAB] OR monkey[TIAB] OR monkeys[TIAB] OR anthropoidea[TIAB] OR anthropoids[TIAB] OR saguinus[TIAB] OR tamarin[TIAB] OR tamarins[TIAB] OR leontopithecus[TIAB] OR hominidae[TIAB] OR ape[TIAB] OR apes[TIAB] OR pan[TIAB] OR paniscus[TIAB] OR "pan paniscus"[TIAB] OR bonobo[TIAB] OR bonobos[TIAB] OR troglodytes[TIAB] OR "pan troglodytes"[TIAB] OR gibbon[TIAB] OR gibbons[TIAB] OR siamang[TIAB] OR siamangs[TIAB] OR nomascus[TIAB] OR symphalangus[TIAB] OR chimpanzee[TIAB] OR chimpanzees[TIAB] OR prosimians[TIAB] OR "bush baby"[TIAB] OR prosimian[TIAB] OR bush babies[TIAB] OR galagos[TIAB] OR galago[TIAB] OR pongidae[TIAB] OR gorilla[TIAB] OR gorillas[TIAB] OR pongo[TIAB] OR pygmaeus[TIAB] OR "pongo pygmaeus"[TIAB] OR orangutans[TIAB] OR pygmaeus[TIAB] OR lemur[TIAB] OR lemurs[TIAB] OR lemuridae[TIAB] OR horse[TIAB] OR horses[TIAB] OR pongo[TIAB] OR equus[TIAB] OR cow[TIAB] OR calf[TIAB] OR bull[TIAB] OR chicken[TIAB] OR chickens[TIAB] OR gallus[TIAB] OR quail[TIAB] OR bird[TIAB] OR birds[TIAB] OR quails[TIAB] OR poultry[TIAB] OR poultries[TIAB] OR fowl[TIAB] OR fowls[TIAB] OR reptile[TIAB] OR reptilia[TIAB] OR reptiles[TIAB] OR snakes[TIAB] OR snake[TIAB] OR lizard[TIAB] OR lizards[TIAB] OR alligator[TIAB] OR alligators[TIAB] OR crocodile[TIAB] OR crocodiles[TIAB] OR turtle[TIAB] OR turtles[TIAB] OR amphibian[TIAB] OR amphibians[TIAB] OR amphibia[TIAB] OR frog[TIAB] OR frogs[TIAB] OR bombina[TIAB] OR salientia[TIAB] OR toad[TIAB] OR toads[TIAB] OR "epidalea calamita"[TIAB] OR salamander[TIAB] OR salamanders[TIAB] OR eel[TIAB] OR eels[TIAB] OR fish[TIAB] OR fishes[TIAB] OR pisces[TIAB]			

	OR catfish[TIAB] OR catfishes[TIAB] OR siluriformes[TIAB] OR arius[TIAB] OR heteropneustes[TIAB] OR sheatfish[TIAB] OR perch[TIAB] OR perches[TIAB] OR percidae[TIAB] OR perca[TIAB] OR trout[TIAB] OR trouts[TIAB] OR char[TIAB] OR chars[TIAB] OR salvelinus[TIAB] OR "fathead minnow"[TIAB] OR minnow[TIAB] OR cyprinidae[TIAB] OR carps[TIAB] OR carp[TIAB] OR zebrafish[TIAB] OR zebrafishes[TIAB] OR goldfish[TIAB] OR goldfishes[TIAB] OR guppy[TIAB] OR guppies[TIAB] OR chub[TIAB] OR chubs[TIAB] OR tinca[TIAB] OR barbels[TIAB] OR barbus[TIAB] OR pimephales[TIAB] OR promelas[TIAB] OR "poecilia reticulata"[TIAB] OR mullet[TIAB] OR mullets[TIAB] OR seahorse[TIAB] OR seahorses[TIAB] OR mugil curema[TIAB] OR atlantic cod[TIAB] OR shark[TIAB] OR sharks[TIAB] OR catshark[TIAB] OR anguilla[TIAB] OR salmonid[TIAB] OR salmonids[TIAB] OR whitefish[TIAB] OR whitefishes[TIAB] OR salmon[TIAB] OR salmons[TIAB] OR sole[TIAB] OR solea[TIAB] OR "sea lamprey"[TIAB] OR lamprey[TIAB] OR lampreys[TIAB] OR pumpkinseed[TIAB] OR sunfish[TIAB] OR sunfishes[TIAB] OR tilapia[TIAB] OR tilapias[TIAB] OR turbot[TIAB] OR turbots[TIAB] OR flatfish[TIAB] OR flatfishes[TIAB] OR sciuridae[TIAB] OR squirrel[TIAB] OR squirrels[TIAB] OR chipmunk[TIAB] OR chipmunks[TIAB] OR suslik[TIAB] OR susliks[TIAB] OR vole[TIAB] OR voles[TIAB] OR lemming[TIAB] OR lemmings[TIAB] OR muskrat[TIAB] OR muskrats[TIAB] OR lemmus[TIAB] OR otter[TIAB] OR otters[TIAB] OR marten[TIAB] OR martens[TIAB] OR martes[TIAB] OR weasel[TIAB] OR badger[TIAB] OR badgers[TIAB] OR ermine[TIAB] OR mink[TIAB] OR minks[TIAB] OR sable[TIAB] OR sables[TIAB] OR gulo[TIAB] OR gulos[TIAB] OR wolverine[TIAB] OR wolverines[TIAB] OR minks[TIAB] OR mustela[TIAB] OR llama[TIAB] OR llamas[TIAB] OR alpaca[TIAB] OR alpacas[TIAB] OR camelid[TIAB] OR camelids[TIAB] OR guanaco[TIAB] OR guanacos[TIAB] OR chiroptera[TIAB] OR chiropteras[TIAB] OR bat[TIAB] OR bats[TIAB] OR fox[TIAB] OR foxes[TIAB] OR iguana[TIAB] OR iguanas[TIAB] OR xenopus laevis[TIAB] OR parakeet[TIAB] OR parakeets[TIAB] OR parrot[TIAB] OR parrots[TIAB] OR donkey[TIAB] OR donkeys[TIAB] OR mule[TIAB] OR mules[TIAB] OR zebra[TIAB] OR zebras[TIAB] OR shrew[TIAB] OR shrews[TIAB] OR bison[TIAB] OR bisons[TIAB] OR buffalo[TIAB] OR buffaloes[TIAB] OR deer[TIAB] OR deers[TIAB] OR bear[TIAB] OR bears[TIAB] OR panda[TIAB] OR pandas[TIAB] OR "wild hog"[TIAB] OR "wild boar"[TIAB] OR fitchew[TIAB] OR fitch[TIAB] OR beaver[TIAB] OR beavers[TIAB] OR jerboa[TIAB] OR jerboas[TIAB] OR capybara[TIAB] OR capybaras[TIAB]) NOT medline[subset])			
	Total: #1 and #2 and #3 and #4	706	13:43:51	04/10/2021

Data base	Descriptors	Items Found	Time	Date
S C O P U S	#1 TITLE-ABS-KEY (“Wound Healing”)	182 349	14:52:15	04/10/2021
	#2 TITLE-ABS-KEY(“myofibroblasts”)	11 935	14:53:19	04/10/2021
	#3 (TITLE-ABS-KEY(Skin) OR TITLE-ABS-KEY(Dermis) OR TITLE-ABS-KEY (“Granulation Tissue”) OR TITLE-ABS-KEY(Epidermis) OR TITLE-ABS-KEY(Keratinocyte*) OR TITLE-ABS-KEY (Integumentary System) OR TITLE-ABS-KEY(Dermatology) OR TITLE-ABS-KEY(Dermoscopy) OR TITLE-ABS-KEY(Skin wounds) OR TITLE-ABS-KEY(Skin	1 393 394	14:54:16	04/10/2021

	injuries) OR TITLE-ABS-KEY(Skin fibrosis) OR TITLE-ABS-KEY(Skin scar*) OR (Skin cicatrix))			
	Total: #1 and #2 and #3	814	14:54:45	04/10/2021
	Keywords Animal Model	260	14:55:20	04/10/2021
Data base	**Descriptors**	**Items Found**	**Time**	**Date**
WEB of SCIENCE	#1 TS=(Wound Healing)	89 211	15:01:15	04/10/2021
	#2 TS= myofibroblasts	11 839	15:04:10	04/10/2021
	#3 TS=Skin OR TS=Dermis OR TS=Granulation tissue OR TS=Epidermis OR TS=Keratinocyte OR TS=Integumentary system OR TS=Dermatology OR TS=Dermoscopy OR TS=Skin wounds OR TS=Skin injuries OR TS=Skin fibrosis OR TS=Skin scar OR TS=Skin cicatrix	718 309	15:04:28	04/10/2021
	#4 Animal ("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSH Terms:noexp] OR "chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSH Terms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSH Terms:noexp] OR "primates"[MeSH Terms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSH Terms:noexp] OR "strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR "catarrhini"[MeSH Terms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "hominidae"[MeSH Terms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongo pygmaeus"[MeSH Terms]) or ((animals[TIAB] OR animal[TIAB] OR mice[TIAB] OR mus[TIAB] OR mouse[TIAB] OR murine[TIAB] OR woodmouse[TIAB] OR rats[TIAB] OR rat[TIAB] OR murinae[TIAB] OR muridae[TIAB] OR cottonrat[TIAB] OR cottonrats[TIAB] OR hamster[TIAB] OR hamsters[TIAB] OR cricetinae[TIAB] OR rodentia[TIAB] OR rodent[TIAB] OR rodents[TIAB] OR pigs[TIAB] OR pig[TIAB] OR swine[TIAB] OR swines[TIAB] OR piglets[TIAB] OR piglet[TIAB] OR boar[TIAB] OR boars[TIAB] OR "sus scrofa"[TIAB] OR ferrets[TIAB] OR ferret[TIAB] OR polecat[TIAB] OR polecats[TIAB] OR "mustela putorius"[TIAB] OR "guinea pigs"[TIAB] OR "guinea pig"[TIAB] OR cavia[TIAB] OR callithrix[TIAB] OR marmoset[TIAB] OR marmosets[TIAB] OR cebuella[TIAB] OR hapale[TIAB] OR octodon[TIAB] OR chinchilla[TIAB] OR chinchillas[TIAB] OR gerbillinae[TIAB] OR gerbil[TIAB] OR gerbils[TIAB] OR jird[TIAB] OR jirds[TIAB] OR merione[TIAB] OR meriones[TIAB] OR rabbits[TIAB] OR rabbit[TIAB] OR hares[TIAB] OR hare[TIAB] OR diptera[TIAB] OR flies[TIAB] OR fly[TIAB] OR dipteral[TIAB] OR drosphila[TIAB] OR drosophilidae[TIAB] OR cats[TIAB] OR cat[TIAB] OR carus[TIAB] OR felis[TIAB] OR nematoda[TIAB] OR nematode[TIAB] OR nematoda[TIAB] OR	6 491 034	15:08:36	04/10/2021

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	ermine[TIAB] OR mink[TIAB] OR minks[TIAB] OR sable[TIAB] OR sables[TIAB] OR gulo[TIAB] OR gulos[TIAB] OR wolverine[TIAB] OR wolverines[TIAB] OR minks[TIAB] OR mustela[TIAB] OR llama[TIAB] OR llamas[TIAB] OR alpaca[TIAB] OR alpacas[TIAB] OR camelid[TIAB] OR camelids[TIAB] OR guanaco[TIAB] OR guanacos[TIAB] OR chiroptera[TIAB] OR chiropteras[TIAB] OR bat[TIAB] OR bats[TIAB] OR fox[TIAB] OR foxes[TIAB] OR iguana[TIAB] OR iguanas[TIAB] OR xenopus laevis[TIAB] OR parakeet[TIAB] OR parakeets[TIAB] OR parrot[TIAB] OR parrots[TIAB] OR donkey[TIAB] OR donkeys[TIAB] OR mule[TIAB] OR mules[TIAB] OR zebra[TIAB] OR zebras[TIAB] OR shrew[TIAB] OR shrews[TIAB] OR bison[TIAB] OR bisons[TIAB] OR buffalo[TIAB] OR buffaloes[TIAB] OR deer[TIAB] OR deers[TIAB] OR bear[TIAB] OR bears[TIAB] OR panda[TIAB] OR pandas[TIAB] OR "wild hog"[TIAB] OR "wild boar"[TIAB] OR fitchew[TIAB] OR fitch[TIAB] OR beaver[TIAB] OR beavers[TIAB] OR jerboa[TIAB] OR jerboas[TIAB] OR capybara[TIAB] OR capybaras[TIAB]) NOT medline[subset])			
	#1 AND #2 AND #3 AND #4	**439**	**15:09:15**	**04/10/2021**

Table S2 - Supporting Information

S2 Table. General characteristics of the experimental models used in all studies included in this systematic review

