UNIVERSIDADE FEDERAL DE VIÇOSA

OxInflammatory response in wound healing and using *Commiphora leptophloeos* leaves and barks to control the OxInflammation

Fernanda Barbosa Lopes
Doctor Scientiae

VIÇOSA - MINAS GERAIS
2024

FERNANDA BARBOSA LOPES

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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-adviser: Manoela M. dos Santos Dias

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Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
L864e
2024

Lopes, Fernanda Barbosa, 1980-
OxInflammatory response in wound healing and using *Commiphora leptophloeos* leaves and barks to control the OxInflamation / Fernanda Barbosa Lopes. – Viçosa, MG, 2024.
1 tese eletrônica (158 f.): il. (algumas color.).

Texto em inglês.
Inclui anexo.
Orientador: Reggiani Vilela Gonçalves.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Biologia Celular e Estrutural, 2024.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2025.064
Modo de acesso: World Wide Web.

1. Inflamação - Aspectos imunológicos. 2. Estresse oxidativo. 3. *Commiphora leptophloeos*. 4. Cicatrização de ferimentos. 5. Citocinas. I. Gonçalves, Reggiani Vilela, 1979-. II. Universidade Federal de Viçosa. Departamento de Biologia Celular e Estrutural. Programa de Pós-Graduação em Biologia Celular e Estrutural. III. Título.

CDD 22. ed. 571.96

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

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APPROVED: October 21, 2024.

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Dedico este trabalho à minha família, pelo amor incondicional, apoio e paciência ao longo dessa jornada.

Aos meus pais e irmãos, que sempre me inspiraram com sua sabedoria, força e dedicação, mostrando-me o valor da educação e da perseverança.

À minha orientadora, Reggiani Vilela Gonçalves, e às minhas coorientadoras, pela orientação, confiança e pela amizade que tornaram este percurso mais leve e enriquecedor.

Aos amigos e colegas que estiveram comigo nos momentos de dúvida e celebração, oferecendo sempre palavras de incentivo.

E, especialmente, a todos que acreditaram em mim quando eu mais precisei acreditar em mim mesma.

Com carinho e gratidão.

ACKNOWLEDGMENTS

Agradeço a Deus, em cujas mãos coloco, todos os dias, a minha vida, meu destino, minhas decisões, por sempre estar presente ao meu lado, nas pequenas coisas, no meu caminhar de aprendizado, em cada benção enviada a mim.

Ao meu esposo Siderlan, meu amor e gratidão não poderiam ser maiores. Nestes 22 anos de matrimônio, você foi mais que um companheiro – foi pai, mãe, professor e meu porto seguro em todos os momentos em que estive ausente. Seu amor incondicional e sua dedicação aos meus sonhos foram fundamentais para que eu pudesse chegar até aqui. Esse momento de realização não é só meu, é nosso. Suas renúncias, envolvimento nas minhas decisões e lutas mostram o quanto este sonho é compartilhado.

Às minhas queridas filhas, Maria Clara e Ana Beatriz, obrigada por compreenderem e aceitarem tantas ausências, por abraçarem a importância do trabalho da mamãe com tanto carinho. Vocês, mais do que ninguém, me inspiram a seguir sempre em frente. Ver o valor que dão à minha dedicação ao ensino e à pesquisa me enche de orgulho.

À minha família: pais, irmãos, cunhados e sobrinhos, cada palavra de incentivo, cada gesto de cuidado com a minha família ao longo desse caminho fez toda a diferença. Obrigada pelo apoio constante, por estarem sempre ao meu lado, e por ajudarem a tornar possível esse momento tão especial.

À minha orientadora Reggiani Vilela Gonçalves, toda a minha gratidão pela oportunidade. Seu apoio, ensinamentos e competência ao longo desse processo foram essenciais para o meu crescimento, não apenas como pesquisadora, mas como pessoa. Sou profundamente grata por sua orientação e, acima de tudo, pela compreensão e paciência ao longo dessa jornada. Mais do que uma orientadora brilhante, você se tornou uma amiga, e essa amizade é um presente que levarei comigo para toda a vida.

À minha coorientadora Mariáurea Matias Sarandy Souza, não tenho palavras suficientes para agradecer por toda a ajuda e apoio que você me deu ao longo do doutorado. Sua orientação, paciência e amizade foram fundamentais para que eu chegasse até aqui. Mais do que uma coorientadora, você foi uma parceira incansável e uma verdadeira amiga em todos os momentos. Cada conselho, incentivo e até os puxões de

orelha foram essenciais para o meu crescimento. Sou profundamente grata por ter tido você ao meu lado nessa jornada. De coração, muito obrigada por tudo!

À minha coorientadora Manoela Maciel dos Santos, quero expressar toda a minha gratidão por sua enorme dedicação e ajuda no meu doutorado. Sua presença nos experimentos, sua paciência e atenção aos detalhes foram essenciais para que eu pudesse concluir essa etapa tão importante. Você me guiou com tanto cuidado e profissionalismo, e isso fez toda a diferença na qualidade do trabalho final. Sou muito grata por todo o apoio que você me deu, especialmente nos momentos decisivos. Muito obrigada por ser uma coorientadora tão dedicada e por acreditar no meu potencial até o último momento!

À professora Silvana Maria Zucolotto Langassner, gostaria de expressar minha profunda gratidão por sua parceria e generosidade. Sua contribuição e colaboração fortaleceu laços, sendo essencial para o sucesso dos estudos. Sua disposição em compartilhar conhecimento e recursos fez uma grande diferença no andamento da pesquisa. Sou imensamente grata por todo o apoio e pela confiança que depositou no meu trabalho.

Aos professores Rômulo Dias Novaes, Giuseppe Valacchi, Mariella Bontempo Freitas, Thiago Antônio de Oliveira Mendes, por todo o auxílio durante o desenvolvimento da pesquisa.

Aos professores Leandro Licursi de Oliveira, Mônica Morais Santos, Silvania Maria Zucolotto Langassner e Mariáurea Matias Sarandy Souza, que fizeram parte da banca da qualificação, gostaria de expressar minha mais sincera gratidão pelo valioso trabalho. A contribuição de cada um foi essencial para a lapidação da minha escrita.

A avaliação cuidadosa e o olhar crítico que trouxeram me permitiram aprimorar e fortalecer a pesquisa, preparando-me de maneira mais sólida para os próximos passos. O tempo e a dedicação de vocês nesse processo foram inestimáveis, e sou profundamente grata por todo o apoio recebido.

Aos amigos que fiz durante o doutorado, quero expressar minha gratidão por cada um de vocês. Em especial, à Néia de Paula, Silvânia Pelinsari e seu esposo Emerson, pelas incontáveis horas de dedicação aos experimentos, pela ajuda incansável em cada etapa do doutorado, e pelos dias, noites e finais de semana que vocês se doaram para que esse trabalho avançasse. Sua amizade, apoio e contribuição foram inestimáveis.

Aos todos os colegas do grupo de pesquisa do Laboratório de Patologia

Experimental (LAPEX), pelo apoio durante o desenvolvimento das atividades laboratoriais. Patrícia Mattosinhos, Leonardo Lopes Silveira, Pedro Henrique Barbosa e Maria Lúcia de Almeida pela amizade, apoio e contribuição durante o desenvolvimento do trabalho. Ninguém faz pesquisa sozinho, nenhum laboratório flui sozinho, a pesquisa e seu avanço só se dão pelo apoio mútuo e o trabalho em equipe. Eu posso dizer, fiz parte de uma excelente equipe! Obrigada a todos.

A Universidade Federal de Viçosa 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.

Por fim, expresso minha gratidão a todos que contribuíram, de forma direta ou indireta, e que não foram mencionados. Meus sinceros agradecimentos.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

“O único lugar aonde o sucesso vem antes do trabalho é no dicionário”.
(Albert Einstein)

ABSTRACT

LOPES, Fernanda Barbosa, D.Sc., Universidade Federal de Viçosa, October, 2024. **OxInflammatory response in wound healing and using *Commiphora leptophloeos* leaves and barks to control the OxInflammation**. Adviser: Reggiani Vilela Goncalves. Co-adviser: Manoela Maciel dos Santos Dias.

Inflammation is the body's response to tissue damage and is characterized by a series of events involving different cell types with the ability to secrete chemical mediators that are key to tissue repair processes. To compile a book, we carried out a literature review and noted that the main cells involved in inflammatory responses are neutrophils, macrophages and other cells of the immune system. The first step in the inflammatory response is the upregulation of genes such as nuclear factor kappa ß (NFK-ß) and the activation of a multi-protein complex known as the inflammasome, which serves as a platform for the activation of caspase-1, which cleaves pro-IL-1-ß and pro-IL-18 into activated interleukin-1 (IL-1ß) and interleukin-18 (IL-18). The secretion of these mediators results in the recruitment of leukocytes, which rapidly produce reactive oxygen and nitrogen species that increase vascular activation and the phagocytic capacity of cells to remove dead cells and debris. In this sense, oxidative stress emerges as a critical link between inflammation and tissue repair, affecting cell migration and consequently the process of tissue repair resolution. Thus, inappropriate activation of the NLRP3 inflammasome can trigger chronic inflammation and tissue damage, establishing a vicious cycle between ROS and the persistence of the inflammatory process (OxInflammation). Understanding these mechanisms is therefore essential for the development of effective therapeutic approaches to promote effective and infection-free tissue repair. Based on this, this study provides a mechanistic theory to explain the process of OxInflammation during tissue repair by analyzing the methodological quality of current evidence. It also attempts to understand how exposure to plant extracts from *Commiphora leptophloeos* can control the process of Oxyinflammation in macrophages. Therefore, a systematic review was conducted according to the PRISMA guidelines, with a structured search of MEDLINE (PubMed), Scopus and Web of Science databases, and 23 original studies were analysed. Bias analysis and study quality were assessed using the SYRCLE tool. The results showed activation of membrane receptors such as IFN-d, TNF-a and toll-like receptors in phagocytes, especially macrophages, during the initial phase of skin wound repair. In addition, positively regulated pathways such as STAT1, IP3 and NF-kß were identified, as well as Ca^{2+} mobilization associated with ROS production and activation of the

inflammasome pathway (NLRP3). The proteolytic consequences of caspase-1 are the cleavage and release of the now active IL-1ß and IL-18, which play roles in immune modulation and vasodilation. These findings are important for understanding the mechanisms involved in skin tissue repair and for developing effective therapeutic approaches. In the second study, the plant *Commiphora leptophloeos*, known as 'Imburana', showed antioxidant and anti-inflammatory properties by negatively regulating the process of OxInflammation, possibly due to the presence of quinic acid tannins and oligomeric B-type procyanidins in the stem bark and the presence of six flavonoid C-glycosides (orientin, isoorientin, vitexin, isovitexin, isoquercitrin and quercetrin) in its leaves. Our results showed =80% inhibition of the free radical DPPH, increased cell viability and protection against protein denaturation, hemolysis, as well as a reduction in ON and H_2O_2 levels and an increase in the cell migration index for both parts of the plant. *Commiphora leptophloeos* extracts showed efficacy in reducing oxidative inflammation, as evidenced by a decrease in the expression of TLR4 and NF-?ß, leading to a decrease in the pro-inflammatory cytokines BAX, IL-6, TNF-a and COX2. At the same time, there was an increase in the expression of NFR2, which helps to control oxidative stress and resolve acute inflammation. However, the expression of HO-1 and HIF-1 decreased, suggesting a cross-regulation between NF-?ß, HO-1 and HIF-1. The results suggest that condensed tannins at stem bark concentrations (50 and 100µl) showed therapeutic potential in vitro by reducing OxInflammation and restoring cellular homeostasis, probably due to the action of condensed tannins, thereby demonstrating their efficacy in vitro. Due to its high concentration of flavonoid C-glycosides, the leaf extract has been shown to possess potent antioxidant properties with cosmetic potential, offering protection against premature aging and damage induced by free radicals, UV rays and pollution. These findings highlight the need for further studies to better elucidate the underlying mechanisms of action.