Reference	Country	Strain	Sex	Age	Weight	Diabetics
Miller et al., 2017	USA	C57BL/6	F	10-12 wk	35-45 g	Genetic
Wong et al., 2019	MAS	SpragueDawley	M	?	200-250g	Induced
Seitz et al., 2010	GER	C57BL/6J	F	6-12 wk	?	Induced
Yan et al., 2018	CHN	ICR	M	6 wk	25g	Induced
Bazrafshan et al., 2014	IRA	SpragueDawley	M	Adult	260-280g	Induced
Cifuentes et al., 2020	SPA	Wistar	F	Adult	183-260g	Induced
Sidhu et al., 1999	IND	C57BL-Swiss albino	F/M	8-10 wk	250–300g	Ind/gen
Wang et al., 2019	USA	BKS	F	11-12 wk	?	Genetic
Kim et al., 2008	JPN	BKS	M	10 wk	?	Genetic
Heit et al., 2012	USA	C57BL/ksj	M	8-10 wk	?	Genetic
Lin et al., 2015	CHN	Wistar	M	?	?	Induced
Demyanenko et al., 2017	RUS	C57BL/ksj	M	9 wk	?	Genetic
Kao et al., 2011	CHN	C57BL/J	M	10-12 wk	?	Genetic
Yan et al., 2020	CHN	C57BL/J	M	6 wk	?	Induced
Huang et al., 2016	JPN	C57BL/ksj	M	10 wk	?	Genetic

Cheing et al., 2014	CHN	SpragueDawley	M	8-10 wk	280-320g	Induced
Liu et al., 2020	CHN	C57BL/6	M	8 wk	?	Induced
Lee et al., 2016	KOR	C57BL/6	M	8 wk	?	Induced

GER = Germany, CHN = China, d = day, SPA= Spain, F = female, g= gram, IND = India, IRA= Iran, KOR = South Korea, M = male, MAL = Malaysia, JPN=Japan, RUS = Russia, USA = United States of America,
wk = week

Table S3 - Supporting Information

Table S3. General characteristics of the wounds in all studies included in this systematic review

	Wound						
Animal model: murine model							
Reference	**Assepsias**	**Biopsia day**	**Site**	**Size**	**Number of wounds for animal**	**Anesthesia (drug and dose)**	**Euthanasia (methods)**
Miller et al., 2017	?	0/3/7/14/21/28	D	8 mm	1	?	?
Wong et al., 2019	?	7/14	D	6 mm	4	?	?
Seitz et al., 2010	EtOH 70%	1/3/5/7/11	D	5 mm	6	Ket/Xyl ?	?
Yan et al., 2018	EtOH 70%	7/14	D	7 mm	1	Chloral hydrate (400 mg/kg)	Cervical deslocation
Bazrafshan et al., 2014	?	3/5/7	D	8 mm	4	?	?
Cifuentes et al., 2020	Sterilized dressing	3/7/14/21	D	15 mm	1	Ket (90 mg/kg)/ Xyl (10 mg/kg)	?
Sidhu et al., 1999	EtOH 70%	4/7/10	D	8 mm	6	Pentobarbitone (30 mg/kg)	?
Wang et al., 2019	?	1/4/7/10	D	8 mm	2	Isoflurane ?	?
Kim et al., 2008	EtOH 70%	7/14	D	10 mm^2	?	Pentobarbitone (30 mg/kg)	Pentobarbitone (30 mg/kg)
Heit et al., 2012	EtOH 70%	2/ 4/ 7	D	10 mm^2	?	Pentobarbitone (60 mg/kg)	?
Lin et al., 2015	?	12	D	6 mm	4	?	?
Demyanenko et al., 2017	EtOH 70%	1/3/5/ 7	D	7 mm	1	Zoletil 50mg/Kg	?
Kao et al., 2011	?	3/7/10/14/21/28	D	10 mm^2	?	?	?
Yan et al., 2020	?	1/5/8/14	D	9 mm	?	Avertin (240mg/Kg)	?
Huang et al., 2016	?	4/7/10/14/28	D	10 mm	?	Pentobarbitone ?	?

Cheing et al., 2014	EtOH 70%	7/10/14/21	D	20 mm	1	Ket (100 mg/kg)/ Xyl (10 mg/kg)	?
Liu et al., 2020	?	5/ 9	D	5 mm	1	Chloral hydrate ?	?
Lee et al., 2016	EtOH 70%	1/4/7/10	D	4 mm	1	?	?

EtOH = ethanol, D= Dorsal, Iso = Isoflurane, Ket = Ketamine, ? = Uninformed,
Xyl = xylazine, Zolaz = Zolazep.

Table 4. Biases analyses (ARRIVE) of the studies.

Title	Miller et al., 2017	Wong et al., 2019	Seitz et al., 2010	Yan et al., 2018	Barzrafshan et al., 2014	Cifuentes et al., 2020	Sidhu et al., 1999	Wang et al., 2019	Kim et al., 2008	Heit et al., 2012	Lin et al., 2015	Demyanenko et al., 2017	Kao et al., 2011	Yan et al., 2020	Huang et al., 2016	Cheing et al., 2014	Liu et al., 2020	Lee et al., 2016	
Accurate and concise description of the article content	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Abstract																			
Background summary, research objectives, methods, principal findings, and conclusions	✔							✔				✔	✔	✔	✔			✔	
Introduction																			
Sufficient scientific background	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Explanation of the experimental approach and rationale	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Objectives																			
Clear primary and second objectives	✔					✔							✔	✔				✔	
Materials and Methods																			
Nature of the ethical review permissions, relevant licenses and national or institutional guidelines for the care and use of animals	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Study design																			
Number of animals per group	✔	✔	✔	✔	✔	✔	✔	✔		✔		✔	✔	✔	✔	✔	✔		
Information on whether the experiment was performed as a blind controlled study																✔	✔		
Experimental procedures																			
Treatment Description	✔	✔		✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Treatment dosage	✔	✔		✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Treatment Duration		✔		✔	✔	✔	✔	✔	✔	✔	✔	✔	✔			✔	✔	✔	
Time of day of treatment administration																			
Experimental animals																			

Information regarding animal species	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Animals' strain																			
Animals' sex	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔		✔	✔	✔	✔	✔	
Animals' Weight range	✔	✔	✔	✔	✔	✔	✔									✔			
Animals' Age	✔		✔		✔		✔	✔	✔	✔		✔	✔	✔	✔	✔	✔	✔	
Description of genetic modification status (Knock-out, transgenic, SPF)	✔	✔	✔		✔	✔	✔	✔				✔		✔	✔	✔	✔	✔	
Information related to previous procedures performed on the animals																			
Housing and husbandry																			
Housing of experimental animals (facility type, cage or housing type, material, number of cage companions)									✔										
Husbandry conditions (breeding program, light/dark cycle, temperature, water)				✔	✔	✔										✔	✔		
Welfare-related assessments and interventions that were carried out before, during, or after the experiment																			
Sample size																			
Total number of animals used in each experimental and the number of animals in each experimental group	✔	✔		✔	✔	✔	✔	✔		✔	✔	✔	✔	✔	✔	✔	✔	✔	
Explanation regarding the decision of the number of animals and details of sample size calculation																			
Allocating animals to experimental groups																			
Full details of how animals were allocated to experimental groups (including randomization or matching)																			
Order in which the animals in the different experimental groups were treated and assessed																			
Experimental outcomes																			
Clear experimental outcomes assessed	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Statistical methods																			
Statistical methods used for each analysis	✔	✔	✔	✔	✔	✔	✔		✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Unit of analysis specifications for each dataset		✔	✔	✔	✔	✔	✔		✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Methods used to assess whether the data met the assumptions of the statistical approach		✔	✔	✔	✔	✔			✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Results																			
Baseline data																			
Description of animals health status, for each experimental group, before treatment	✔			✔				✔	✔										
Number analyzed																			

Number or animals in each group included in each analysis (absolute numbers)	✔	✔	✔	✔	✔	✔	✔	✔		✔		✔	✔	✔	✔	✔	✔	✔	
Animals or data not included in the analysis (and explanation for the exclusion)																			
Outcomes and estimation																			
Information (Mean= Standard Deviation)	✔	✔	✔	✔	✔	✔	✔		✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Adverse events																			
Information regarding mortality of experimental animals (Mean=Standard Deviation)																			
Modifications to the experimental protocols made to reduce adverse events																			
Discussion																			
Interpretation /scientific implications																			
Interpretation of the results, taking into account the study objectives and hypotheses, current theory and relevant studies	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	
Comments on the study limitations (sources of bias, limitations of the animal model, imprecision associated with the results)																		✔	
Generalisability /translation																			
Comments on how the findings are likely to translate to other species or systems, including relevance to human biology				✔	✔	✔	✔	✔	✔	✔	✔	✔	✔			✔		✔	
Funding																			
List of funding sources and the role of the funder(s) in the studt			✔	✔	✔	✔					✔	✔		✔	✔	✔	✔	✔	
Results of total	**21**	**20**	**18**	**23**	**24**	**24**	**21**	**19**	**19**	**20**	**18**	**23**	**21**	**22**	**21**	**25**	**23**	**24**	

CHAPTER III

TRICHILIA SILVATICA EXTRACTS MODULATE THE OXINFLAMMATORY RESPONSE: AN *IN VITRO* ANALYSIS[1]

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ABSTRACT

Ethnopharmacological relevance: Plants belonging to the Meliaceae family, such as *Trichilia silvatica* C. DC., known as catiguá-branco, have attracted considerable interest in phytochemical research due to their diverse and significant secondary metabolites. *T. silvatica* has traditionally been employed in Brazilian medicine to treat inflammatory disorders. Moreover, studies have reported its antioxidant and antimicrobial properties, highlighting its potential therapeutic applications. **Aim of the study:** This study aimed to evaluate the potential of *Trichilia silvatica* leaf and stem extracts in modulating OxInflammation in RAW264.7 macrophage cells following exposure to lipopolysaccharide (LPS) or hydrogen peroxide (H_2O_2) and to elucidate the underlying mechanisms of action. **Material and methods:** The phytochemical composition of the extracts was characterized using thin-layer chromatography (TLC), HPLC equipped with a reversed-phase Hypersil C-18 column, and spectrophotometric method. Their antioxidant activity was evaluated using the 2,2-difenil-1-picrilhidrazil (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assays. Cell viability was assessed via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, alongside the determination of catalase (CAT) and superoxide dismutase (SOD) enzyme activities, as well as nitric oxide (NO) production in cells treated with the extracts and subsequently stimulated with H_2O_2. Gene expression levels of Factor nuclear kappa B (NF-κB), Ciclooxygenase 2 (COX-2), Tumor necrosis factor alpha (TNF-α), Interleukin 10 (IL-10), and Hypoxia-inducible factor-1 (HIF-1) were quantified using RT-qPCR. **Results:** *Trichilia silvatica* extracts revealed the presence of terpenes/steroids, coumarins, condensed tannins, and phenolic acids, including

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1 Article published in the Journal of Ethnopharmacology in 12/06/2025.

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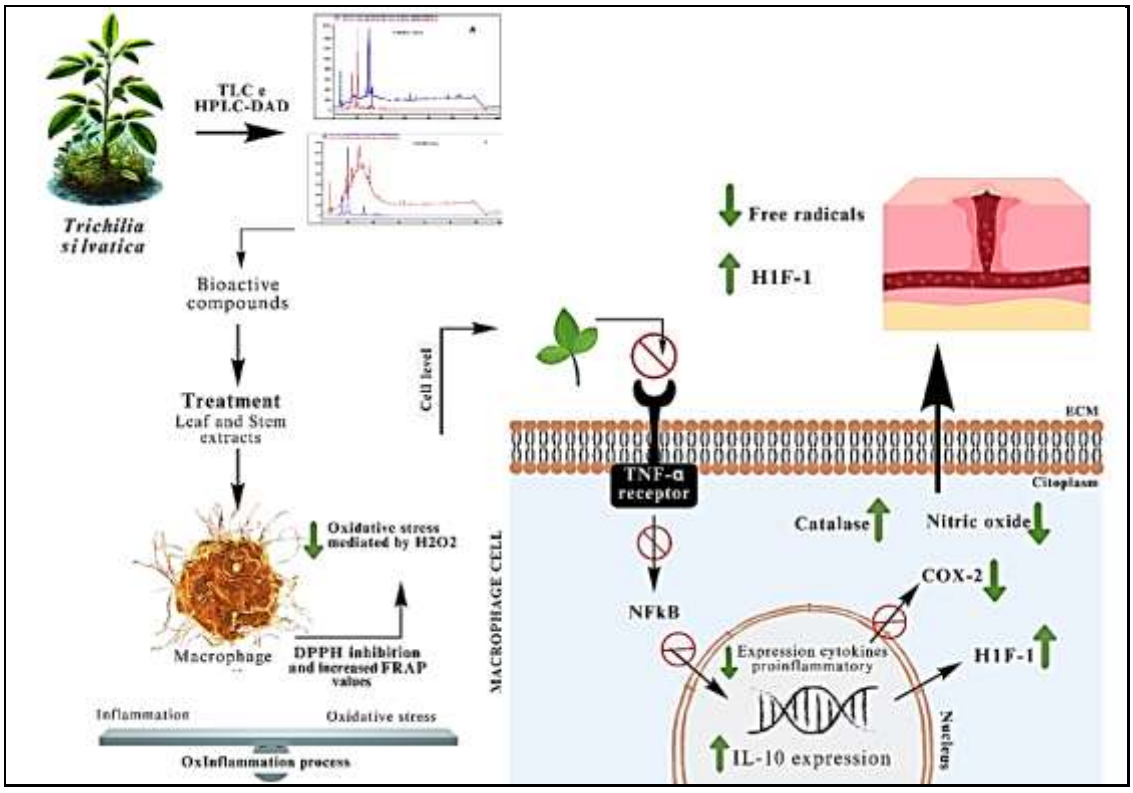
chlorogenic and caffeic acids. The findings indicate that the leaf extract at 100 µg/ml and the stem extract at 100 µg/ml and 250 µg/ml preserved or enhanced cell viability, conferring protection against H_2O_2-induced oxidative stress. These concentrations significantly increased CAT activity, whereas SOD activity remained unaffected. Nitric oxide production was significantly reduced when cells were treated with 100 µg/ml and 250 µg/ml of both leaf and stem extracts. Moreover, FRAP value revealed an increase in antioxidant capacity at 250 µg/mL. Both leaf and stem extracts, at 100 µg/mL and 250 µg/mL, exhibited a DPPH radical scavenging capacity exceeding 50% and downregulated the expression of pro-inflammatory cytokines, including NF-κB, TNF-α, and COX-2. Notably, the leaf extract at 250 µg/ml and the stem extract at 100 µg/mL upregulated the expression of IL-10 and H1F1. **Conclusions:** These findings indicate that *Trichilia silvatica* extracts exhibit notable antioxidant activity, as evidenced by greater than 75% inhibition of DPPH radicals and elevated FRAP values. Additionally, the extracts demonstrated anti-inflammatory properties by downregulating key pro-inflammatory mediators, including TNF-α, NF-κB, and COX-2, while upregulating the anti-inflammatory cytoline IL-10 and enhancing enhancing tissue oxygenation and nutrient supply through increased expression of HIF-1. These effects highlight the potential of *T. silvatica* extracts as therapeutic agents for managing inflammatory diseases associated with oxidative stress, thereby supporting their traditional medicinal use.

Keywords: *Trichilia silvatica*; Inflammation; antioxidant activity; Oxidative stress; Phytochemical analysis.

Graphical Abstract



Illustrative scheme of the antioxidant and anti-inflammatory effects of *Trichilia silvatica* extract on macrophages subjected to oxidative stress mediated by H_2O_2. The extract demonstrated antioxidant activity by neutralizing the damage caused by H_2O_2 and reducing the expression of pro-inflammatory cytokines (NF-κβ, TNF-α, and COX-2) while promoting the expression of anti-inflammatory cytokines (IL-10), contributing to cellular protection against oxidative damage and inflammation.

1 INTRODUCTION

The use of plants to develop new compounds to treat different diseases has been growing rapidly. Today, it represents a promising tool for controlling the development of inflammatory diseases (Najmi et al., 2022). Some plants with significant biological activities have shown promising results regarding their protective capacity. However, there is still a gap in understanding the main pathways activated after plant extract exposure, identifying the concentration of the extracts, and necessary pharmacological and toxicological research to ensure the safety of these plants' use (Tabach, 2022).

The genus *Trichilia,* belongs to the *Meliaceae* family and has attracted significant interest within Brazilian Flora due to the biological activities of its secondary metabolites (Riyadi et al., 2023). In traditional medicine, various *Trichilia* species have been utilized in different preparations, including maceration, topical application, infusion and decoction to exert their antimicrobial, antiviral, insecticidal, anti-proliferative and antioxidant properties (Da Silva et al., 2018; Meneguelli et al., 2020). While several studies have supported the applications of certain Trichilia species, demonstrating that extracts derived from its leaves and bark exhibit significant anti-inflammatory and antioxidant activities (Da Silva et al., 2021; Kamdem et al., 2012; Martins et al., 2018; Park et al., 2020), specific studies focusing on the *T. silvatica* remain limited. *T. silvatica,* popularly known as catiguá-branco*,* has been traditionally used for its anti-inflammatory properties, particularly in treating rheumatic conditions. Experimental studies have shown that these extracts reduce leukocyte migration and attenuate edema formation in arthritis models, which may underlie their efficacy in managing inflammatory processes; anti-inflammatory effects were studied in carrageenan-induced paw edema (Da Silva et al., 2018). Furthermore, *T. silvatica* has been reported to possess antimicrobial activity against *Staphylococcus aureus*, *Streptococcus salivarius* and *Streptococcus mutans*, as well as potential antitumour effects (Figueiredo, 2010).

Several species of the genus *Trichilia* are predominantly found in tropical regions, including Brazil. Phytochemical investigations of *Trichilia* species have led to the isolation and identification of different bioactive compounds, such as monoterpenes, diterpenes, sesquiterpenes, triterpenes, limonoids, steroids, coumarins, lignans, flavonoids, phenolic acids, and lactones. *T. silvatica* has been reported to contain a variety of protolimonoids, limonoids, and terpenoids, many of which exhibit potential therapeutic properties (Ji et al., 2015; Zohora et al., 2024). These bioactive metabolites have demonstrated significant anti-inflammatory and antimicrobial activities, which are particularly relevant in mitigating OxInflammation.

Therefore, the *Trichilia* species can contribute to the development of therapies for inflammatory conditions, especially controlling the Free radicals, Reactive oxygen Species (ROS), and inflammatory cytokines release. The continuous and reciprocal interaction between oxidative stress and inflammation in cells and tissue is known OxInflammation (Lopes et al., 2024). OxInflammation occurs due to the excessive production of reactive oxygen species related to endogenous antioxidant defense mechanisms. The oxidative stress leads to the development and maintenance of inflammation and contributes to the pathophysiology of various debilitating diseases, such as cardiovascular disease, diabetes, cancer, or neurodegenerative processes (Steven et al., 2019) ring the inflammation, Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) work as signaling molecules that are critical in all inflammation phases (Mandal et al., 2022). An imbalance in the production of these markers will promote prolonged or unregulated inflammation, impair tissue regeneration, and lead to chronic complications (Janakiram et al., 2021). It is important to highlight that inflammation is not a negative phenomenon, as it is necessary to maintain a constant defense to preserve the integrity of tissues and organs (Medzhitov, 2021). However, this response must be well-regulated and promptly resolved once the harmful agent is eliminated, which is achieved through the activation of an anti-inflammatory response by immune cells (Ao et al., 2021).

On the other hand, the development of chronic inflammation needs to be avoided, especially because many cells accumulate at the site of injury, leading to the secretion of cytokines by inflammatory cells, especially pro-inflammatory ones. In this process, molecules such as interleukins IL-1, IL-2, IL-6, IL-17, and TNF (tumor necrosis factor) are released, promoting the activation of macrophages, neutrophils, and mast cells while also stimulating the expression of adhesion molecules (Furtado et al., 2019). In Chronic inflammatory disease, there is an imbalance between pro-inflammatory and anti-inflammatory compounds, with pro-inflammatory compounds in greater abundance. Therefore, a sustained oxidative response can lead to cellular damage due to the overproduction of ROS, which can also recruit additional inflammatory cells, further increasing the production of pro-inflammatory and oxidant compounds and exacerbating cellular damage (Morosi et al., 2021). Therefore, it is desirable to identify molecules and new compounds like the phenolic compound to avoid the development of chronic inflammation in different diseases. This has been included in the priorities to improve human health quality (Rahman & Helvie, 2022).

To fill the gaps and understand the mechanisms of action of a plant extract in a specific tissue, it is necessary to conduct basic research using a cell model to establish the relationship between inflammation and oxidative factor dysregulation, along with their consequences after

T. silvatica extract exposure. Analyzing antioxidant activity, cytoprotective capacity, nitric oxide production, antioxidant enzymatic activity, and the action of this extract after stress induced are essential to understand the mechanisms action of the *T. silvatica* extract and consequently to offer a new, innovative, and promising perspective for developing effective and economically viable treatments to control the progression of the inflammatory diseases. It is the first step to develop a potent antioxidant and anti-inflammatory compound that could be used in future therapies to improve the quality of human life. Therefore, this study investigates the complexity of inflammatory and oxidative stress pathways and their influence in RAW264.7 macrophage cells after stress promoted by H_2O_2 exposure and the action of the *T. silvatica* extract. Furthermore, we look to define which part of the plant is most promising as an antioxidant and anti-inflammatory. In addition, this study aimed to evaluate the effects of *T. silvatica* extract on inflammatory mediators (NF-κβ, TNF-α, COX-2, IL-10), antioxidant enzymes (SOD, CAT), and HIF-1α expression, in order to explore potential interactions involved in the modulation of the OxInflammation process.

2 MATERIAL AND METHODS

2.1 Plant Material

Trichilia silvatica C. DC. plant samples were collected from São José de Almeida, Jaboticatubas (S 19º 25' 809" W 43º 48' 206"), Minas Gerais, Brazil. The plant material was identified, and a voucher specimen (PAMG 56319) was deposited at the Herbarium of the Plant Analysis and Microbiology Laboratory (PAMG) of the Agricultural Research Company of Minas Gerais (EPAMIG). The plant name has been checked with http://www.theplantlist.org on 15 January 2024. This study received authorization for access and remittance of genetic material for scientific research by the SISGEN (Registration A3EE9E9).

2.2 Preparation of the Extracts

The collected material was separated into leaves (TSL) and stems (TSS). Both materials were dried in an air-circulating oven at 40 ºC and ground into a fine powder. The stems (134 g) and leaves (240 g) were separately extracted by percolation using an EtOH solution (8:2) until exhaustion at room temperature. The extracts were concentrated under reduced pressure in a

rotary evaporator at 40 ºC and then lyophilized. The extract was ressuspended in dimethyl sulfoxide (DMSO) and normalized to a maximum concentration of 0.4 % DMSO in cell culture.

2.3 Phytochemical Screening by TLC and Exploratory Profile by HPLC

The phytochemical screening of extracts (10 mg/mL) was performed by thin-layer chromatography (TLC) on silica gel 60 F254 aluminum plates using eluents and spray reagents, according to Wagner & Bladt (2001), as described in Table 1. An aliquot (10µL) of each solution was applicated and the reference standards (1 mg/mL) were used as a positive control of the chromatographic conditions. The analyis was performed in duplicate.

Table 1 **-** Conditions of thin-layer chromatography

Mobile phase	Spray reagent	Reference standard	Special metabolites evaluated
Toluene: chloroform: ethanol (8:8:1)	Anisaldeyde-sulphuric acid	Lupeol	Terpenes/steroids
Ethyl acetate :formic acid:acetic acid: water (20:2:2:5).	NP/PEG reagent	Rutine, caffeic acid and chlorogenic acid	Flavonoids and simple organic acid
Ethyl acetate:methanol:water (8:1:1)	Vanilin-sulphuric acid reagent	catechin	Condensed tanins
Ethyl acetate:methanol:water(8:1:1)	Ferric chloride 3%	Galic acid	Phenolic compounds
Toluene:chloroform:ethanol (8:8:1)	Potassium hidroxide 5%	Coumarin	Coumarins

HPLC-grade organic solvents (Sigma-Aldrich, USA) and ultrapure water (Milli-Q system) were employed. The samples were prepared at 10 mg/mL in acetonitrile water (8:2), were filtered through 0.45 µm PTFE membranes and injected into the HPLC system (Agilent Technologies 1200 series) equipped with a diode array detector (Agilent Technologies, USA). The separation was achieved on a reversed-phase column Hypersil C-18 column (250 × 4.6 mm, 5 µm). Elution was carried out with water (A) and ACN (B), both with 0.1% formic acid in a linear gradient elution from 5 to 95% of B in 60 min. The peaks were detected using UV absorbance, λ of 254 nm, the flow rate of 0.8 mL/min, 30 °C, and an injection volume of 20 µL. The UV spectra of the major peaks were included in the chromatograms.

2.4 Phytochemical Analysis of Total Phenolic, Flavonoid, Proanthocyanidin Content, and Content of Chlorogenic and Caffeic Acid in the Ethanolic Extracts in Leaves and Stems of *T. Silvatica*

The total phenolic compound content (TPC) was determined following the method described by Sartori et al. (2014) with adaptations. A calibration curve was prepared using pyrogallol at 20 to 100 µg/mL concentrations. The absorbance of the mixture was measured at 760 nm using a UV–VIS spectrophotometer (Multiskan GO, Thermo Scientific), with methanol used as the blank. All assays were performed in triplicate, and the total phenolic content was expressed in mg pyrogallol per gram of dry extract, based on the standard curve ($A = 93.83C - 0.05$; $R^2 = 0.9988$).

The total flavonoid content (TFC) was determined using a spectrophotometric method, according to (Nurcholis et al., 2021). A calibration curve was constructed with quercetin in 50% methanol at 20 to 100 µg/mL concentrations. Absorbance was measured at 420 nm using the same spectrophotometer (Multiskan Go, Thermo Scientific), with methanol as the blank. All assays were conducted in triplicate, and the results were expressed in mg quercetin per gram of the dry extract, based on the standard curve ($A = 40.86C - 0.02$; $R^2 = 0.9983$).

The proanthocyanidin assay was performed using catechin as a standard at a spectrometric method, according to Nakamura et al. (2003). The calibration curve was constructed using a catechin solution. The samples were mixed with 100 µL of 1% vanillin in sulfuric acid. The absorbance was measured at 500 nm after 15min. TSS was evaluated at the same methodology. Methanol was used as the blank. The results were expressed as catechin equivalents (CAE) in mg per gram (g) of extract. The assay was performed in triplicate using a standard curve $A = 23.71C – 0.0181$; $R^2 = 0{,}9926$.