Keywords: inflammation; oxidative stress; oxInflammation; cytokines; wound healing; imburana; *Commiphora leptophloeos*.

RESUMO

LOPES, Fernanda Barbosa, D.Sc., Universidade Federal de Viçosa, outubro de 2024. **Resposta OxInflamatória na cicatrização de feridas e uso de folhas e cascas de *Commiphora leptophloeos* para controlar a OxInflamação**. Orientadora: Reggiani Vilela Goncalves. Coorientadora: Manoela Maciel dos Santos Dias.

A inflamação é uma resposta do organismo a lesões teciduais caracterizadas por uma sequência de eventos que envolvem vários tipos celulares e que tem a capacidade de secretar mediadores químicos que são a chave nos processos de reparo tecidual. Para a confecção de um livro, realizamos uma revisão de literatura, observamos que as principais células envolvidas nas respostas inflamatórias são neutrófilos, macrófagos e outras células do sistema imune O primeiro passo da resposta inflamatória é a upregulation de genes como fator nuclear de Kappa ß (NFK-ß) e a ativação de um complexo multiproteico conhecido como inflamassoma que serve como uma plataforma para ativação da caspase-1, que realiza a clivagem de pró-IL1-ß e pró-IL-18 em Interleucina 1 (IL-1) e Interleucina 18 (IL-18) ativadas. A secreção destes mediadores, resulta no recrutamento de leucócitos que rapidamente produzem espécies reativas de oxigênio e nitrogênio, que aumentam a ativação vascular e a capacidade fagocítica de células eliminando células mortas e restos debris. Neste sentido, o estresse oxidativo emerge como um elo crítico entre inflamação e o reparo tecidual, afetando a migração celular e consequentemente o processo de resolução do reparo tecidual. Assim, a ativação inadequada do inflamassoma NLRP3 pode desencadear uma inflamação crônica e dano tecidual, estabelecendo um ciclo vicioso entre ROS, e a persistência do processo inflamatório (OxInflamação). Portanto, entender esses mecanismos é essencial para desenvolver abordagens terapêuticas eficazes para promover um reparo tecidual eficaz e livre de infecções. Baseado nisto, este estudo fornece uma teoria mecanicista para explicar o processo de Oxinflamação durante o reparo tecidual através de uma análise da qualidade metodológica da evidência atual. Adicionalmente, tenta entender como a exposição a extratos vegetais obtidos de *Commiphora leptophloeo*s pode controlar o processo de OxInflamação em macrófagos. Assim, uma revisão sistemática foi conduzida seguindo as diretrizes PRISMA, com uma busca estruturada nas bases de dados MEDLINE (PubMed), Scopus e Web of Science, analisando 23 estudos originais. A análise de viés e a qualidade do estudo foram feitas usando a ferramenta SYRCLE. Os resultados mostraram a ativação de receptores de membrana como IFN-d, TNF-a e receptores do tipo Toll-like em fagócitos, especialmente macrófagos, durante a fase inicial do

reparo de feridas cutâneas. Além disso, foram identificadas vias reguladas positivamente, como STAT1, IP3 e NF-kß, e a mobilização de Ca^{2+} associada à produção de ROS e ativação da via do inflamassoma (NLRP3). As consequências proteolíticas da caspase-1, são a clivagem e liberação das IL-1ß e IL-18 agora ativas, que desempenham papéis na modulação imunológica e vasodilatação. Essas descobertas são essenciais para entender os mecanismos envolvidos no reparo tecidual cutâneo e para desenvolver abordagens terapêuticas eficazes. No segundo estudo, a planta *Commiphora leptophloeos*, conhecida como "Imburana", apresentou propriedades antioxidantes e anti-inflamatórias regulando negativamente o processo de oxinflamação, possivelmente devido à presença de taninos de ácido quínico e procianidinas oligoméricas do tipo B na casca do caule, e a presença de seis flavonoides C-glicosídeos (orientina, isoorientina, vitexina, isovitexina, isoquercitrina e quercetrina) em suas folhas. Nossos resultados mostraram =80% de inibição do radical livre DPPH, aumento da viabilidade celular, e proteção contra desnaturação proteica, hemólise, além de redução dos níveis de ON e H_2O_2 e aumento do índice de migração celular por ambas partes da planta. Os extratos de *Commiphora leptophloeos* mostraram eficácia na redução da inflamação oxidativa, como evidenciado por uma diminuição da expressão de TLR4 e NF-?ß, levando a uma diminuição das citocinas pró-inflamatórias BAX, IL-6, TNF-a e COX2. Ao mesmo tempo, verificou-se um aumento da expressão de NFR2, que ajudou a controlar o stress oxidativo e a resolver a inflamação aguda. No entanto, a expressão de HO-1 e HIF-1 diminuiu, sugerindo uma regulação cruzada entre NF-?ß, HO-1 e HIF-1. Os resultados sugerem que taninos condensados nas concentrações casca do caule (50 e 100µl) apresentaram potencial terapêutico in vitro, reduzindo a oxinflamação e restaurando a homeostase celular, provavelmente devido à ação dos taninos condensados, demonstrando assim sua eficácia in vitro. Devido à sua alta concentração de flavonoides C-glicosídeos, o extrato da folha demonstrou possuir propriedades antioxidantes potentes com potencial cosmético, oferecendo proteção contra o envelhecimento prematuro e os danos induzidos por radicais livres, raios UV e poluição. Essas descobertas destacam a necessidade de mais estudos para elucidar melhor os mecanismos de ação subjacentes.

Palavras-chave: inflamação; estresse oxidativo; oxinflamação; citocinas; cicatrização de feridas; imburana; *Commiphora leptophloeos*

Sumário

CHAPTER 01

Interplay of Oxidative Stress and Inflammation in Skin Wound Healing: Potential of Plant-Derived Antioxidants

Fernanda Barbosa Lopes

PREFACE

Dear Readers,

I am pleased to present the chapter 'Oxidative Stress and Inflammation in cutaneous wound healing.' This chapter will be part of a book collection to be published by the Laboratory of Experimental Pathology, the Postgraduate Programme in Cellular and Structural Biology (Department of General Biology), and the Postgraduate Programme in Animal Biology (Department of Animal Biology) of the Federal University of Viçosa. The book aims to publish literature reviews of research in the field of Biological and Health Sciences, with a focus on Histopathology and Histomorphology, mainly in the study of the biotechnological potential for the development of drugs that promote skin, liver, and lung repair, associated or not with diabetes. The author, Fernanda Lopes, has a degree in Biology from Santa Marcelina College, a Master's in Animal Biology, and a PhD in Cellular and Structural Biology from the Federal University of Viçosa, provides a comprehensive overview of the inflammatory, proliferative, and remodeling phases, highlighting the interaction between oxidative stress and inflammation in the healing process.

This chapter provides a detailed analysis of the skin's functions, the importance of the inflammatory phase in healing, the plasticity of macrophages, and the role of the inflammasome in skin wound inflammation. In addition, the author discusses the influence of antioxidants, promising therapies, and the interaction between oxidative stress and inflammation in wound healing.

Readers are encouraged to read this chapter to gain an in-depth understanding of the biological mechanisms involved in wound healing and explore emerging therapeutic strategies to optimize this vital process. The author's multidisciplinary approach, experience in clinical analysis, and active methods in biology and chemistry ensure a comprehensive and up-to-date perspective on the subject.

I hope that this chapter will be a valuable source of knowledge and inspire new research and advances in wound healing.

PRESENTATION

The interplay between oxidative stress and inflammation in the wound-healing process is critical because these processes significantly impact all healing phases. Oxidative stress, characterized by the excessive production of reactive oxygen species, can cause cell damage and delay cell migration and proliferation necessary for proper healing. On the other hand, inflammation plays a paradoxical role, essential for initiating the healing process, but can lead to chronic complications if not adequately regulated. Fernanda Barbosa Lopes, Biologist, Master in Animal Biology and PhD in Cellular and Structural Biology, is the author of scientific articles in scientific research published in high-impact journals. The author's specific contributions to the content of this book come from her previous research and publications on the morphophysiology of enteroendocrine cells, a systematic review of OxInflammation in cutaneous wound healing, solid experience in clinical analysis, and experience in the field of antioxidants, wound healing, and inflammatory processes. Therefore, understanding and targeting this interaction through therapeutic strategies, such as using plant extracts rich in antioxidants and anti-inflammatory compounds, is crucial in improving wound healing outcomes and preventing complications associated with tissue repair disorders. This topic is essential for developing effective therapeutic approaches and better understanding the mechanisms underlying the wound-healing process.