The contents of the chlorogenic and caffeic acid in the samples were estimated by external calibration method in HPLC-DAD system. The calibration curve was constructed using a standard solution (100 to 500µg/mL). The elution was carried out with water (A) and ACN (B), both with 0.1 % formic acid in a linear gradient elution from 5 to 65 % of B in 45 min, the flow rate of 0.8 mL/min; 23 °C. Aliquots of each dilution (10 µL) were analyzed via HPLC, and each determination was injected twice. For each standard, the corresponding chromatogram was obtained, and a graph was constructed from the mean of the peak areas plotted against the concentration of the standard. The assay was performed in triplicate. The limits of detection (LOD) and quantitation (LOQ) were determined (INMETRO, 2010)

according to Equations 1 and 2, respectively, where σ is the standard deviation of response of lowest level of analytical curve and S is the slope of the calibration curve:

$$\text{Equation 1: } LOD = \frac{3.3x\sigma}{S}$$

$$\text{Equation 2: } LOQ = \frac{10x\sigma}{S}$$

Three stock solutions of TSL and TSS at 20 mg/mL in acetonitrile water (8:2) were prepared. The samples were filtered through 0.45 µm PTFE membranes and injected (10 µL) into the HPLC system. The mean analytical curve obtained was used to determine the concentration of caffeic and chlorogenic acids in the sample solutions. Results were then expressed in g of caffeic acid µg/g extract and chlorogenic acid/g extract.

2.5 DPPH Radical Assays

In the present study, the *T. silvatica* extracts were evaluated at concentrations of 50, 100 and 250 µg/mL. Aliquots of 50 µL of each extract concentration, 250 µL of 2,2-difenil-1-picrilhidrazil (DPPH•) was added, and the samples were analyzed after 30 minutes of reaction, with absorbance readings taken in a microplate at 517 nm (Musa et al., 2013) . Ascorbic acid at 100 µg/mL, prepared in methanol was used as a reference standard. The results were expressed as % inhibition according to the following equation:

$$\% \text{ Inhibition} = ((A_{DPPH} - A_{EXTRACT})/A_{DPPH})) \times 100$$

Where A_{DPPH} is the absorbance of the DPPH• solution, and $A_{EXTRACT}$ is the absorbance of the sample. This assay evaluates the extract's antioxidant capacity, specifically its ability to neutralize the DPPH• radical through electron or hydrogen transfer.

2.6 FRAP Assay

The total antioxidant capacity was also estimated using the Ferric Reducing Antioxidant Power (FRAP) method as described by (Benzie & Strain, 1996), employing TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) as the substrate. This method reduces a ferric complex, 2,4,6-tripyridyl-s-triazine (Fe^{3+}-TPTZ), to its ferrous form (Fe^{2+}-TPTZ). *T. silvatica* extracts were evaluated at concentrations from 50, 100 and 250 µg/mL. For the analysis, 10 µL of sample/standard was added to 190 µL of FRAP solution in polystyrene microplates, which were incubated in the dark for 7 minutes at 37 °C. The control used was a buffer + FRAP solution

(blank control). Ascorbic acid at 100 µg/mL, prepared in acetate buffer, was used as a reference standard. Absorbance readings were performed using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific) at a wavelength of 593 nm. The FRAP value obtained for the total antioxidant capacity was based on the standard curve of ferrous sulfate in the range of 0 to 0.75 µM.

2.7 Cell Viability

The effect of the *T. silvatica* extracts was evaluated on RAW264.7 macrophage cell viability. Initially, cells suspended in a complete DMEM medium were seeded in a 96-well plate at a concentration of 1×10^5 cells per well for the RAW 264.7 cell assay, with a final volume of 200 µL. The plate was incubated for 24 hours at 37 °C with 5% CO_2 to allow cell adhesion to the plate. The culture medium was carefully removed from the plate, and 200 µL of the extracts at 50 µg/mL, 100 µg/mL and 250 µg/mL was added to each well.

The control (100% growth) was conducted with cells cultured only in a medium. The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (0.5 mg/mL) (Sigma-Aldrich, Germany) was added to each well, and the cells were incubated for 1 hour at 37 °C. The formed formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm. The MTT assay was performed to determine cell viability, as previously described by Villalvilla et al. (2016).

2.8 Protective Capacity and Cell Viability After Induction of Stress with Hydrogen Peroxide (H_2O_2)

Initially, RAW264.7 macrophages were treated for 24 hours with different concentrations of the leaf and stem extract (50, 100 and 250 µg/mL). Following this treatment, the cells were stimulated with 1 mM H_2O_2 for 3 hours and then incubated for 1 hour with MTT to evaluate the protective capacity of the extracts.

2.9 Catalase (CAT) and Superoxide Dismutase (SOD) Activities

RAW 264.7 macrophages were incubated for 24 hours to promote cell growth. Subsequently, the cells were treated with the extracts for 24 hours and then exposed to 0.75 mM of H_2O_2 for 3 hours. Thereafter, the supernatant was collected for nitric oxide analysis, and the

cells were collected using PBS with 1% Triton 100X and stored at -80ºC for further CAT and SOD activity analyses.

A sodium and potassium phosphate buffer (50 mM, pH 7.4) was used to prepare the reagents for CAT activity analysis. An aliquot of 5 µL of the sample was added to the wells, followed by 100 µL of the 20 mM H_2O_2 solution. After 3 minutes of reaction, 150 µL of ammonium molybdate was added. A standard curve of H_2O_2 was performed (from 0.078 mM to 20 mM). The absorbance was measured at 374 nm (Aebi 1984). Enzymatic activity was expressed in units of CAT per mg of protein.

For SOD activity analysis, a volume of 30 µL of the sample was added to 99 µL of phosphate buffer (0.2 M, pH 8.0), 6 µL of MTT (1.25 mM), and 15 µL of pyrogallol (100 µM) in a 96-well plate, and incubated for 15 minutes at 40 ºC. The analysis was performed in triplicate. The standard control and the blank were prepared similarly without adding the sample, using 129 µL and 144 µL of buffer, respectively. Pyrogallol was not added to the blank. Following incubation, the reaction was stopped with 150 µL of DMSO (1.25 mM), and the absorbance of samples was read at 540 nm, (Dietrich-Muszalska & Rabe-Jablonska 2005), using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific). Enzymatic activity was expressed in units of SOD per mg of protein.

It is important to mention that for oxidative stress and gene expression analyses, we selected two concentrations (100 and 250 µg/mL) based on the most promising results observed in preliminary dose-response experiments. These concentrations were chosen to balance biological relevance with cell viability and to highlight the most effective responses.

2.10 Nitric Oxide Analysis

The nitric oxide dosage was performed according to the Griess method (Sigma Aldrich, Germany) to indirectly determine NO release, as previously described by Villalvilla et al. (2014). RAW264.7 cells, suspended in complete DMEM medium, were seeded in 96-well plates at a concentration of 1×10^5 cells per well, with a total volume of 1 mL. The plate was maintained in an incubator at 37ºC with 5% CO_2 for 24 hours. After this period, the medium was removed, and the DMEM supplemented with 10% fetal bovine serum (FBS) containing the treatment with the extract at concentrations of 100 and 250 µg/mL was added for another 24 hours.

For the positive control, cells were maintained with only colorless DMEM and stimulated with H_2O_2, while the negative control proceeded without H_2O_2 stimulation. An

aliquot of 50 µL of the supernatant was collected and transferred to a 96-well plate. A volume of 50 µL of the Griess reagent (Sigma-Aldrich) was added to each well. After 10 minutes of reaction in the dark, the absorbance was measured using a plate reader set to a wavelength of 570 nm.

The nitric oxide concentration was calculated from the standard curve of sodium nitrite in the concentration range of 150 µM to 10 µM. The results were expressed as nitric oxide concentration.

2.11 Gene Expression Analysis

The gene expression of pro-inflammatory and anti-inflammatory cytokines was determined using quantitative reverse transcription PCR (qRT-PCR) . The primer sequences used are presented in Table 2. Macrophages (2.5×10^5 cells/well) were seeded in 6-well plates and treated with DMEM medium containing 10% FBS, along with *T. silvatica* extract at concentrations of 100 and 250 µg/mL for leaves and stems. The cells were incubated at 37 °C with 5% CO_2 for 24 hours. Following incubation, the cells were stimulated with 10 µg/mL of LPS for 4 hours and then harvested for total RNA extraction using TRI Reagent® (Sigma-Aldrich). The samples were stored in 1.5 mL microtubes at -80 °C. RNA was isolated according to the manufacturer's protocol. The concentration and quality of the extracted RNA were assessed using a µDrop Duo plate with a Multiskan SkyHigh spectrophotometer (Thermo Fisher Scientific). The extracted RNA (1000 ng) was reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific). RT-qPCR was performed using PowerTrack™ SYBR™ Green Master Mix (Thermo Fisher Scientific) on a QuantStudio™ 3 real-time PCR system (Thermo Fisher Scientific). The β-actin was used as a housekeeping gene, and the relative standard curve method was employed for quantitative data analysis. Negative control (CRTL-): mRNA from untreated macrophage cells; positive control (CRTL+): mRNA from LPS-stimulated macrophage cells.

Table 2 **-** Primer Sequences

Gene	*Forward*	*Reverse*
NF-κβ	5'-GCT GCC AAA GAA GGA CAC GAC A-3'	5'-GGC AGG CTA TTG CTC ATC ACA G-3'
COX-2	5'-TGC ACT ATG GTT ACA AAA GCT GG-3'	5'-TCA GGA AGC TCC TTA TTT CCC TT-3'
TNF-α	5'-TAT GGC TCA GGG TCC AAC TC-3'	5'-CCC ATT TGA GTC CTT GAT GG-3'
HIF-1	5'-CGA AGT TAC AG CTT TCC GAC CAG-3'	5'-GTT TGT GTC GGT CAG CAC CAC T-3'
IL-10	5'-TTA ATA AGC TCC AAG ACC AAG G-3'	5'-CAT CAT GTA TGC TTC TAT GCA G-3'

Mice primer sequences: NF-κβ: Nuclear factor-kappa B; COX-2: Cyclooxygenase; TNF-α: Tumor necrosis factor-a; HIF-1: Hypoxia-inducible factor 1-alpha; IL-10: Interleukin 10.

2.12 Statistical Analysis

All results were expressed as the mean of three independent triplicate experiments. Furthermore, the coefficient of variation between each triplicate was less than 0.2, which means homogeneity of the results. Statistical analyses of data were performed using one-way ANOVA followed by Tukey's post-hoc test, using GraphPad Prism software, version 8.0 (GraphPad Prism Software Company, 2018, San Diego, California, USA). A significance level of 5% was adopted.

3 RESULTS

3.1 Phytochemical Content Results of *Trichilia Silvatica*

The results of the spectrophotometric assays for the determination of the total phenolic, flavonoid, and proanthocyanidins contents are presented in Table 3. The TSS extract exhibited a slightly higher total phenolic content than TSL, which is in agreement with previous results found by Da Silva et al. (2018). Conversely, the flavonoid content was higher in TSL than in TSS, while the proanthocyanidin levels were comparable between the two extracts. *T. silvatica* leaf (TSL) and stem (TSS) extracts were obtained in 12.8% and 9.20% yields, respectively.

Table 3 – Secondary metabolites in the hydroalcoholic extracts of leaves (TSL) and stems (TSS) from *T. silvatica* and quantification of total phenolic compounds, flavonoids, and proanthocyanidins content

	TSL	**TSS**
Secondary metabolites	Terpene/steroids, flavonoids, phenolic acids, coumarins, proanthocyanidins	Terpene/steroids, flavonoids, phenolic acids, coumarins, proanthocyanidins
Total phenolic compound content	75.6 ± 2.1 GAE/g	95.5 ± 8.1 GAE/g
Total flavonoid content	48.47 ± 2.286 QE/g extract	5.822 ± 0.842 QE/g extract
Proanthocyanidin content	1.1324 ± 0.0647 Cat/g extract	1.1894± 0.0428 Cat/g extract

The phytochemical screening performed by TLC (Figure 1) indicated the presence of triterpenes/steroids, organic acids, and coumarins in both TSL (leaf) and TSS (stem) extracts. Notably, flavonoids were exclusively detected in the TSL extract. The reference standards were used as a positive control of the chromatographic conditions.

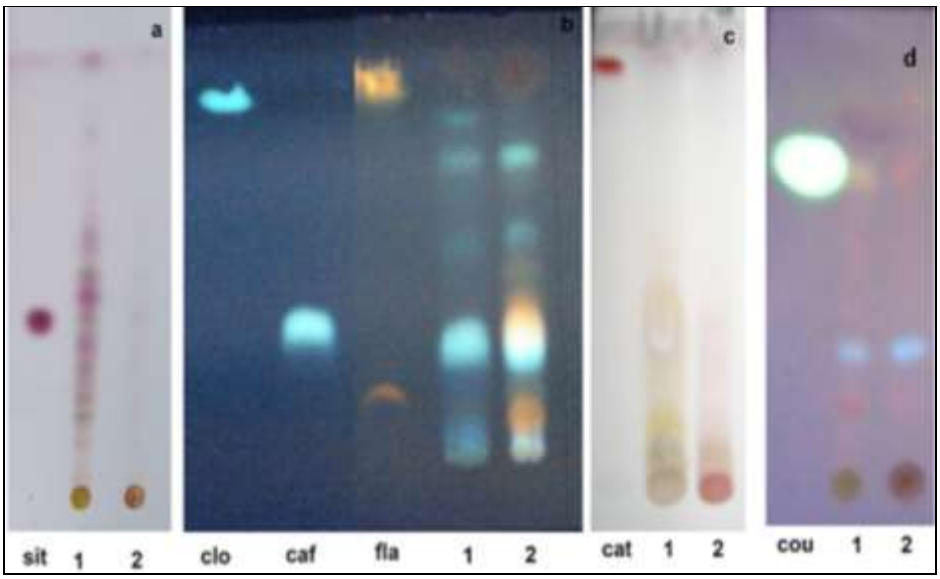


Figure 1 - Chromatographic profiles obtained by TLC. Samples sit, sitosterol, clo, chlorogenic acid, caf, caffeic acid, fla, mixture of quercetin and rutin, cat, catechin, cou, coumarin, 1 TSS (*T. silvatica* stem extract), 2 TSL (*T. silvatica* leaf extracts). 2a **Chromatographic system** - Tol: $CHCl_3$:EtOH (8:8:1); Spray reagent anisaldehyde-sulphuric acid; 2b **Chromatographic system** - AcOEt: Formic acid: Acetic acid: W (20:2:2:5); Spray reagent NP/PEG. The plate was visualized under UV light at λ = 366 nm; 2c **Chromatographic system** - AcOEt:MeOH: Water (8:1:1); Spray reagent vanillin-sulphuric acid; 2d **Chromatographic system** - Tol: $CHCl_3$:EtOH (8:8:1) Spray reagent KOH 5% (EtOH).

TLC analysis indicate that both extracts (TSL and TSS) are constituted by terpenes/steroids, caffeic and chlorogenic acids and coumarins.

The fingerprinting of the ethanolic extract from leaves (TSL) and stems (TSS) of *T. silvatica* were obtained by HPLC-DAD at 254 nm and 325 nm, as shown in Figures 2 and 3. In the Figure 2, peaks along the chromatogram at 254 nm are mainly concentrated between Rt = 2.00 and 20.00 min, indicating the presence of a polar substances in the extract. The analysis of the major peaks at 13.09, 14.493 and 14.575 showed UV spectra characteristics of flavonoids (λ_{max} at 255 and 325 nm). In the chromatogram at 355 nm were observed two peaks (Rt 8.221 and 10.885min) which showed UV spectra characteristics of phenol carboxilic acid (λmax at 220, 240, 325 nm). At the chromatogram of TSS (Figure 3) obtained to 254 nmm, the peaks are mainly concentrated between Rt = 2.00 and 20.00 min. The one major peak at 10.071 min showed UV spectra characteristic of phenol carboxilic acid. The chromatogram at 355 nm to TSS (Figure 2) showed a similar profile similar to chromatogram of TSL.

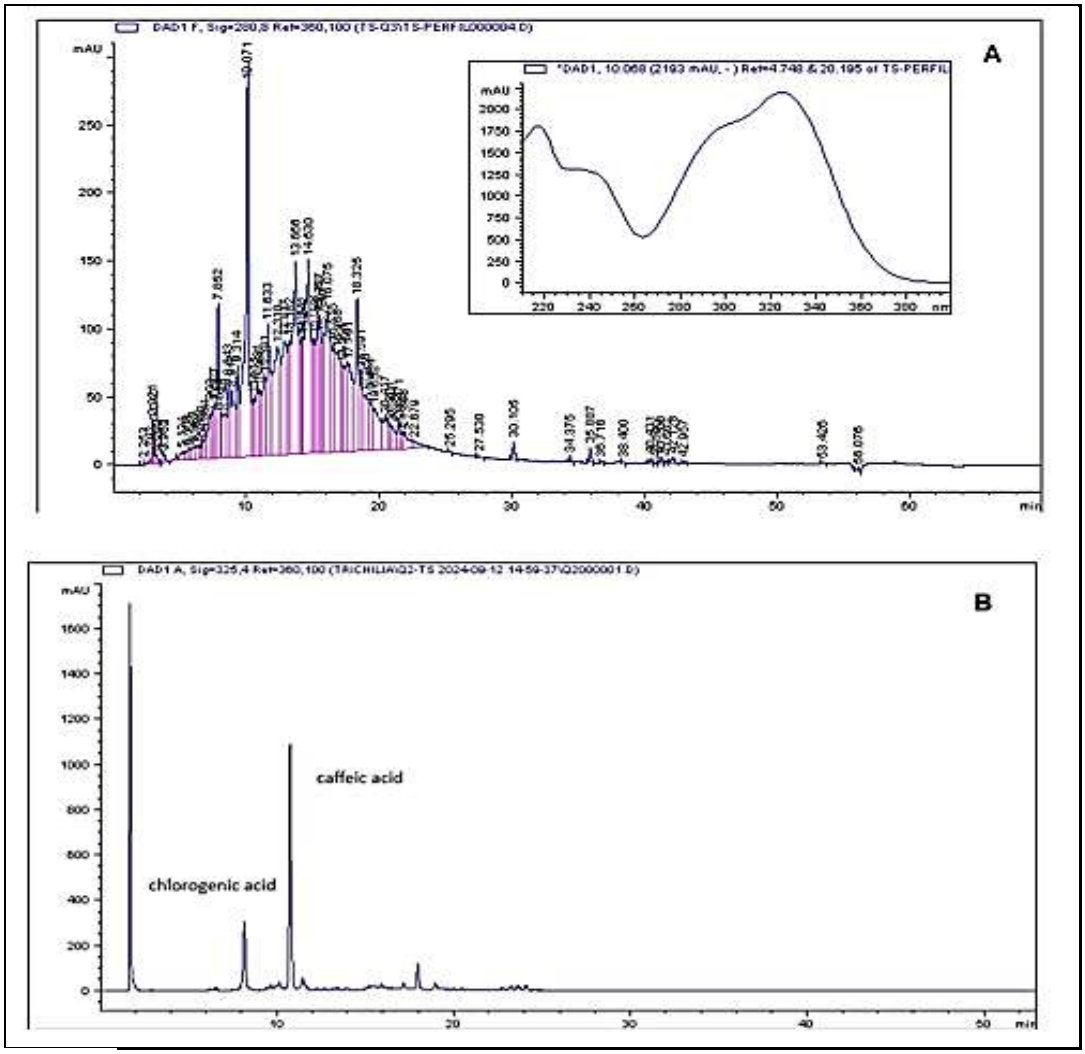


Figure 2 - The fingerprinting of the ethanolic extract from stems of *T. silvatica.* Chromatographic profiles by RP-HPLC-DAD detected at λ 280 nm (**A**) and 325 nm (**B**).

The content of caffeic acid and chlorogenic acid was quantified in both extracts from *T. silvatica*. The caffeic acid (Caf, tr = 10.878min) was determined in TSS (184.14± 7.29µ/mL, 9.207 ± 0.36 µg of Caf/mg TSS) and in TSL (241.78 ± 7.91 µg/mL; 12.04 ± 0.38 µg de Caf/mg TSL) calculated using the standard curve A = 24.375C + 120.67, R^2 = 0.9995. The chlorogenic acid (Chl, tr = 8.214min) was determined in TSS (107.43 ± 3.90 µg/mL, 5.402 ± 0.09 µg Chl/mg TSS) and TSL (187.37 ± 5.98 µg/mL; 9.25 ± 0.26 µg Chl/mg TSL) by analytical curve, Area = 14.81C – 110.61, R^2 = 0.9978 (Figure 3). The limits of detection (LD) and quantitation (LQ) to caffeic acid were 4.67 µg/mL and 14.73 µg/mL, respectively. The limits of detection (LD) and quantitation (LQ) to chlorogenic acid were 10.05 µg/mL and 30.36 µg/mL, respectively. The content of evaluated phenol carboxilic acids in the sample were above the LQ.

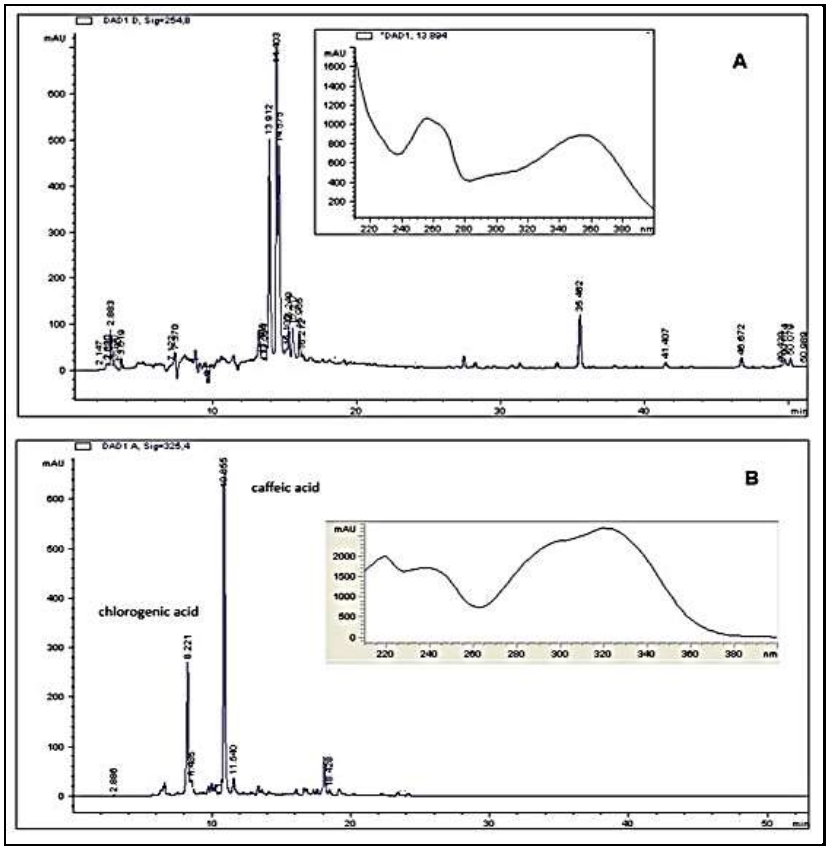


Figure 3 - The fingerprinting of the ethanolic extract from leaves of *T. silvatica* Chromatographic profiles obtained by HPLC-DAD detected at λ 254 nm (**A**) and 325 nm (**B**).

3.2 DPPH Assay

The leaf extract at 250 µg/mL demonstrated the highest capacity to inhibit the DPPH radical after 30 minutes (83 % inhibition) (Figure 4A). Furthermore, stem extracts at 100 and

250 µg/mL also exhibited the highest capacity for DPPH radical inhibition after 30 minutes (over than 75 % inhibition) (Figure 4B).

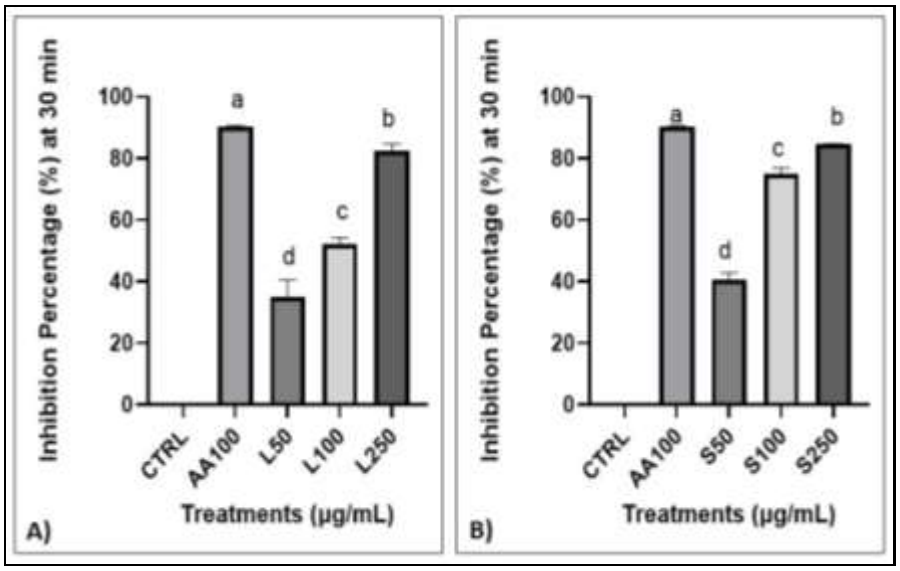


Figure 4 - The antioxidant activity of the extracts the *T. silvatica* at 50, 100 and 250 µg/mL was measured using the DPPH assay. Panel (A) shows the percentage of DPPH radical inhibition by the leaf, while panel (B) displays the percentage of DPPH radical inhibition by the stem, all after 30 minutes of reaction. CTRL: cell + DMEM; AA100: Ascorbic Acid at 100 µg/mL Data are expressed as mean ± standard error. According to Tukey's test, means followed by the same letter are not significantly different ($p > 0.05$).

3.3 FRAP Assay

The results were expressed as FRAP values, indicating that both extracts exhibited a high antioxidant capacity at both concentrations tested, with TSS showing higher activity than TSL extract (Figure 5).

3.4 Effect of *Trichilia Silvatica* on the Viability of RAW264.7 Cells

Different concentrations of *T. silvatica* leaf and stem extracts were tested. Cell viability was found to be satisfactory for both leaf and stem. The leaf groups L50 and L100 and stem groups S50 and S100 showed considerable cell viability, with the best results presented by group S250 (Figure 6).