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

(H_2O_2)	Hydrogen peroxide
(O_2^{--}):	Superoxide radical
(O_3):	Ozone
(^{-}OH)	Hydroxyl radical
($ONOO^-$)	Peroxynitrite anion
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxyanisole
Ca^+	Calcium ion
CASP	Caspase
CAT	Catalase
CoQ10	Ubiquinone
CoQH2	Ubiquinol
DAMPS	Danger-associated molecular sensor
DNA	Desoxyribonucleic acid
EGF	Epidermal growth factor
eNOS	Endothelial NOS
FGF-2	Fibroblast growth factor 2
FLG	Filaggrin
FLIP	FLICE-like inhibitory protein
GSDMD	Gasdermin D
GST	Glutationa s-transferases
HIF-1	Hypoxia-inducible factor-1

HO-1	Heme oxygenase 1
HSP90	Heat shock protein 90
IFN-γ	Interferon-gamma
IKK	Iκκ kinase
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
iNOS:	Inducible NOS
IP-10	IFN-γ-inducible protein 10
IP3 pathway	Inositol 1,4,5-triphosphate
I	Involucrin
K^+	Potassium ion
LOR	Loricrin
LPS	Lipopolysaccharide
M1	Macrophages phenotype type 1
M2	Macrophages phenotype type 2
MAPKs	MAP kinases
MAVS	Mitochondrial antiviral signalling protein
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Mononuclear phagocyte colony-stimulating factor
MIP-1α	Macrophage inflammatory protein-1 alpha
MMP	Metalloproteinase
mtNOS	Mitochondrial NOS
NADPH	Nicotinamide adenine dinucleotide phosphate
Na-K-ATPase	Sodium–potassium pump
NF-κβ	Nuclear factor kappa κβ

NK	Natural killer cells
NLR's	NOD-like receptors
NLRP3	NOD-like receptor protein 3
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthases
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
Nrf-2	Nuclear factor erythroid 2-related factor 2
Ox-mtDNA	Oxidised mtdna
P2X7	Purinergic receptor p2x7
PAMPS	Pathogen-associated molecular patterns
PDGF	Platelet-derived growth factor
PRRs	Pattern recognition receptors
RNS	Reactive nitrogen species
ROS	Reactive oxygen Species
SOD	Superoxide dismutase
TGF-β	Transforming growth factor beta
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
Treg	T regulatory cells
Trm	T memory resident cells
UVB	Ultraviolet B
VEGF	Vascular endothelial growth factor
VIM	Vimentin

ABSTRACT

As the largest organ in the human body, the skin is susceptible to various injuries that can trigger pathways associated with pain and inflammation. In addition, this sensitivity to external factors also makes it susceptible to damage that compromises its structure and function. Skin wound healing is a dynamic process involving complex interactions between molecules, cells, and soluble mediators. It begins with an inflammatory response, followed by tissue proliferation and remodeling phases. Inflammation plays a crucial role in this process, involving the production of pro-inflammatory cytokines, reactive oxygen Species (ROS), and reactive nitrogen species (RNS), which are essential for resolving the inflammatory process and improving the wound healing process. The imbalance between pro- and anti-inflammatory mechanisms can delay healing by exacerbating inflammation. Oxidative stress is emerging as a critical link between inflammation and wound healing, affecting cell migration and proliferation and the expression of inflammatory mediators that are very important to controlling tissue regeneration. Antioxidants from plant extracts have been investigated for their anti-inflammatory and antioxidant properties, showing potential in regulating the inflammatory response and oxidative stress during the healing process. Understanding these mechanisms is essential for developing effective therapeutic approaches to promote wound healing and prevent complications associated with tissue repair disorder.

1. INTRODUCTION

More than just an outer layer, the skin is a complex system of different layers and structures that perform various functions. Playing a vital role in maintaining the body's homeostasis, it regulates body temperature, protects against pathogens, and excretes metabolic waste, among other functions. Its high sensitivity to physical stimuli can alter its chemical and biological properties, activating specific pathways associated with pain and inflammation [1,2]. However, this same sensitivity also makes it vulnerable to damage caused by external factors, such as wounds, burns, and metabolic diseases like diabetes [3]. This damage can compromise the structure and functions of the skin, often resulting in challenging prognoses for tissue recovery [4,5].

1.1 Skin Structure

To fully understand the complex physiological functions of the skin, it is crucial to understand its anatomical structure and the different layers that make it up (Fig. 1). Skin is derived from ectoderm (epidermis) and mesoderm (dermis, subcutis). Each layer performs

specific functions and interacts harmoniously to maintain homeostasis [6]. The epidermis is the outermost layer of the skin, composed mainly of keratinized epithelial cells, the keratinocytes, organized into several layers. The keratinocyte is responsible for producing keratin, a protein that gives the skin mechanical resistance and impermeability. Like macrophages, they can exert and modulate the inflammatory response in the skin, as they are constantly exposed to environmental insults [7], although their specific mechanisms and roles may vary. The dermis, the middle layer of the skin just below the epidermis, is made up of interstitial elements such as collagen fibers and elastic tissue, as well as a ground substance and various cells, including fibroblasts, mast cells, plasma cells, lymphocytes, and histiocytes. It also contains blood vessels, lymphatic vessels, sensory nerves, including sensory receptors, among them are the free nerve endings, such as nociceptors that detect tissue damage, and specialized lamellated corpuscles such as Pacinian's, which detect vibrations, and Meissner's, responsible for touch and pressure sensations. Collagen, especially types I and III, are the main structural component of the dermis and are deposited by fibroblasts [8], cells that play an essential role in skin homeostasis, wound healing, and hair follicle formation. The dermis also contains histiocytes and antigen-presenting cells, which phagocytize foreign substances and present them to T lymphocytes, contributing to immune defense. Mast cells near the blood vessels release histamine during allergic reactions and are fundamental to the immune response. Thus, the dermis provides structural and sensory support and performs vital functions in nourishing the epidermis, regulating body temperature, and providing immune protection [9,10]. The subcutis, also known as the subcutaneous layer, is the deepest layer of the skin. It comprises unilocular adipose tissue and loose connective tissue; as a thermal insulator and energy reserve, the production of hormones such as leptin plays a fundamental role in regulating appetite. In addition, the subcutis plays an essential role in fixing the skin to the underlying tissue and absorbing mechanical shocks [9,11].

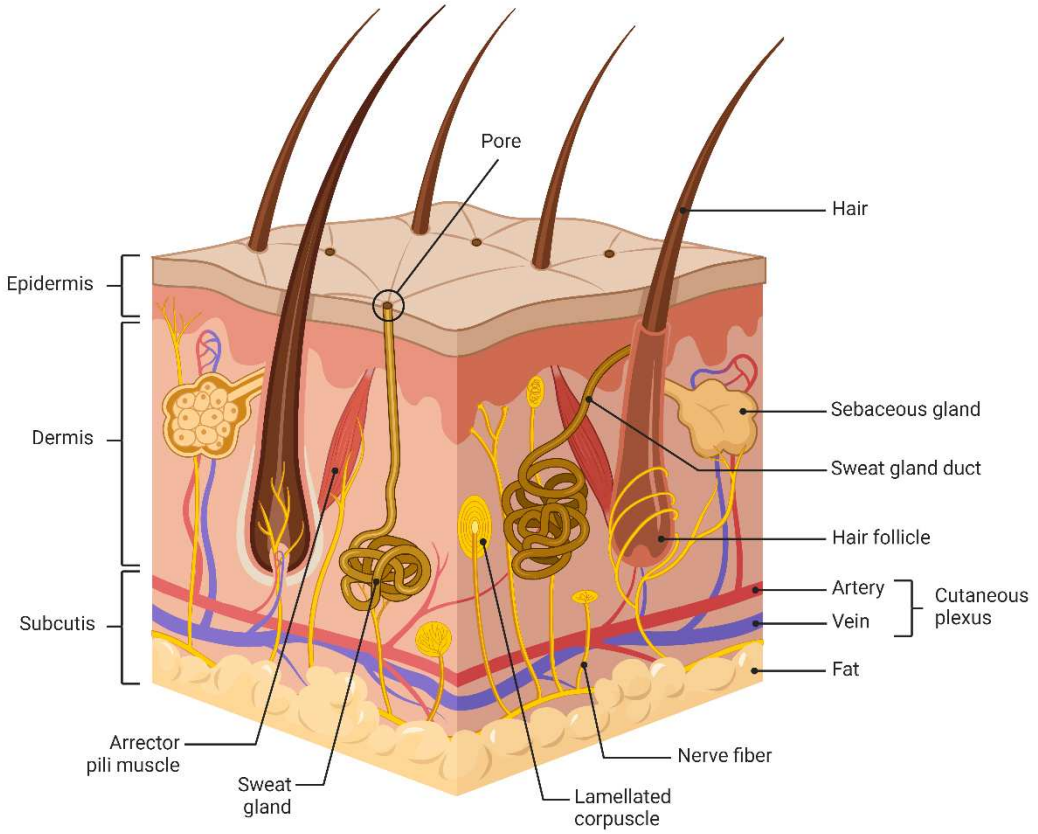

Figure 1: Schematic representation of skin: The skin comprises three primary layers. The outermost layer, the epidermis, provides a protective barrier against pathogens and environmental factors. Underneath is the dermis, where sweat and sebaceous glands, hair follicles, blood vessels, sensory nerves and receptors (e.g., lamellated corpuscles) are located. The innermost layer, the subcutis, contains fat and cutaneous plexus. The skin's hair, muscles (arrector pili muscle), and nerves contribute to sensory perception, thermoregulation, and protection against UV rays. Collectively, these components ensure the skin's health, function, and resistance. Image: Biorender.com (2024) - Anatomy of the skin.

1.2 Skin Functions

The skin plays a fundamental role in maintaining the human body's homeostasis. The following topics will describe the main skin functions:

- Protective Barrier: The skin plays a vital role as a protective barrier against external agents, whether physical, chemical, immunological, or microbial. The epidermis is essential to this defense and is mainly composed of keratinocytes, which make up 95% of its cells. The other 5% is made up of melanocytes, Langerhans cells, and Merkel cells, which also contribute to this protection [10]. These cells produce pro-inflammatory cytokines in response to external threats and act in the presentation of antigens to T lymphocytes to activate the adaptive immune response. In addition, keratinocytes can produce antimicrobial peptides that fight various pathogens [12];

- Thermoregulation: The skin is an important regulator of body temperature, helping the body to maintain an adequate internal thermal range for optimal cell function. In the dermis, sweat glands (eccrine and apocrine) are responsible for most thermoregulation, by secreting sweat which dissipates heat as it evaporates on the surface of the skin. When the body is exposed to high temperatures, the blood vessels in the skin dilate, allowing more excellent blood circulation and heat dissipation through the skin's surface [13,14;]. In addition, the erector muscles of the hair contract, causing piloerection (shivering). This mechanism, although more effective in animals with dense fur, aims to trap a layer of warm air close to the surface of the skin, helping to insulate the body against heat loss. In this way, the skin acts as a sophisticated thermoregulation system to cope with extreme environmental variations [15,16;]

- Sensation: The skin is a highly sophisticated sensory organ, full of specialized nerve endings that detect a variety physical of stimuli from the external environment in addition to stimuli from the internal environment (e.g., capillary colloid osmotic pressure, ATP, uric acid, danger-associated molecular sensor - DAMPS). All epidermal cells (keratinocytes, melanocytes, Langerhans cells, and Merkel cells) express sensor proteins and neuropeptides that regulate the neuroimmune cutaneous system; consequently, they must play a role in the epidermal sensory system [17,18]. These issues are essential for our interaction with the environment and alert us to possible dangers. In addition to thermoregulation, the skin is a complex sensory organ full of specialized receptors that allow the body to respond to environmental stimuli [19,20]. Nociceptors detect painful stimuli and warn of possible injury [21]. Thermoreceptors detect changes in temperature, triggering adaptive responses to cold or heat. Pacinian corpuscles, located in the deep dermis, specialize in detecting deep pressure and vibration [15]. Meissner's corpuscles, found in the most sensitive areas of the skin such as the fingertips, respond to light tactile stimuli and movement [22]. The free nerve endings in the hair follicle, on the other hand, detect the movement of hairs, thus increasing tactile sensitivity [20–22];