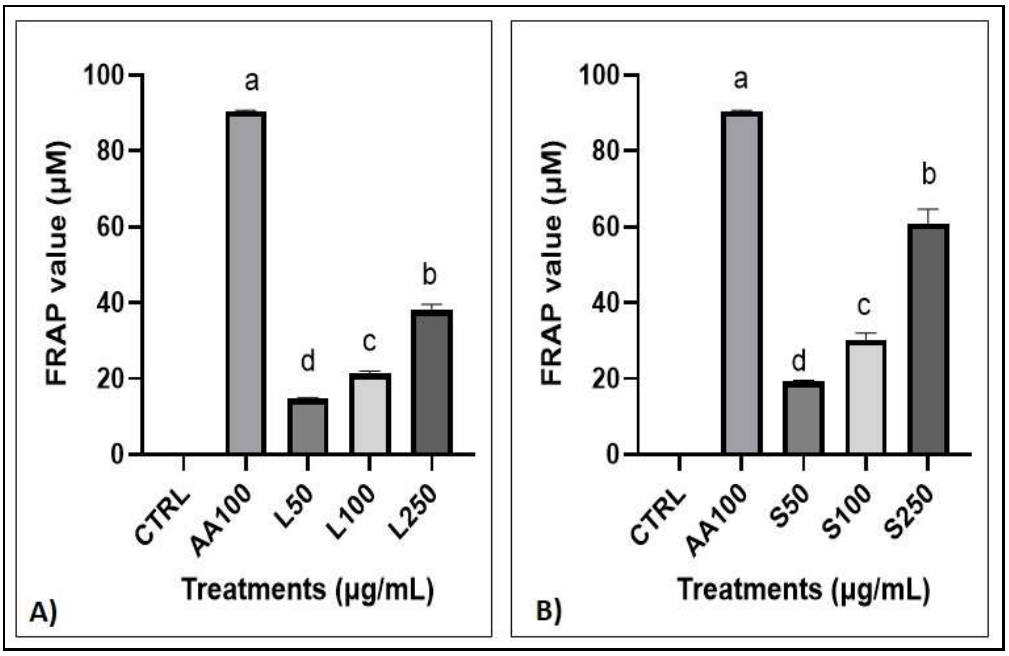


Figure 5 - FRAP values were obtained for the control containing only the acetate buffer and the treatments with extracts of *T. silvatica* leaves (L) and stems (S) at concentrations of 50, 100 and 250 µg/mL. The data (n = 3) are expressed as mean and standard error. According to Tukey's test, means followed by the same letter the same letter do not significantly differ ($p > 0.05$).

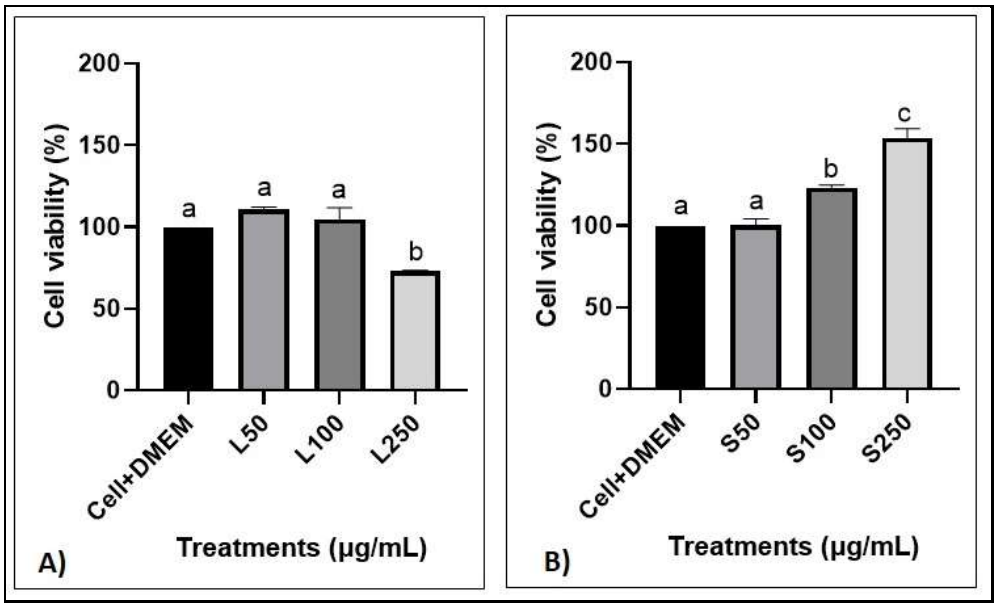


Figure 6 **-** Cell viability under the effect of *T. silvatica.* RAW264.7 macrophages were treated with DMEM/10% FBS and different concentrations of leaves and stems: 50, 100 and 250 µg/mL. The negative control consisted solely of cells with DMEM/10% FBS. The data (n = 3) are expressed as mean ± standard error. Means followed by the same letter do not differ significantly ($p > 0.05$) according to ANOVA followed by Tukey's post-hoc test.

3.5 Effect of *Trichilia Silvatica* on the Viability of RAW264.7 Cells and Antioxidant Capacity After H_2O_2 Exposure

The leaf and stem extracts at concentrations of 100, and 250 μg/mL increased cell viability compared to H_2O_2 -stimulated cells without previous treatment. The stem extract at 50 μg/mL also presented a significant increase ($p<0.05$) (Figure 7).

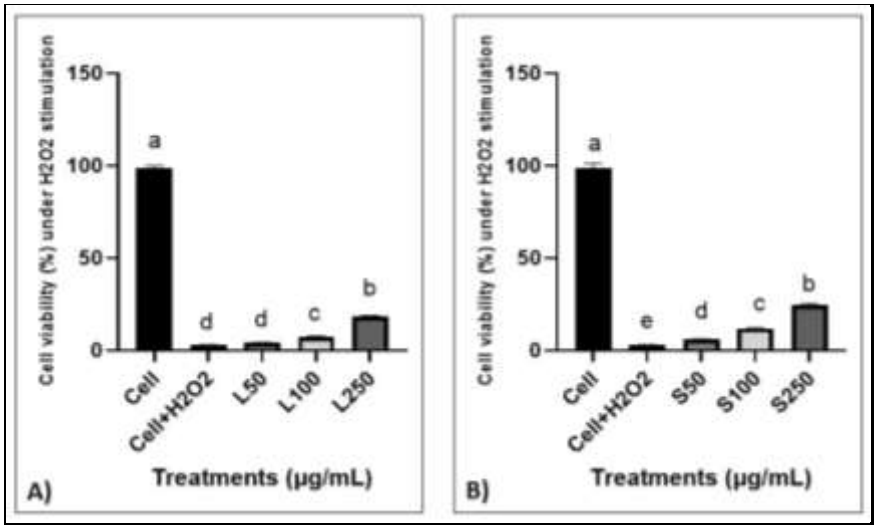


Figure 7 - Cell viability after hydrogen peroxide- induced oxidative stress, using the *T. silvatica* leaf (A) and stem (B) at concentrations of 50, 100 and 250μg/mL; negative control (CTRL-)(cell + DMEM) and positive control (CTRL+) (cell + DMEM + H_2O_2). The data (n = 3) are expressed as mean ± standard error. Means followed by the same letter are not significantly different ($p > 0.05$) according to the Tukey test.

3.6 Catalase Activity

Our results showed an increase in catalase enzyme activity in the L100, S100, and S250 groups after exposure to H_2O_2 compared to H_2O_2 -stimulated cells without previous treatment (Figure 8).

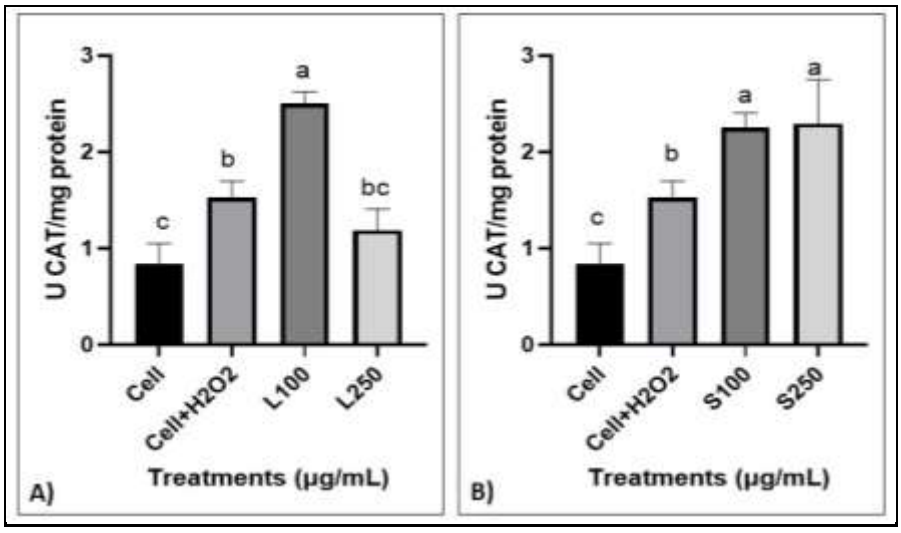


Figure 8 - Catalase activity after macrophage cells exposure to different treatments: cell + DMEM; cell + DMEM + H_2O_2; *T. silvatica* leaf and stem extracts at concentrations of 100 µg/mL and 250 µg/mL; stem extract at concentrations of 100 µg/mL and 250 µg/mL. The data (n = 3) are expressed as mean ± standard error. Means followed by the same letter are not significantly different ($p > 0.05$) by Tukey's test.

3.7 Superoxide Dismutase Activity

No significant difference between most of the treatments evaluated regarding the activity of the enzyme superoxide dismutase (SOD). Only the leaf extract at a concentration of 250 µg/mL reduced SOD activity compared to H_2O_2 -stimulated cells without previous treatment.

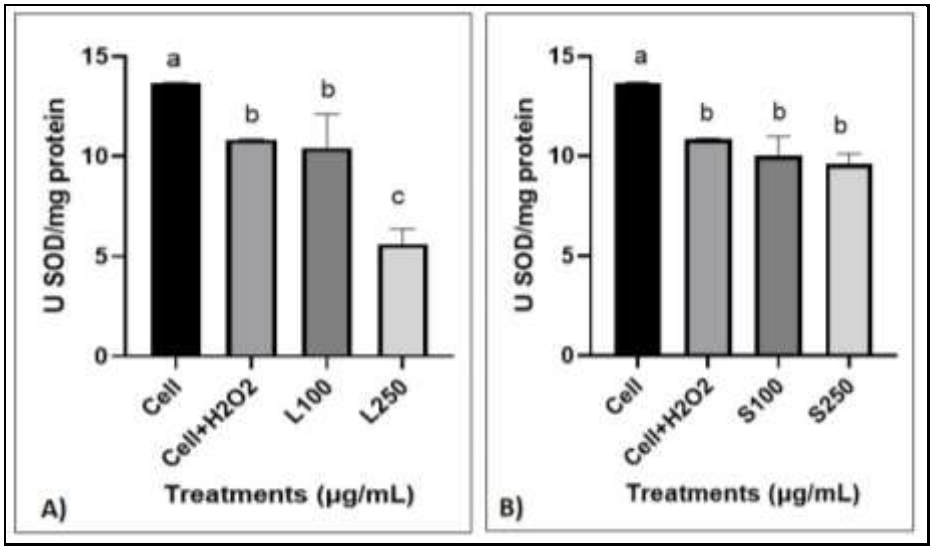


Figure 9 - Superoxide dismutase activity was measured after macrophage cells were exposed to different treatments: cell + DMEM; cell + DMEM + H_2O_2; *T. silvatica* leaf and stem extracts at concentrations of 100 µg/mL and 250 µg/mL. The data (n = 3) are expressed as mean ± standard error. Means followed by the same letter are not significantly different ($p > 0.05$) according to the Tukey test.

3.8 Nitric Oxide Analysis

The leaf and stem extracts at 100, and 250 µg/mL exhibited a reduction in nitric oxide levels compared to H_2O_2 -exposed cells without previous treatment (Figure 10).

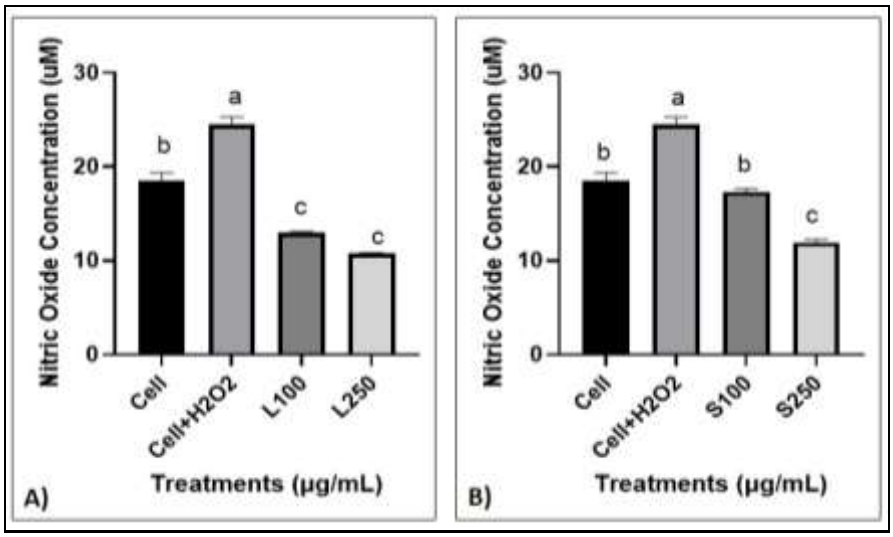


Figure 10 - Nitric Oxide Concentration after Exposure of Macrophage Cells to Treatments: cell + DMEM; cell + DMEM + H_2O_2; *T. silvatica* leaf and stem extracts at concentrations of 100 µg/mL and 250 µg/mL. The data (n = 3) are expressed as mean ± standard error. Means followed by the same letter are not significantly different ($p > 0.05$) according to the Tukey test.

3.9 Gene Expression Analysis

3.9.1 Expression of pro-inflammatory marker genes

The L100 and L250 groups showed a decrease in the gene expression of NF-κB, COX-2, and TNF-α compared to the CTRL+ group. Regarding the stem groups, for NF-κB, a decrease was noted in the S100 group compared to the CTRL+ group (Figure 11). Furthermore, the S100 and S250 groups exhibited a reduction in the gene expression of COX-2 and TNF-α compared to the CTRL+ group.

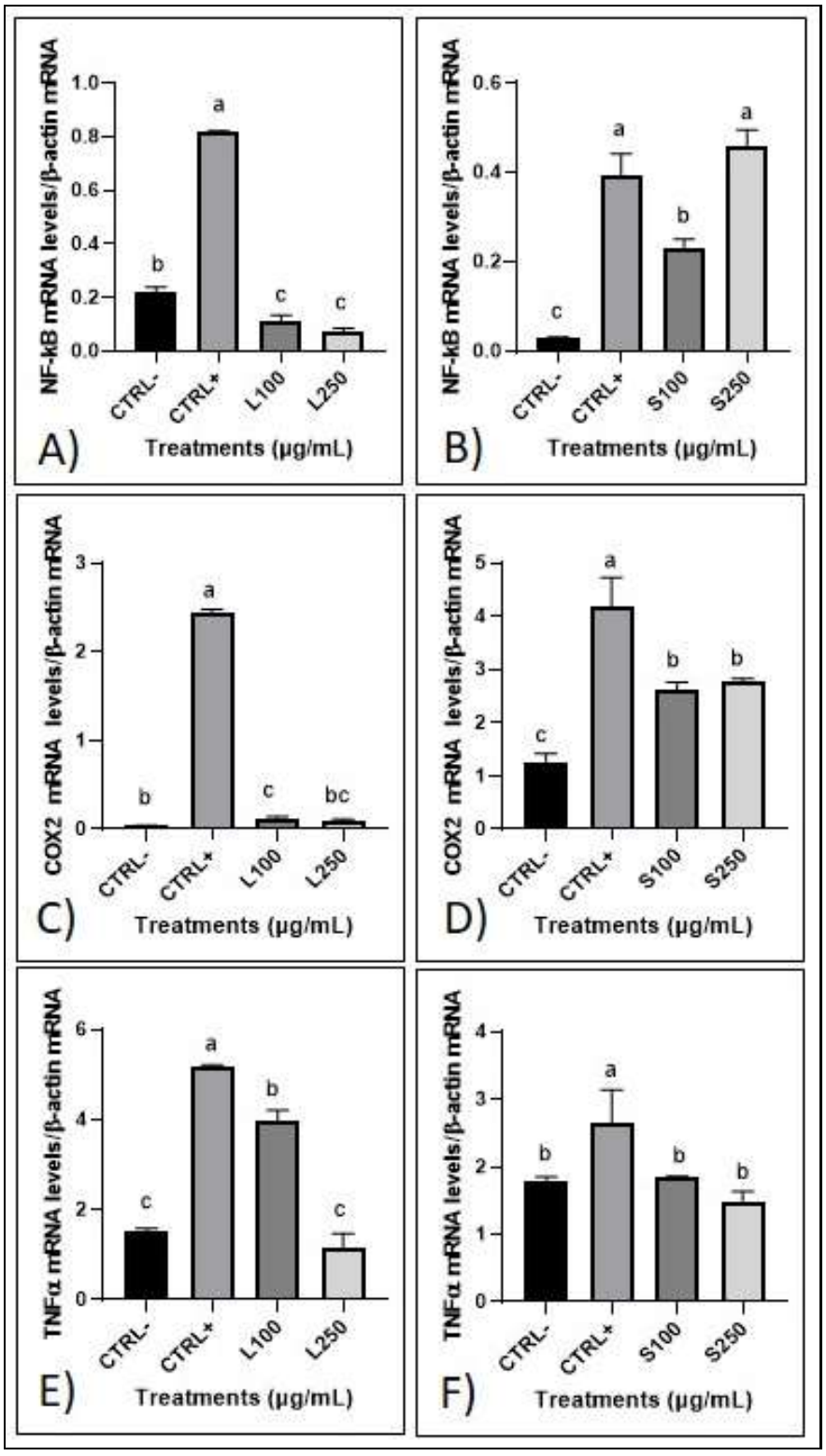


Figure 11 - mRNA expression levels of pro-inflammatory genes — NF-κB (A-B), COX-2 (C-D), TNFα (E-F) — following treatment with *T. silvatica* leaf (L) and stem (S) extracts at concentrations of 100 and 250 µg/mL, compared to untreated cells (CTRL-) and cells stimulated with 10 µg/mL LPS (CTRL+). The data (n = 3) are expressed as mean ± standard error. Means followed by the same letter are not significantly different ($p > 0.05$) according to the Tukey test.

3.9.2 Expression of anti-inflammatory marker genes

The L250 and S100 groups showed an increase in IL-10 gene expression compared to the CTRL+ group, while a decrease in IL-10 gene expression was observed in the L100 and S250 groups compared to the CTRL+ group. Regarding HIF-1, no significant differences were found between the L100, L250, and CTRL+ groups. In the groups exposed to the stems, an increase in gene expression was observed in the S100 and S250 groups compared to the CTRL+ group (Figure 12).

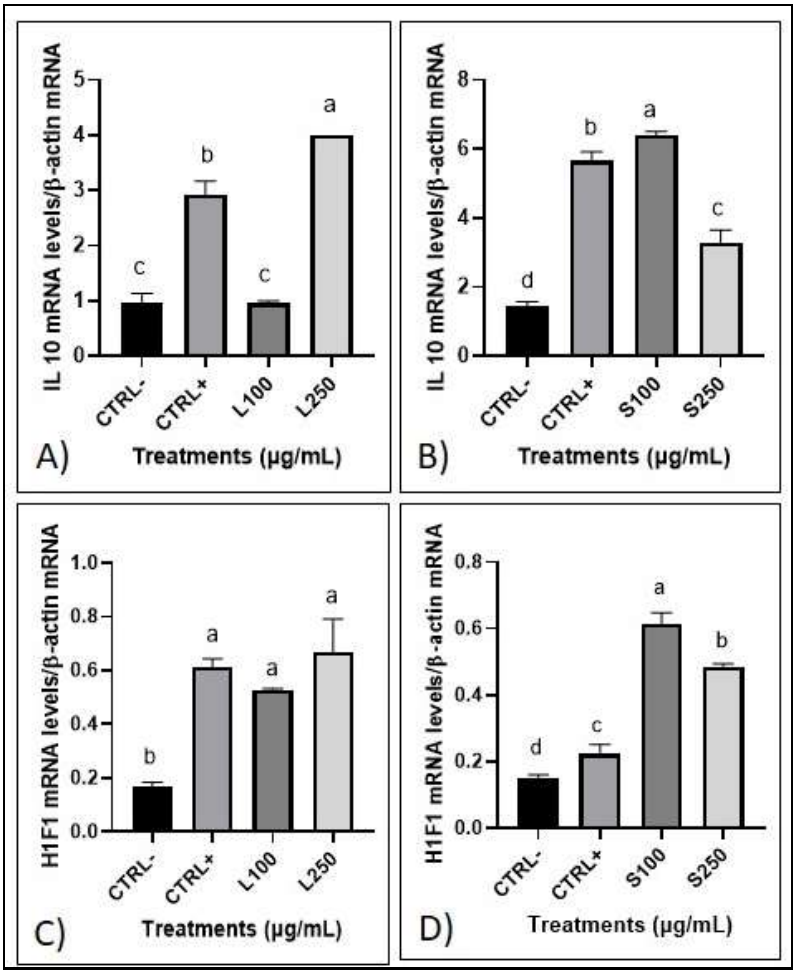


Figure 12 - Results of mRNA expression levels of anti-inflammatory genes — IL-10 (A-B), HIF-1 (C-D) — following treatment with *T. silvatica* leaf (L) and stem (S) at concentrations of 100 and 250 µg/mL, compared to untreated cells (CTRL -) and cells stimulated with 10 µg/mL LPS (CTRL+). The data (n = 3) are expressed as mean ± standard deviation. Statistical difference compared to the control group: means followed by the same letter are not significantly different ($p < 0.05$) according to one-way ANOVA with Tukey post-hoc test.

4 DISCUSSION

Trichilia silvatica is a plant garnering significant interest in Brazilian flora due to its therapeutic and medicinal properties attributed to the bioactive compounds in its secondary metabolites. Among these compounds, terpenoids (triterpenes, sesquiterpenes, limonoids, and steroids) and polyphenols (flavonoids and tannins) stand out for their considerable anti-inflammatory and antioxidant potential. These bioactive compounds are primarily concentrated in stems, leaves, and seeds (Da Silva et al., 2018; Passos et al., 2021).

The chemical characterization of plant extracts under analysis is a very important step in the phytochemical study. The content of special metabolites can be affected by several factors, including seasonal changes, extraction procedures and preparation of the extracts. Therefore, phytochemical analysis and quantification of special metabolites and chemical or biological markers play a crucial role in the evaluation of the quality of plant extracts, since the biological activity observed for them is directly related to their phytochemical profile (Li et al., 2013). Several techniques, including spectrometric and chromatographic methods, can be used for the characterization and/or standardization of the sample. In this study, some characterization methods were based on methods accepted by official compendia (pharmacopoeias) and widely accepted. Phytochemical analysis by TLC and HPLC demonstrated the presence of terpenes, phenolic acids (caffeic and chlorogenic acid), flavonoids, coumarins, and tannins. This result agrees with previous studies described by Da Silva et al. (2021). To obtain data on the quantitative chemical composition of the extracts produced, the quantification of total polyphenols and total tannins in the extracts was performed using a spectrophotometric method. Both extracts analysed (TSS and TSL) showed significant levels of phenolics, flavonoids, and proanthocyanidins. In addition, the development of an analytical methodology for quantification by HPLC-DAD of the caffeic acid and chlorogenic acid (chemical markers) was performed. The content of phenolic acids in TSL were higher than the TSS. The antioxidant activity of *T. silvatica* extracts (TSL and TSS) can be associated with a synergistic interaction between the phenolic compounds (phenolic acids, flavonoids, coumarins, and tannins).

The results obtained in this study demonstrated that both stem and leaf extracts of *Trichilia silvatica* have significant antioxidant activity and a protective effect on cells exposed to H_2O_2. These findings are particularly relevant as they indicate the potential of this extract as a natural therapeutic agent and support their potential in pharmaceutical formulation in the future.

Furthermore, the high levels of phenolics, flavonoids, proanthocyanidins, chlorogenic and caffeic acids, terpenes, and coumarins underscore the bioactive potential of these extracts (Saini et al., 2024). This differential distribution of phenolic compounds across plant parts suggests variability in antioxidant efficacy (Eseberri et al., 2022). Considering these compounds, we evaluated the antioxidant and antiinflammatory potential of *T. silvatica* stem and leaf extracts. The persistent and interconnected dynamic between oxidative stress and inflammatory processes in cells and tissues is referred to as OxInflammation (Lopes et al., 2024). This phenomenon arises from the exacerbated generation of reactive oxygen species, surpassing the capacity of endogenous antioxidant defense mechanisms. For a better understanding, evaluating the antioxidant activity of substances capable of neutralizing ROS is essential. Tests such as DPPH and FRAP assays are commonly used to determine the antioxidant efficacy of these compounds, providing valuable information about their ability to eliminate free radicals. The DPPH assay, revealed high antioxidant capacity, likely due to the elevated concentration of phenolic compounds in the stems and leaves, consistent with results from (Mazutti et al., 2016), who demonstrated that plant parts rich in phenolic compounds, such as stems, exhibit greater antioxidant activity by acting as electron donors to the DPPH radical. This happens because phenolic compounds are characterized by one or more aromatic rings with hydroxyl groups (-OH), which gives them a high electron donation capacity and the ability to neutralize with reactive oxygen species (ROS) (Dai & Mumper, 2010). The study conducted by Perumal et al. (2020) on *T. emetica*, highlights its significant anti-inflammatory, antioxidant, and antibacterial properties, which are attributed to its high content of secondary metabolites, particularly phenolic compounds, flavonoids, and terpenoids. These bioactive molecules exhibit antioxidant activity not only through their ability to donate hydrogen atoms or electrons but also by forming stable radical intermediates, thereby inhibiting the oxidation of various food components, particularly fatty acids and oils. These properties closely resemble those observed in *T. silvatica*, where high phenolic content and antioxidant activity were similarly associated with reductions in inflammatory markers. These findings reinforce the therapeutic potential of the *Trichilia* genus, but especially of *T. silvatica* species, providing scientific support for its traditional use in managing Oxinflammation-related diseases.