- Vitamin D synthesis: Exposure to sunlight, specifically ultraviolet B (UVB) radiation, by melanocytes, dendritic cells derived from the neural crest and located in the basal layer of the skin. They play a crucial role in synthesizing vitamin D in the skin, which is essential for the absorption of calcium and phosphorus, helping maintain bone health and mineral balance [23–25];

- Excretion: Sweating is the process by which the body excretes small quantities of inorganic compounds, such as water, salts, and organic compounds. As well as helping to regulate body temperature, perspiration also plays a role in removing toxins from the body and

maintaining electrolyte balance, such as the secretion and reabsorption of sodium, potassium, chlorine, and calcium, modulated by Na-K-ATPase and influenced by the hormonal control of aldosterone [26];

- Immunity: When the skin is damaged, the immune system is activated to initiate the healing process of wounds and damaged tissues [27]. The effector cells of the innate immune system, such as macrophages, neutrophils, dendritic cells, and natural killer (NK) cells, protect the body. These cells use processes such as phagocytosis, release of inflammatory mediators, activation of the complement system, and production of specific proteins to defend the body. These processes are triggered by specific stimuli, such as pathogen-associated molecular patterns (PAMPs), which bind to pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs). This interaction is not adaptive and does not allow for the generation of new receptors in addition to those already present in the genetic code [28], however, more recent evidence indicates that innate immunity can also be trained [29–31];

- Wound healing: A critical process that occurs skin the integrity is compromised due to wounds, cuts, burns [32,] or acute and chronic skin diseases. Such diseases affect millions of people worldwide each year and results from a poorly regulated tissue repair response in healthy tissue. This response includes several elements, such as inflammation, angiogenesis, matrix deposition, and cell recruitment [33,34], which will be discussed in detail in this chapter.

[35]The skin's response to pain, inflammation, and burns involves intricate mechanisms that include activating specific pathways and the modulation of intercellular junctions [35–37.] Impaired skin barrier function in conditions such as type 2 diabetes has been associated with increased levels of advanced glycation end products, highlighting the systemic impact on skin health [38]. In addition, the skin microbiome has been found to influence skin barrier function, with symbiotes bacteria playing a role in maintaining skin health [12].

2. TISSUE REPAIR MECHANISMS

Tissue repair is a vital process that aims to restore the integrity and functionality of tissues after an injury and is essential for the survival of multicellular organisms. This complex process involves a series of biochemical, cellular and molecular events that take place in different temporal and spatial phases. In the inflammatory phase, the initial response to injury is crucial for limiting tissue damage, preventing infection, and initiating repair, involving platelets activation, immune cells recruitment, and synthesis of extracellular matrix by connective tissue cells.

The dynamic phases of the repair process involve a series of complex events that occur in response to tissue injury. Traditionally, this process is divided into 4 stages, Hemostasis, inflammation, proliferation, and remodeling (Fig. 2). While this division helps understand the predominant characteristics of each phase, it is important to recognize that the different stages of repair are interconnected and influenced by various chemical mediators and cellular events. In this text, we will explore each of the phases of the repair process in detail, highlighting both the macroscopic and histological aspects and the influence of chemical mediators.

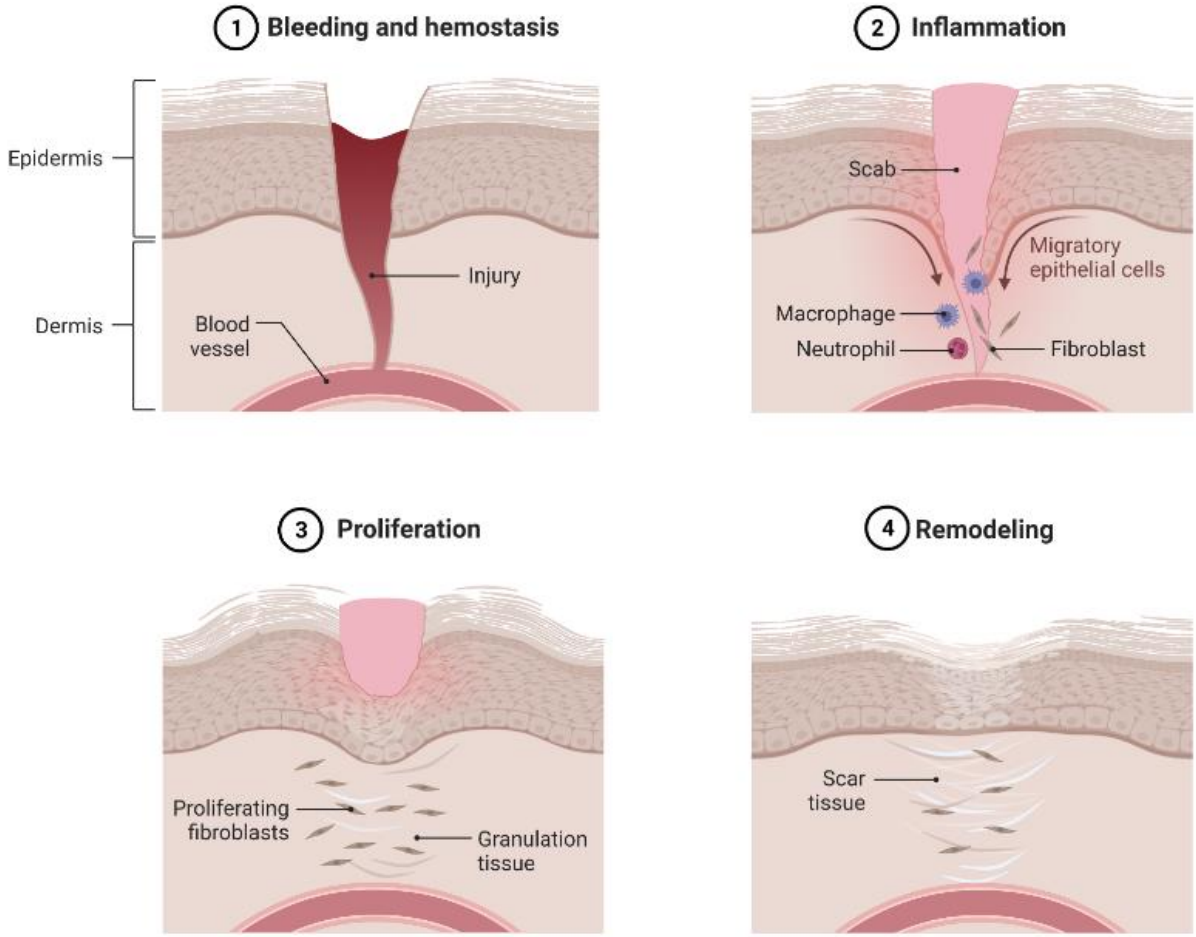

Figure 2: Wound healing is a complex, well-orchestrated process characterized by four distinct yet overlapping phases. The process begins with hemostasis, where clot formation and platelet activation rapidly arrest bleeding. This process is followed by the inflammatory phase, characterized by immune cell infiltration to combat potential infections and facilitate the clearance of cellular debris. Subsequently, the proliferative phase is activated, marked by fibroblast proliferation, angiogenesis, and the formation of granulation tissue to support tissue regeneration. The final phase is tissue remodeling, during which the extracellular matrix is restructured, and the tissue regains its tensile strength through scar tissue formation. A comprehensive understanding of these sequential biological processes is essential for developing effective wound management strategies. Image: Biorender.com (2020) - Wound healing.

2.1 Molecular and Cellular Dynamics in Tissue Repair

The complex processes involved in tissue repair involve a multitude of biochemical, cellular and molecular events that unfold in different temporal and spatial phases (Fig. 2). Central to these processes is the balance between different molecular mediators, including cytokines, chemokines and growth factors, which are critical for the transition between different phases of healing (Fig. 3). Transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) are particularly important as they play a key role in regulating cell proliferation and the formation of new blood vessels, which are essential for effective wound healing [39–41]. TGF-β not only promotes fibroblast proliferation and extracellular matrix production, but also facilitates the recruitment of macrophages and endothelial cells to the wound site, thereby enhancing angiogenesis and tissue remodeling [40–42].

Macrophages are essential in all phases of wound healing and show remarkable plasticity in their functional roles. During the inflammatory phase, macrophages adopt a pro-inflammatory phenotype (M1), which is essential for initiating the healing process by removing debris and pathogens. As healing progresses, they transition to a pro-regenerative phenotype (M2), which supports tissue repair and resolution of inflammation [40,41,43]. This transition is critical and failure to properly switch from M1 to M2 can lead to chronic wounds characterized by persistent inflammation and inadequate healing [43].

Recent studies have highlighted the metabolic reprogramming of macrophages, in particular with the production of itaconate, a metabolite that accumulates in macrophages due to disruption in the Krebs cycle derived from cis -aconitate by the enzyme cis -aconitate decarboxylase (ACOD1), encoded by the immunoresponsive gene 1 (Irg1), which can modulate inflammatory responses and promote tissue repair [44–46]. The cellular dynamics of wound healing also involve key players such as fibroblasts, keratinocytes, endothelial cells and mast cells. Fibroblasts are primarily responsible for the synthesis of collagen and extracellular matrix, which are essential for structural integrity during healing. They also regulate wound contraction, a critical process for minimizing scarring [40,47] . Keratinocytes contribute significantly to re-epithelialization, a critical step in restoring skin barrier function [40,43]. Mast cells, on the other hand, release mediators such as histamine and cytokines that not only initiate the inflammatory response, but also facilitate angiogenesis and tissue remodeling [40,47,48]. The interaction between these cells and biochemical mediators is highly interdependent, underlining the complexity of the tissue repair process. Disruption or imbalance in this intricate network can lead to pathological outcomes such as hypertrophic scars or keloids, which are usually the

result of excessive fibroblast activity and collagen deposition [40,42,48]. Recent advances in molecular biology and bioengineering are paving the way for innovative therapeutic strategies aimed at optimizing tissue regeneration and minimizing pathological scarring. These approaches include the modulation of growth factors and the development of engineered tissues that mimic the natural extracellular matrix [49,50]. In addition, the role of epigenetic regulation in wound healing has attracted attention as it influences gene expression patterns essential for cellular functions during the healing process. Epigenetic modifications, including DNA methylation and histone modifications, can affect the behavior of fibroblasts and keratinocytes, thereby influencing overall healing outcomes [51,52]. The balance between pro- and anti-inflammatory responses, the role of key growth factors such as TGF-β and VEGF, and the intricate interactions between different cell types are essential for effective tissue repair. Continued research into the molecular basis of these processes will undoubtedly lead to better therapeutic strategies for wound treatment and improved healing outcomes.