The evaluation of the total antioxidant capacity of *T. silvatica* leaf and stem extracts using the FRAP (Ferric Reducing Antioxidant Power) assay revealed their significant ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), reflecting a robust electron-donating capability. This reduction process underscores the potential of the extracts to mitigate damage by neutralizing reactive oxygen species (ROS) and free radicals. The antioxidant activity observed

may be attributed to the interaction between phenolic compounds in the extracts and ferric ions, facilitating their conversion to Fe^{2+}. The efficiency of iron reduction implies the presence in the extracts of reducing mediators such as flavonoids, which are the main electron donors (Youl et al., 2023). A study conducted by Maroyi (2024) on *T. dregeana* extracts demonstrated that the ferric ion reduction observed in the FRAP assay highlights the ability of these compounds to function as primary antioxidants, effectively interrupting oxidative chain reactions and stabilizing ROS. The consistency between these findings and our results on *T. silvatica* suggests a shared mechanism of action, in which phenolic-rich fractions participate in redox reactions and contribute to the neutralization of ROS. The next step in our investigation was to evaluate the impact of *T. silvatica* extract on cellular metabolism through cell viability analyses. Our results indicate that leaf and stem extracts of *T. silvatica* preserved cell viability at the tested concentrations. Leaf extract at 250 µg/mL, exhibited over 70% cell viability under normal conditions, which generally is considered indicative of a non-cytotoxic effect (ISO 10993-5:2009). Furthermore, treatment with both extracts at concentrations of 100 and 250 µg/mL resulted in significantly higher cell viability compared to cells exposed to H_2O_2 alone, indicating a potential modulatory effect on cellular responses as an antioxidant compound. Thus, cell viability results reinforce the hypothesis that our extract at the doses evaluated does not induce significant cytotoxicity and can be used without compromising cellular integrity. However, although the available literature does not provide detailed information on cytotoxic effects at concentrations above 250 µg/mL, we conducted an additional test at 500 µg/mL and observed a 70% reduction in RAW 264.7 cell viability, indicating a cytotoxic effect at this concentration (data not shown). These findings highlight the importance of conducting further investigations to define the safety margins and cytotoxic thresholds of *T. silvatica* extract across a broader concentration range, particularly for potential therapeutic applications. The preservation of cellular viability in RAW264.7 macrophages is particularly relevant, given their critical role in immune regulation and the oxinflammation response. It is important to highlight that the macrophage is considered a primordial cell in the organization and outcomes of acute inflammation, in which there is a greater production of free radicals, ROS, and pro-inflammatory cytokines. The RAW 264.7 cell line that was used in our study remains the best option because it allows us to activate cellular plasticity during the oxidative and inflammatory process. For example, using this model, it is possible to see the capacity of the cells to adapt and modify their phenotypic and functional characteristics according to the local environment, including exposure to extracts. This adaptability is crucial for maintaining tissue homeostasis and ensuring an effective immune response under different conditions. The process of

macrophage plasticity is orchestrated by a variety of extracellular signals, including cytokines, lipid mediators, and interactions with neighbouring cells in the microenvironment, all of which influence macrophage activation and differentiation. Therefore, the macrophages allow us to understand the mechanisms activated after extract exposure to control the acute inflammation and consequently oxidative stress after the respiratory burst.

Our results also showed a significant decrease in nitric oxide (NO) concentration in samples treated with leaf and stem extracts. The observed decrease in NO suggests that chemical molecules in the extracts may regulate NO synthesis by impacting NOS activity or expression, as seen in other plant extracts with anti-oxyinflammatory properties (Okonogi et al., 2018). Other species of the *Trichilia* genus, such as *T. martiana*, have demonstrated antioxidant activity by inhibiting NO production and reducing oxidative stress in LPS-stimulated macrophages (Park et al., 2020). However, this pathway was never studied using the *T. silvatica* and we believe that the results presented in this study can mitigate damage by inhibiting leukocyte migration and inflammation. The bioactive compounds in these extracts, including flavonoids and terpenoids, assist in reducing free radicals and preventing ROS-induced cellular damage. The modulation of oxidative stress markers by *Trichilia* extracts reinforces their therapeutic potential for treating diseases related to oxidative damage, such as chronic inflammation (Da Silva et al., 2018). These findings highlight the role of polyphenol-rich extracts, such as those from *T. silvatica* leaves, in maintaining cell viability and combating oxidative stress (Gullón et al., 2020).

Regarding the enzyme activity, our results demonstrate a significant increase in catalase activity in cells treated with different concentrations of *T. silvatica* extracts from leaf and stem tissues. Catalase is an enzyme crucial for breaking down hydrogen peroxide and protecting cells from oxidative damage (Shi et al., 2022). Then, their increased activity can result from the antioxidant effect of the bioactive compounds present in the extracts (Naz et al., 2023; Ullah et al., 2020). However, no difference was observed regarding superoxide dismutase (SOD) activity. Yang et al. (2013) reported that variations in extract concentrations result in variable modulation of SOD activity, with some compounds promoting an increase in antioxidant activity while others lead to its inhibition. This suggests that catalase activation may be a more pronounced strategy against oxidative stress induced by *T. silvatica*. At the same time, SOD stability reflects a more balanced regulatory response to different concentrations of extracts, corroborating the complexity of interactions between plant compounds and cellular antioxidant pathways (Park et al., 2011). Furthermore, the absence of impact on SOD suggests that the

generation of superoxide radicals was sufficiently controlled by the anti-inflammatory effect, reducing the need for further activation of this enzyme.

The inflammatory response is largely modulated by TNF-α, which plays a crucial role in the OxInflammation process, initiating and amplifying inflammatory responses. TNF-α induces the production of reactive oxygen species (ROS) and activates transcription factors, such as NF-κβ, which regulate the expression of pro-inflammatory cytokines and enzymes, such as COX-2 and iNOS. Increased TNF-α levels are often associated with chronic inflammation and tissue damage, contributing to the progression of several inflammatory diseases (Lopes et al., 2024). Interestingly, Bernardo et al. (2022) demonstrated that the *Trichilia* genus has a significant inhibitory effect on the control of oxidative stress and some inflammatory markers, including a reduction in nitrite levels. These findings corroborate our results, which showed a decrease in TNF-α following treatment with the extracts, further supporting studies such as that of Moura (2012), which demonstrated the anti-inflammatory effect of Aloe vera. The NF-kβ is a transcription factor essential in regulating pro-inflammatory genes, being an important target in controlling the inflammatory response (Barnabei et al., 2021; Zhang et al., 2021). Furthermore, the downregulation of the COX-2 gene observed in the present study reinforces the importance of *T. silvatica* extracts in controlling persistent inflammation mediated by this enzyme. COX-2 is directly involved in the maintenance of chronic oxy-inflammation (Szlasa et al., 2023), and its regulation, observed after treatment with plant extracts, demonstrates the potential of these compounds to modulate critical inflammatory pathways. The relationship between inflammatory mediators, such as NF-κβ and COX-2, is well established in the literature (Ambati & Jachak, 2021), and the activation of NF-κβ after pro-inflammatory stimuli leads to the expression of cytokines, chemokines, and COX-2 (Desai et al., 2018). It is essential to highlight that the best experimental model to understand the cellular mechanisms, cellular biology, and the relationship between Oxidative stress and inflammation after extract exposure is *in vitro*. In our study, we tried to identify the main pathways at the molecular level activated after *T. Silvatica* exposure, and this type of study has never been done before. The cellular pathways activated are a primary study to understand the potential benefits of a new compound in controlling the OxInflammation process and consequently to improve the quality of life of people during the treatment of inflammatory diseases.

The results for IL-10 and HIF-1 indicate complementary responses to exposure to *T. silvatica* extracts. IL-10 expression showed a concentration-dependent regulation, with a downregulation observed at lower concentrations (100 µg/mL leaf and 250 µg/mL stem) and upregulation at higher concentrations (250 µg/mL leaf and 100 µg/mL stem). This pattern may

be associated with the modulation of macrophage activation and the resolution of inflammation. These effects likely stem from the ability of macrophages to differentiate into distinct subsets, mainly pro-inflammatory M1 macrophages, which are predominant at the initial injury site and anti-inflammatory M2 macrophages, which emerge later at the recovery stage, promoting tissue healing (Singampalli et al., 2020). This variation may reflect a dynamic regulation of the immune response, in which different concentrations of bioactive compounds promote different inflammatory modulation profiles. No difference in HIF-1 expression was observed in cells treated with leaf extract compared to the positive control. However, increased HIF-1 expression was verified in cells treated with stem extract, suggesting that stem extract may activate pathways associated with the hypoxic response. Therefore, according to our results, we can suggest that HIF-1 may induce IL-10 production, as reported by Meng et al. (2018), who demonstrated the contribution of HIF-1 to IL-10 synthesis in B cells. Furthermore, we hypothesize that stem extract exerts a protective effect, possibly mediated by cellular adaptation to chronic oxidative stress, which follows the increased expression of HIF-1 under stress conditions (Pan et al., 2021). Thus, *T. silvatica* extracts appear to act differently, modulating inflammatory and adaptive cellular responses to stress. In this context, the results obtained in this study demonstrated that the stem and leaf extracts of *T. silvatica* exhibit significant antioxidant activity and protective effects on cells stimulated with LPS or H_2O_2. These findings are particularly relevant because they highlight the potential of *T. silvatica* extract as a natural therapeutic agent with anti-inflammatory and cytoprotective properties, contributing to the modulation of OxInflammatory responses. Therefore, this knowledge may pave the way for the formulation of new natural drugs within the pharmaceutical field to improve the quality of Human life, as an antioxidant and anti-inflammatory promising compound.

5 CONCLUSION

Trichilia silvatica extracts revealed the presence of terpenes/steroids, coumarins, condensed tannins, and phenolic acids, including chlorogenic and caffeic acids. These extracts demonstrated significant antioxidant activity, as evidenced by over 75% inhibition of the DPPH radical and increased FRAP values. Additionally, our findings indicate that *T. silvatica* extracts modulate oxidative stress and inflammatory pathways, by downregulating NF-κB, TNF-α, and COX-2, while upregulating IL-10 and HIF-1 expression, suggesting improved tissue oxygenation and nutrient supply. Collectively, these results support the traditional medicinal use of *T. silvatica* in therapeutic approaches for managing inflammatory conditions.

6 LIST OF ABBREVIATIONS

AA, Ascorbic acid

ACN, Acetonitrile

AcOEt, Ethyl acetate

ANOVA, Analysis of variance

CAE, Catechin equivalents

CAT, Catalase

$CHCl_3$, Chloroform

CO_2, Carbon dioxide

COX-2, Ciclooxigenase-2

CTRL +, Control positive

CTRL -, Control negative

DMEM, Dulbecco's Modified Eagle's Medium

DMSO, Dimethyl sulfoxide

DPPH, 2,2-Diphenyl-1-picrylhydrazyl

DNA, Deoxyribonucleic acid

ELISA, Enzyme-Linked Immunosorbent Assay

EtOH, Ethanol

FBS, Fetal bovine serum

FRAP, Ferric Reducing Antioxidant Power

GAE, Gallic acid equivalent

H_2O_2, Hydrogen peroxide

HPLC, High performance liquid chromatography

H1F-1, Hypoxia-inducible factor-1

IL-1, Interleukin-1

IL-1β, Interleukin-1β

IL-2, Interleukin-2

IL-6, Interleukin-6

IL-10, Interleukin-10

IL-17, Interleukin-17

KOH, Potassium hydroxide

LPS, Lipopolysaccharide

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MeOH, Methanol

NFκB, nuclear factor kappa beta

NO, Nitric Oxide

NOS, Nitrogen oxide Sintase

NP/PEG, Natural products-polyethylenglycol reagent

OxInflammation: Oxidative Stress and Inflammation

PCR, Polymerase Chain Reaction

PTFE, Polytetrafluoroethylene

q-PCR, Quantitative Polymerase Chain Reaction

Rf, Retention factor

RNA, Ribonucleic acid

RNS, Reactive nitrogen species

ROS, Reactive oxygen species

Rt, Retention time

SOD, Superoxide dismutase

TCL, Thin-layer chromatography

TSL, *Trichilia sylvatica* leaf

TSS, *Trichilia silvatica* stem

TNF-α, tumor necrosis factor alpha

UV-VIS, Visible ultraviolet

7 ACKNOWLEDGEMENTS

The authors would like to thank the Research Support Foundation of the State of Minas Gerais (Brazil) FAPEMIG (processes APQ-03519-22, APQ-04164-22, APQ-00373-21 and

BIP-00213-24) the National Council for Scientific and Technological Development (CNPq) (310413/2023-0), and the Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil) (Finance Code 001) for their financial support and Andreia Fonseca Silva by collected and identified the vegetal species.

8 AUTHORSHIP CONTRIBUTIONS

LLS and MMSD contributed to the conceptualization and design of the study, data acquisition, analysis, and interpretation, as well as writing the manuscript. SP and RAP were involved in data acquisition, analysis, and interpretation. ASBC contributed with data and extract aquisition. VLA contributed to extract acquisition, physicochemical characterization, and critical revision of the manuscript. RG contributed to the conceptualization and design of the study, supervision, data interpretation, critical revision of the manuscript, and funding acquisition.

9 DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest related to this study.

10 DATA AVAILABILITY

All acquired data are systematically presented within the text. For additional inquiries, please direct them to the corresponding author.

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