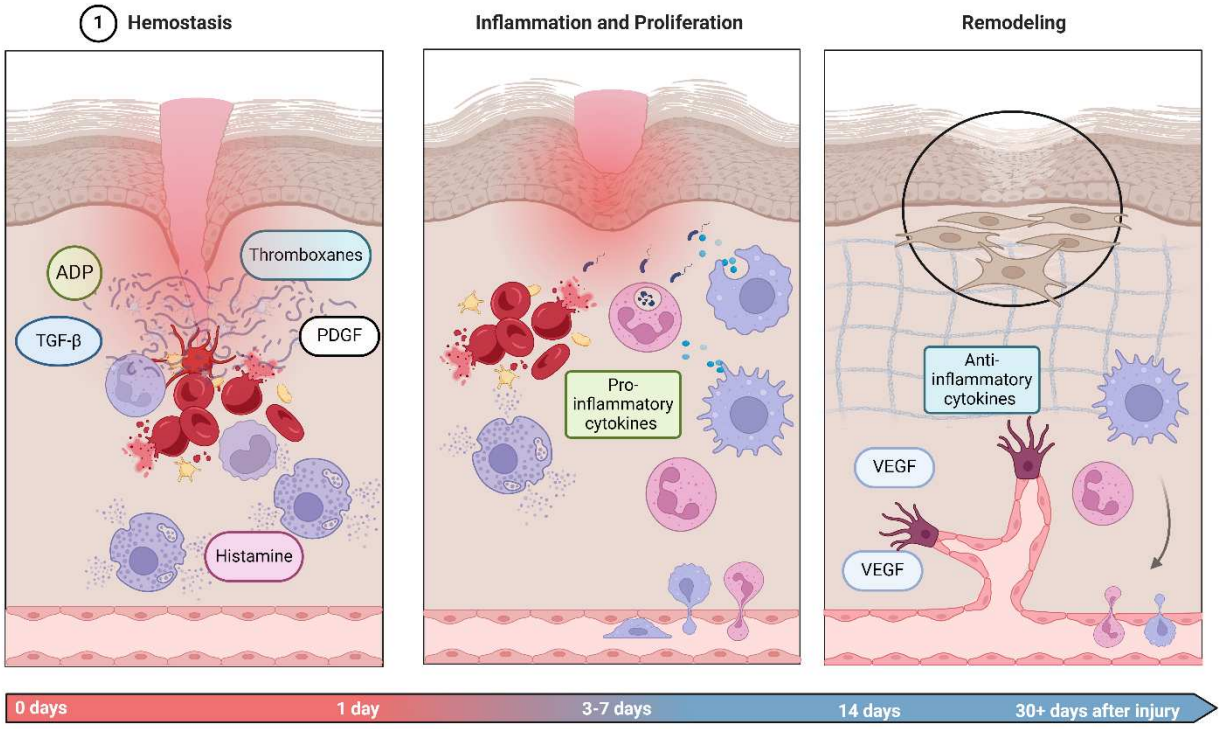

Figure 3: Hemostasis (phase 1): Injured blood vessels and initial vasoconstriction, reducing blood flow. Platelets adhere to the exposed extracellular matrix and form the hemostatic plug. Fibrinogen begins to form fibrin, which stabilizes the clot. Thromboxane's, adenosine diphosphate (ADP) and growth factors - plaque-derived growth factor (PDGF) and transforming growth factor beta (TGF-beta) - are released from platelets. Mast cells in the dermis release histamine to initiate vasodilation and increase vascular permeability. Inflammation (phase 2): Cellular recruitment of neutrophils and macrophages to the injured

area, attracted by signals from mast cells and platelets. Pro-inflammatory cytokines are released and act to recruit immune cells, and macrophages phagocytose cell debris and combat microorganisms. Proliferation (phase 3): Re-epithelialization begins as keratinocytes migrate and cover the wound, restoring the epidermal barrier. Fibroblasts synthesize collagen and incorporate it into the extracellular matrix. At the same time, angiogenesis begins with the action of VEGF (vascular endothelial growth factor). Remodeling (phase 4): Collagen is remodeled by fibroblasts and the extracellular matrix matures, culminating in the gradual reduction of the vessels formed during proliferation. Authorial image Biorender (2024) - Skin Wound Healing Process in phases.

A skin lesion that goes beyond the epidermal layer involves a series of complex events. After the injury, blood and lymphatic vessels are traumatized (Fig. 3), triggering coagulation cascades mediated by the coagulation factors present in the injured skin. The injured blood and lymphatic vessels activate coagulation cascades mediated by the coagulation factors of the injured skin, resulting in rapid and temporary vasoconstriction. After this initial moment, vasodilation allows platelets to be trapped in the clot in the area of vascular damage to form a hemostatic plug [53]. The platelets then release thromboxane's and ADP, which promote platelet aggregation and stabilize the clot, thereby initiating the healing process [33,54,55].

2.2 Hemostasis

The first phase of skin repair, the hemostasis phase, plays a fundamental role in the initiation of the healing process and occurs immediately after tissue injury. The main goal of this phase is to stop bleeding and stabilize the affected area, preventing significant blood loss and creating a favorable environment for the subsequent repair phases [56,57]. Macroscopically, this phase is characterized by rapid vasoconstriction of the injured blood vessels, reducing blood flow. This is followed by platelet aggregation, where platelets adhere to the exposed extracellular matrix, forming an initial hemostatic lesion. These plaques release chemical mediators such as thromboxane's and ADP, which intensify aggregation and prolong vasoconstriction [58] .

Simultaneously, a coagulation cascade is activated, resulting in the conversion of fibrinogen to fibrin, which stabilizes the clot and forms a structural network that supports the wound and provides a basis for the next phases of tissue repair [59] . Histologically, hemostasis involves not only the action of platelets, but also the participation of resident skin cells such as fibroblasts, endothelial cells and especially mast cells, which play a critical role [60]. Fibroblasts, when activated by chemical signals, begin to prepare the environment for future tissue components, while endothelial cells are directed towards reorganization of blood vessels and

regeneration of microcirculation in the wound [61] . Mast cells, which are widely distributed in the dermis, are essential in this process, releasing mediators such as histamine, heparin and cytokines. Histamine promotes immediate vasodilation and subsequently increases vascular permeability, facilitating the recruitment of inflammatory cells to the injured area [62] .

In addition, mast cells influence the transition to the inflammatory phase and act as important modulators of the healing process [63]. Finally, plaques release growth factors such as plaque-derived PDGF and TGF-β, which coordinate the migration of fibroblasts and other repair cells to the site of injury [51]. This phase is therefore a healthy process that not only controls bleeding, but also initiates a series of molecular and cellular events essential for healing [56,57]. Hemostasis thus represents an integrated phase in which different cells and chemical mediators work synergistically to prepare the tissue for the subsequent phases of inflammation, periodicity and tissue remodeling [56,57].

2.3 Inflammation phase

The inflammation phase is the body's sequential response to injury and is essential for the technical repair process. This phase begins immediately after the injury and aims to limit tissue damage, eliminate contracted agents, and initiate the healing process. The injury leads to the activation of immune system cells, including leukocytes such as neutrophils and macrophages. These cells are recruited to the site of the injury by chemical signals, such as cytokines and chemokines, released by the damaged cells and surrounding tissues[28].

After stabilization hemostasis, the inflammatory phase begins. During this phase, vasodilation is mediated by substances such as histamine, prostaglandins and leukotrienes, primarily released by mast cells, facilitating increased blood flow to the injury site [64,65]. This enhanced circulation allows neutrophils and macrophages to migrate to the wound, where they play a crucial role. Neutrophils are the first cells to arrive at the injury site, where they phagocytose pathogens and cellular debris. Shortly thereafter, macrophages assume a pivotal role in clearing debris, orchestrating the transition to the proliferative phase, and secreting pro-inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) [64,65]. Throughout the wound healing process (Fig. 4), neutrophil accumulation peaks during the initial inflammatory phase, decreases after 4 days, and persists until the end of a first week. Cañedo-Dorantes & Cañedo-Ayala (2019), report in their study that, macrophages, on the other hand, increase in number during the inflammatory phase, reach their peak concentration in the proliferative phase and progressively decline during the remodeling phase, remaining the most abundant cell throughout all phases, being the most

abundant cell in of wound repair. These mediators (cytokines, chemokines, growth factors and ROS) are essential for promoting the inflammation necessary for re-epithelialization and fibroblast recruitment. Fibroblasts, also recruited during inflammation, initiate collagen production, which is essential for tissue structure and regeneration [55,56]. At the same time, by releasing histamine, mast cells promote vasodilation and increase vascular permeability, facilitating the arrival of more immune cells at the wound site [55,56]. Prostaglandins and leukotrienes produced by various inflammatory cells further amplify this response, while cytokines such as IL-1, IL-6 and TNF-α further promote inflammation and stimulate collagen production for the healing process [64,66].

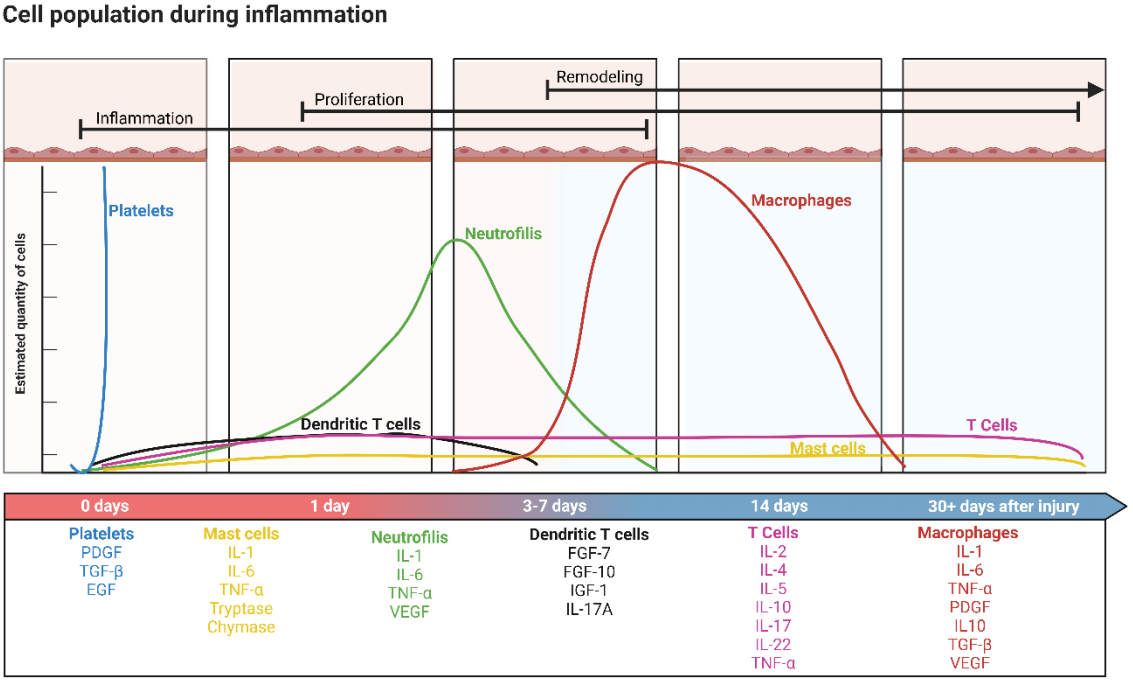

Figure 4: During the initial inflammatory phase, the number of neutrophils at the injury site increases significantly. However, this number begins to decrease after four days, with a gradual reduction over the course of a week [67]. Macrophages also increase in number during the inflammatory phase, peak during the proliferative phase, and gradually decline in the remodeling phase. The number of resident cells in the skin, such as dendritic cells, mast cells, and T cells (Trm -resident memory T cells, and Treg – regulatory T cells), is not yet fully understood. It is known that the skin contains a large population of memory T cells, which play a critical role protecting against potential reinfection [68]. Further studies are needed to clarify the exact role of these cells in the healing process. The approximate duration of each phase of tissue repair is shown in the bars at the bottom of the graph [61,69]. Authorial image, Biorender (2024) - Cell population during inflammation.

During inflammation, there is a balance between proinflammatory mediators (e.g., pro-inflammatory IL's, TNF, prostaglandins, and leukotrienes), and anti-inflammatory mediators'

(e.g., interleukin-10 -IL-10, interleukin-1 receptor antagonist -IL-1RA, lipoxins and resolvins). This balance is important to limit the excessive production in the mediators involved in the inflammation and the beginning of the resolution process. The expression of integrins is triggered by externalization through the Weibel-Palade corpuscle and/or the synthesis of new integrin molecules, especially in response to the presence of IL-1 and TNF-α [70]. Leukocyte extravasation and migration (Fig. 5) are dependent on chemokines such as interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1), which are produced at infection sites and bind to proteoglycans in the extracellular matrix and similar molecules on the surface of endothelial cells [71]. IL-8, released by activated macrophages, attracts neutrophils, which are stimulated to penetrate the inflamed tissue, while MCP-1 recruits' monocytes, T cells, NK cells, and dendritic cells later [28,72].

Although inflammation is commonly associated with tissue damage, it represents a crucial part of the healing process. The phases of inflammation, both physiologically and functionally, are integral to the mechanistic response required for establishing a favorable environment conductive to tissue homeostasis and healing.

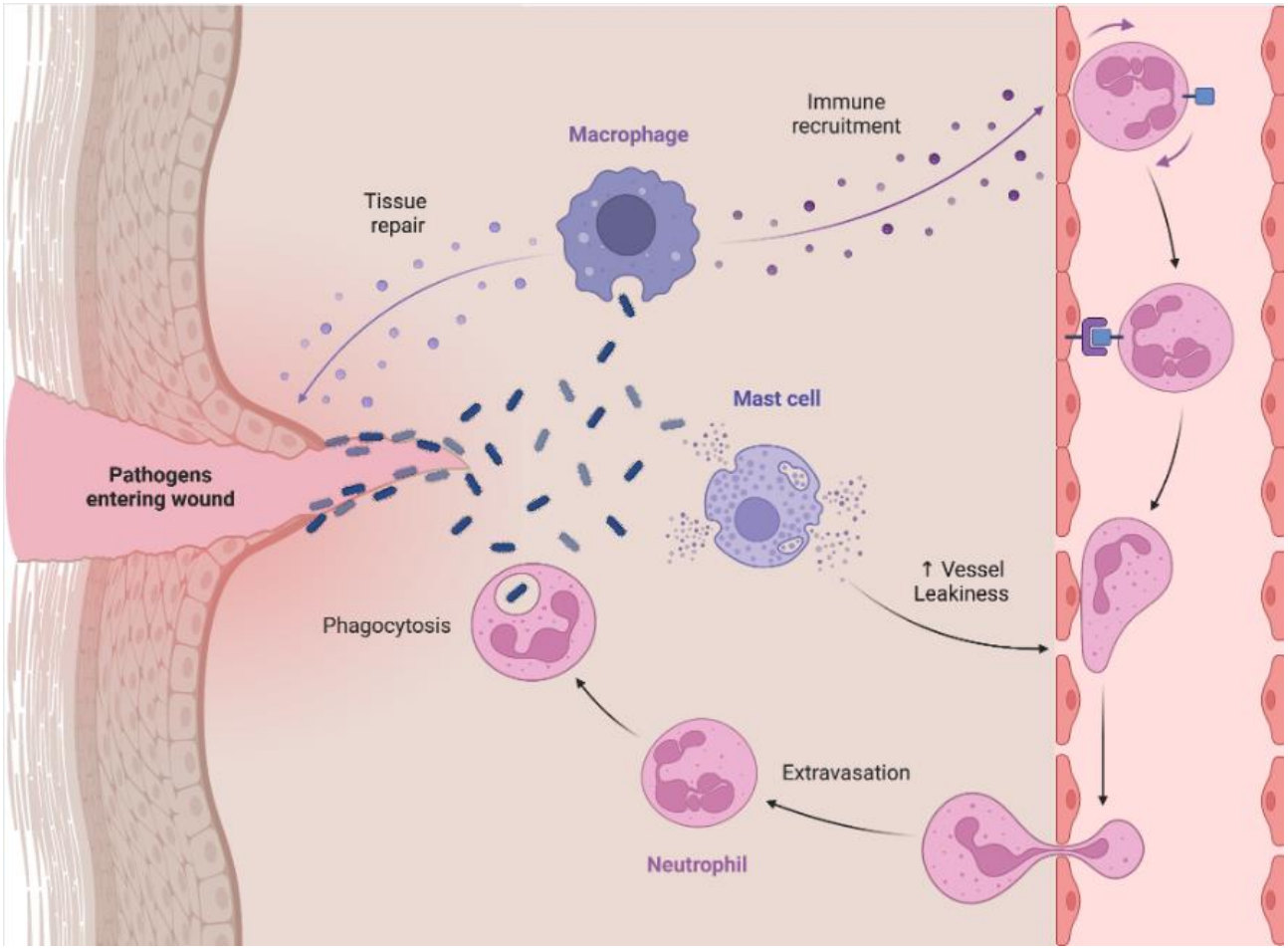

Figure 5: Upon pathogens entry into a wound, macrophages release cytokines, chemokines, and lipid mediators, which act as chemical signals to recruit leukocytes, like neutrophils. These neutrophils bind to specific receptors on endothelial cells, facilitating their adhesion. Concurrently, mast cells interact with pathogens and release mediators that enhance vascular permeability. This increased permeability allows neutrophils to undergo extravasation, migrating from the bloodstream into the site of infection. Once at the infection site, neutrophils initiate phagocytosis to eliminate the foreign material. Image creator: Danielle Penk, Biorender.com (2020) - Neutrophil Recruitment Pathway.

2.4 Proliferation phase

During the proliferation phase, cells begin to multiply and rebuild the damaged tissue (Figs. 2-4). Fibroblasts, specialized cells responsible for extracellular matrix synthesis, migrate to the injury site where they produce collagen, particularly type III, and fibronectin. These extracellular matrix components plays a crucial role in providing structural support to the regenerating tissue, creating an environment for subsequent remodeling, and ensuring the integrity of the healing process [32]. Simultaneously, neovascularization, the process of new blood vessel formation, occurs, facilitating the supply of nutrients and oxygen to the proliferating cells [33]. During this process, the extracellular matrix is gradually reorganized and strengthened, contributing to the restoration of the tissue's structural integrity and function [70]. The main growth factors released by fibroblasts include epidermal growth factor (EGF), which acts in the chemotaxis and proliferation of fibroblasts and basal keratinocytes; and TGF-β, responsible for stimulating fibroblasts and myofibroblasts to produce collagen [73]. Fibroblasts, in response to extracellular soluble signals such as cytokines (IL-1, TNF-α) and growth factors (TGF-β1, PDGF, EGF and fibroblast growth factor 2 - FGF-2), play vital roles in wound healing [69]. They proliferate, migrate and adjust protein producing to the granulation tissue, where they synthesize collagen, replacing the temporary fibrin matrix.

Angiogenesis, given its complexity, is notably regulated by a single growth factor, VEGF, which plays a predominant role in this process [74]. Key events in angiogenesis include increased vascular permeability, extravascular deposition of fibrin, disruption of the vascular wall with removal of pericytes (undifferentiated mesenchymal cells) similar to fibroblasts, found in small blood vessels (near endothelial cells) and connective tissue. After tissue damage, pericytes are able to differentiate and form new blood vessels and connective tissue cells [75]. In addition, fragmentation of the basement membrane and extracellular matrix by proteases, migration of endothelial cells through the remodeled extracellular matrix, endothelial cell proliferation and the formation of sprouts [76]. During this phase, various therapeutic interventions have been explored to enhance the healing process, such as laser therapy, platelet-rich plasma, ozone

therapy, and the application of silver nanoparticles [77–79]. These approaches have demonstrated the potential to accelerate healing, promote re-epithelialization and angiogenesis, and contribute to the effective recovery of injured tissues.

2.5 Remodeling phase

The remodeling phase (figs 2-4), also known as scar maturation, is crucial to the healing process and may last for months or even years. During this phase, cellular activity and vascularization decrease, accompanied by a reduction in VEGF expression, signaling the end of new vessel formation and the onset of scar tissue stabilization [32]. Remodeling of the extracellular matrix involves the replacement of type III collagen with type I collagen, which enhances the tissue's resistance [32,34]. This dynamic process, mediated by fibroblasts and growth factors, ensures the integrity and functionality of the scar tissue [80].

Concurrently, there is a reduction in the number of inflammatory cells, such as macrophages and neutrophils, which undergo apoptosis and are eliminated from the wound site. Wound contraction persists, with myofibroblasts contracting the extracellular matrix around the wound[81], thereby reducing the size of the scar [81]. In addition, angiogenesis decreases as the new blood vessels are remodeled, becoming more mature and resistant [74]. The final stage involves the formation of a scar, which, although it may be functionally and aesthetically acceptable, never fully restores the structure and function of the original tissue [32,33].

3. THE IMPORTANCE OF THE OXINFLAMMATION PROCESS IN THE WOUND HEALING

The importance of redox balance in wound healing is essential for understanding the mechanisms underlying this complex biological process. Redox balance, which refers to the dynamic equilibrium between the production of reactive oxygen (ROS) and nitrogen species (RNS) and the body's antioxidant capacity, plays a crucial role in regulating healing. Reactive oxygen species, such as superoxide and hydrogen peroxide, are naturally produced during cellular processes and play key roles in cell signaling and immune defense. However, an imbalance in their production can lead to oxidative stress, compromising the integrity of the healing tissue. Both endogenous and exogenous antioxidants are crucial in neutralizing these reactive species and maintaining the redox balance.

On the other hand, inflammation plays a paradoxical role in wound healing. Although an initial inflammatory response is necessary to initiate the healing process, prolonged or unregulated inflammation can impair tissue regeneration and lead to chronic complications.

Proper modulation of the inflammatory response, with the coordinated transition of macrophages from the pro-inflammatory phenotype (M1) to the anti-inflammatory phenotype (M2), is crucial for effective healing [82–87].

In this context, the concept of process of OxInflammation is a pre-pathological state characterized by well-documented chronic and systemic oxidative stress, which is associated in a vicious circle with mild subclinical chronic inflammation. The occurrence of long-term persistent oxidative stress contributes to the generation of a permanent loss of adaptive homeostatic response capacity, stabilizing and reinforcing a chronic induction of a pro-inflammatory state [88].. This imbalance leads to the development of a state of oxidative stress with significant biological consequences [89]. OxInflammation plays a critical role in the development and maintenance of inflammation, contributing to the pathophysiology of various debilitating diseases, such as cardiovascular disease, diabetes, cancer or neurodegenerative processes [90]. In addition, ROS and RNS respectively, are critical in all phases of inflammation, promoting the release of signaling molecules by damaged tissues. These danger signals are detected by innate immune receptors such as TLRs [91] and NOD-like receptors (NLRs) [92], which activate the NOD-like receptor protein 3 (NLRP3) inflammasome [90]. This activation triggers signaling pathways that stimulate the adaptive immune response necessary for addressing the injury and repairing the tissue.

3.1 Oxidative stress in skin wounds

The production of metabolic by-products is part of cellular metabolism. However, under conditions of cellular stress, it significantly impacts all phases of the wound healing process. During the initial inflammatory phase, increased ROS production contributes to the elimination of invading microorganisms and facilitates the cell signaling required to initiate the immune response [93,94]. However, OxInflammation, the degradation of extracellular matrix components and cellular lipids and DNA occurs [95], along with damage to blood vessels, and delayed cell migration to the site of injury [96]. In addition, ROS can inactivate growth factors and pro-inflammatory cytokines, further damaging tissue regeneration. During remodeling phase, the continued presence of oxidative stress can affect the function of fibroblasts and keratinocytes, key cells involved in collagen synthesis and wound re-epithelialization, respectively [97]. They can interfere with the formation of new blood vessels, an essential process for supplying nutrients and oxygen to healing tissues. During the remodeling phase, excessive ROS activity can lead to a disorganized extracellular matrix, resulting in abnormal wound repair, such as hypertrophic scars or keloids [33].

3.2 Free radical formation

Oxidative stress is characterized by an imbalance between the production of ROS and the antioxidant capacity of the body to neutralize them. These ROS include radicals such as the hydroxyl radical ($^{\cdot}OH$), superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), as well as other non-radical species such as the peroxynitrite anion ($ONOO^-$) and ozone (O_3) [96,98] under physiological conditions. ROS and RNS play a critical role in the regulation of cell survival [99]. Under normal conditions, moderate levels of ROS/RNS act as signaling molecules to promote cell stimulation and survival. However, excessive increases in these species can lead to cellular damage and trigger cell death [95,100]. Exogenous and endogenous factors, such as mechanical trauma, ischemia, infection, ultraviolet radiation, redox-active metals like copper and iron, membrane-associated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and inflammation can induce oxidative stress. This stress impairs tissue regeneration and prolongs the healing process [95,101].

A. Reactive Oxygen Species (ROS)

ROS are a class of oxygen-rich free radicals characterized by their high reactivity, attributed to the presence of unpaired electrons. ROS are primarily generated as by-products of cellular metabolism, particularly within mitochondrial electron transport chain. Superoxide ($O_2^{\cdot-}$) is produced by the partial reduction of molecular oxygen during cellular respiration. It can be converted to hydrogen peroxide (H_2O_2) by enzymes such as superoxide dismutase (SOD) or react with metal ions to form the hydroxyl radical ($^{\cdot}OH$) by the Fenton or Haber-Weiss processes [102]. The generation of these species can occur in different cellular compartments by specific mechanisms:

- Mitochondria: During aerobic respiration, most ROS are generated as by-products of the electron transport chain [103]. The loss of electrons within this chain leads to the formation of superoxide anion (O_2^-), which can occur at different stages of the mitochondrial respiratory chain, particularly at specific complexes such as complex I (NADH dehydrogenase) and complex III (cytochrome bc1) [104]. This superoxide acts as a precursor to other reactive oxygen species, triggering a cascade of reactions that can disrupt cellular homeostasis and contribute to oxidative stress.

- NADPH oxidases (NOX): These specialized enzymes play an important role in the immune response and signaling processes by generating ROS through the transfer of electrons from NADPH to oxygen, resulting in the production of superoxide [105].

- Xanthine oxidase: During purine metabolism, this enzyme is responsible for generating superoxide [106]. In this process, xanthine oxidase catalyzes the conversion of hypoxanthine and xanthine to uric acid. This reaction that involves the transfer of electrons from the substrate to molecular oxygen, resulting in the formation of superoxide (O_2^-) [107].

- Cytochrome P450 enzymes: Certain cytochrome P450 enzymes can generate ROS during their metabolic reactions. One of the main pathways is the transfer of electrons from the substrate to molecular oxygen during cytochrome P450-catalyzed oxidation [108]. This electron transfer can lead to the formation of ROS, including O_2^-, H_2O_2 and OH^- [109].

Other sources of endogenous ROS include lipid oxidation by the enzyme's lipoxygenases and cyclooxygenases, as well as the leukocyte-mediated inflammatory response [95,99,110]. In addition, external factors such as pollution, alcohol consumption, exposure to tobacco smoke, heavy metals, industrial solvents, pesticides, certain drugs and radiation significantly contribute to ROS generation [93].

B. Reactive Nitrogen Species (RNS)

Reactive nitrogen species (RNS) are highly reactive nitrogen-containing molecules that play an important role in cell signaling and homeostasis. Like ROS, RNS can have both beneficial and harmful effects on cells, depending on their levels and the cellular context [94].

RNS are mainly produced by the enzymatic activity of nitric oxide synthases (NOS), which catalyze the conversion of L-arginine to nitric oxide (NO) [95]. NO^- is a key signaling molecule involved in several physiological processes, including vasodilation, neurotransmission and the immune response. However, NO^- can also react with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), a highly reactive RNS that can cause cell damage [95,111]. The production of RNS, particularly NO^-, occurs in different cellular compartments and tissues [93,95,111]:

- Endothelial cells: Endothelial NOS (eNOS) produces NO^- in blood vessels, playing a key role in regulating vascular tone and blood flow.

- Neuronal cells: Neuronal NOS (nNOS) generates NO^- in the nervous system, where it acts as a neurotransmitter and modulates synaptic transmission.

- Immune cells: Inducible NOS (iNOS) is expressed in immune cells such as macrophages and plays a role in the immune response, producing NO^- to fight pathogens.

- Mitochondria: Mitochondrial NOS (mtNOS) resides in mitochondria, where it plays a role in regulating mitochondrial function and redox signaling.

In general, RNS, particularly NO^-, are important signaling molecules that regulate various physiological processes. However, deregulation of RNS production or excessive RNS levels

can lead to oxidative and nitrosative stress, contributing to cell damage and the pathogenesis of various diseases [93,95].

4. INFLAMMATION IN WOUND HEALING

4.1 Acute inflammation

Acute inflammation usually lasts only a few days, functioning as a transient and self-limiting process. In the context of wound healing, the inflammatory phase is well orchestrated and usually lasts approximately 1 to 3 days and can extend up to 7 days [69]. Several key proteins involved in the formation of the epidermal barrier that are directly related to the inflammatory process, including vimentin (VIM), filaggrin (FLG), loricrin (LOR) and involucrin (IVL). During this period, processes such as cutaneous neurogenesis, hemostasis, recruitment of neutrophils and monocyte-derived macrophages to the injury site occur, establishing the conditions necessary for the subsequent repair of the damaged tissue [33,69].

Acute inflammation is typically divided into three distinct phases:

(1) Vascular phase: Tissue damage triggers the release of vasoactive mediators such as histamine, serotonin and prostaglandins, which induce vasodilation and increase vascular permeability [69]. This results in increased blood flow to the injury site and the extravasation of blood cells and plasma proteins into surrounding tissues. Vimentin (VIM) is essential in this phase, as it facilitates the migration of fibroblasts and immune cells to the injury site, a process critical for the initiation of the tissue repair [112]. As a key component in the inflammatory response and tissue regeneration, vimentin is often used as a marker in studies of wound healing[113,114].

(2) Cellular phase: Leukocytes, mainly neutrophils and monocytes, are attracted to the injury site by chemical gradients such as chemokines and tissue degradation products [70]. Neutrophils release mediators such as TNF-α, IL-1β and IL-6, which amplify the inflammatory response and stimulate VEGF and IL-8 for an appropriate repair response [33]. In the cellular phase, cell recruitment is intensified and proteins such as FLG and IVL are activated[115,116]. These proteins are essential for maintaining the integrity of the epidermal barrier and protecting the healing tissue from external pathogens. Three days after injury, circulating monocytes migrate into the tissue where they differentiate into macrophages, which are responsible for phagocytosing larger particles and releasing pro-inflammatory mediators (IL-1, IL-6, IL-17, TNF-α) and potent mediators such as TGF-β, TGF-α, basic FGF, PDGF and VEGF [33,69];

(3) Repair and resolution phase: As the injury is resolved and the offending agents are eliminated, the process transitions into the repair and resolution phase. Loricrin (LOR) plays

acritical role in restoring the integrity of the epidermal barrier[117]. This protein is essential for the resistance and impermeability of the newly formed tissue, ensuring that the regenerated skin maintains its protective functions against external threats[116,117]. During this phase, apoptotic leukocytes and cellular debris are removed by macrophages, and stem cells and fibroblasts are recruited to initiate tissue repair [118]. Additionally, macrophages differentiate into M1 and M2 subtypes, with the M2 phenotype playing a crucial role in the resolution of inflammation [69]. As a result, acute inflammation is resolved, and the damaged tissue is repaired and replaced by scar tissue.

Metabolic dysfunction, such as diabetes, or molecular changes induced by hyperglycemic conditions, may promote the transition from acute to chronic inflammation, leading to a prolonged and dysregulated inflammatory response. Under these conditions, the integrity of the skin is not properly restored, which can lead to complications such as ulcers, pathological fibrosis, vascular disease, neuropathies and immune system dysregulation [69,119].

4.2 Chronic inflammation

Chronic inflammation in wounds represents a prolonged and unregulated inflammatory response that can interfere with the healing process, resulting in persistent tissue damage and delayed recovery. Although acute inflammation is critical for the initiation of healing, the transition to chronic inflammation can occur under certain circumstances, such as persistent infection, extensive tissue damage and underlying comorbidities such as diabetes, obesity [119,120] and autoimmune diseases [28]. These conditions can exacerbate tissue damage by further compromising tissue vascularization and nutrient delivery, thereby prolonging the inflammatory state [33,69,70]. Moreover, an imbalance between oxidants and antioxidants molecules can negatively affect signaling pathways, leading to the expression of pro-inflammatory cytokines (IL-1, TNF-α, IL-6, Interferon-gamma - IFN-γ), chemokines such as macrophage Inflammatory Protein-1 alpha (MIP-1α), IFN-γ-inducible Protein 10 (IP-10) and proteolytic enzymes (neutrophil elastase, and metalloproteinase's MMP-1, MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13)[121]. This dysregulated response contributes to a hostile microbial environment characterized by unbalanced proteolytic activity, which can result in the degradation of key growth factors and structural proteins, such as type I collagen, that are essential for tissue repair [121].

Chronic inflammation directly affects fibroblasts, key cells in wound healing, by altering their ability to respond to soluble extracellular signals. Inflammatory cytokines such as IL-1, TNF-α and TGF-β1, which are essential for modulating fibroblast activity, can become

deregulated in a chronically inflammatory environment. Furthermore, communication between different cell types, such as platelets, macrophages, endothelial cells and keratinocytes, can be disrupted, impairing fibroblast signaling and activation. This dysfunction in fibroblast regulation results in unbalanced collagen synthesis, inadequate replacement of the provisional fibrin matrix and impaired myofibroblast formation [69,122]. The ability of fibroblasts to sense and respond to mechanical stress may also be compromised, leading to inappropriate responses to tissue stress and deformation. For example, signaling mediated by vimentin, an intermediate filament, may be disrupted, failing to signal TGF-β and impairing epithelial-mesenchymal transition and subsequent re-epithelialization [69,123].

This review will focus specifically on the actions of macrophages and their plasticity in the context of inflammation.

5. PLASTICITY OF M1 and M2 MACROPHAGES

Macrophage plasticity is a pivotal characteristic of these phagocytic cells of the innate immune system, enabling their response to various stimuli. Their functional plasticity allows macrophage to adapt and modify both their phenotypic and functional characteristics based on the local microenvironment [124]. This adaptability is crucial for maintaining tissue homeostasis and ensuring an effective immune response under different conditions. The process of macrophage plasticity (Fig. 6) is orchestrated by a variety of extracellular signals, including cytokines, lipid mediators and interactions with neighboring cells in the microenvironment, all of which influence macrophage activation and differentiation [125].

The M1 phenotype, also known as pro-inflammatory macrophages, is mainly activated by stimuli such as lipopolysaccharide (LPS) and IFN-γ and plays a fundamental role in the innate immune response, eliminating pathogens and promoting inflammation [85]. In addition, it can induce the activation of NF-κβ and immunological effectors associated with M1 macrophages, such as the production of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6, along with high levels of nitric oxide generated by iNOS [126]. These cells show increased microbicidal activity and antigen presentation capacity [127]. In summary, M1 macrophages are present in the pro-inflammatory phase, promoting cytotoxicity, DNA and tissue damage, exacerbating stimuli for M1 polarization, contributing to vicious feedback and evolving into a chronic wound[128].

Conversely, M2 macrophages represent a diverse population activated by various stimuli, including cytokines such as IL-4, IL-13, IL-10 and growth factors like mononuclear phagocyte colony-stimulating factor (M-CSF). These macrophages are often associated with

anti-inflammatory processes, tissue repair and modulation of the immune response. They play a critical role in the resolution of inflammation, wound healing, regulation of angiogenesis and remodeling of extracellular tissues. M2 macrophages encompass several subpopulations of, each with distinct characteristics and functions. For instance, M2a macrophages, activated by IL-4 and IL-13, are primarily involved in tissue repair and allergic responses, while M2b macrophages, activated by immune complexes and LPS, play a key role in modulating the immune response [90]. In summary, M2 macrophages are prevalent during the resolution and anti-inflammatory phases of inflammation, contributing to immune suppression, physiological signaling to M1macrophages, and tissue healing [128].

Macrophages receive signals from various immune system cells, including T lymphocytes, dendritic cells and mast cells, which influence macrophage activation and phenotypic polarization [129]. Additionally, genetic factors, such as polymorphisms in genes associated with immune responses, can modulate how macrophages respond to both pro- and anti-inflammatory stimuli [130].

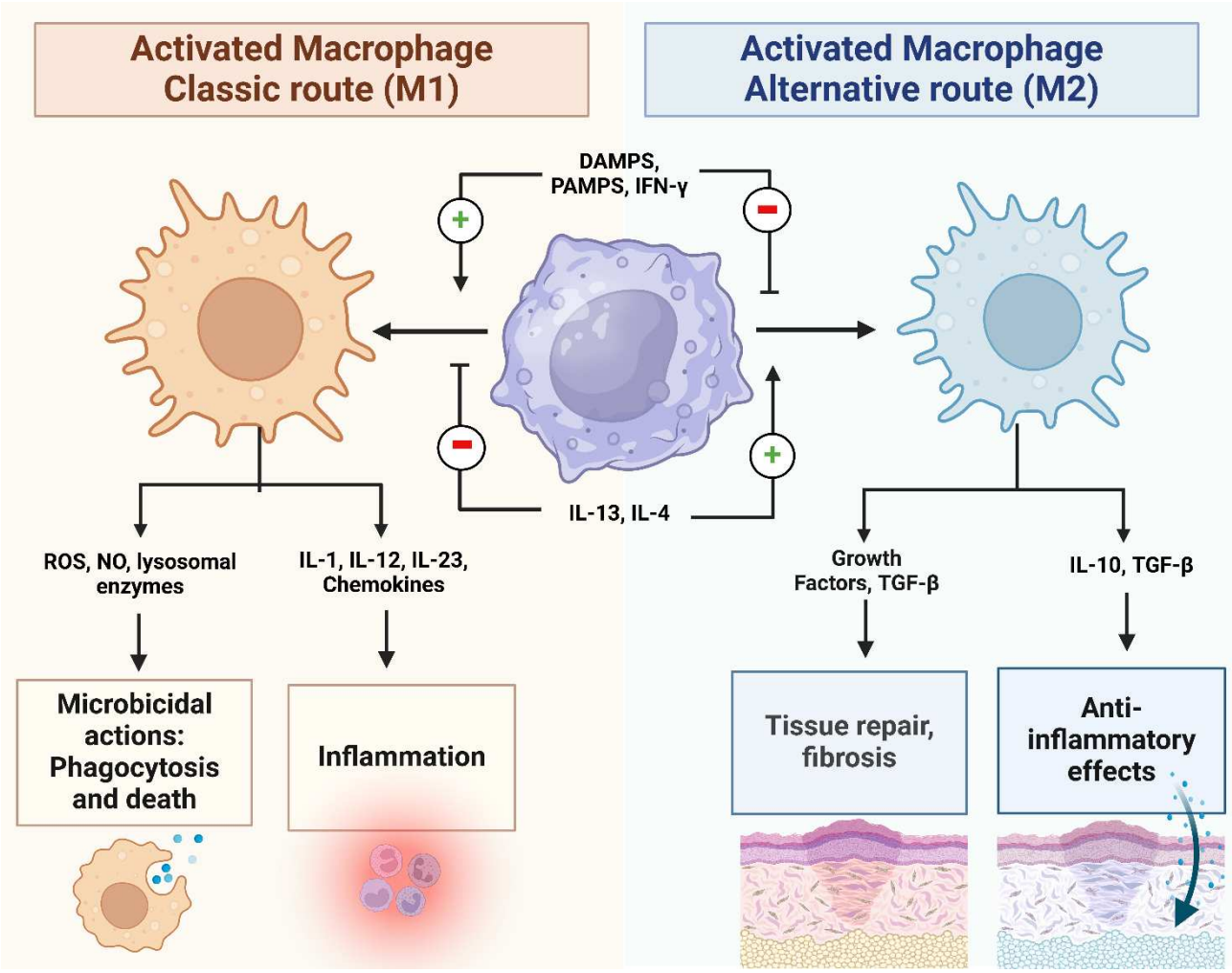

Figure 6: Macrophages Plasticity. The transition from M1 to M2 macrophages is characterized by reduction of inflammation and promotion of tissue repair. Key processes involved in this transition include the regulation of apoptosis, immune system suppression, and the stimulation of angiogenesis and extracellular matrix synthesis, all of which contribute to the resolution of inflammation and the restoration of tissue integrity. Authorial image, Biorender (2024) - Macrophages Plasticity.

5.1 The role of macrophages in the modulation of inflammation

M1 macrophages are activated by exposure to PAMPs and DAMPs, triggering NF-κβ activation and the transcription of pro-inflammatory cytokines such as IL-1 and IL-6 (Fig. 6). This activation also stimulates the NLRP3 inflammasome, leading to the generation of ROS and amplification of the inflammatory response. On the other hand, the transition to M2 macrophages is induced by the synergistic expression of IFN-γ, Toll-like receptors and TNFα/β, leading to the expression of anti-inflammatory cytokines such as TGF-β1 and IL-10. This process is accompanied by the downregulation of proteins associated with the acute phase of inflammation, such as C-reactive protein and fibrinogen. Additionally, there is a decrease in the expression of matrix metalloproteinases and an increase in the activity of antioxidant enzymes, such as catalase (CAT) and Glutatione S-Transferases (GST), which help mitigate oxidative stress and facilitate tissue repair.

However, dysregulation of macrophage activation and polarization can contribute to the development of chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis and cancer. Studies have highlighted the critical importance of the balance between M1 and M2 macrophages in regulating the onset, progression, and resolution of inflammation. This balance highlights the multifaceted roles of macrophage in immune responses, tissue repair and disease the pathogenesis. Macrophage plasticity is essential for maintaining tissue homeostasis and effectively responding to inflammatory challenges.

6. THE ROLE OF THE INFLAMMASOME IN CUTANEOUS WOUND INFLAMMATION

The inflammasome is an intracellular multi-protein structure that function as a sensor of danger signals, including tissue damage, microbial infection and oxidative stress (Fig. 7). It is typically composed of PAMPs or DAMPs, an adaptor protein and an effector enzyme, usually caspase-1 (CASP-1). The activation of CASP-1 leads to the cleavage and activation of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-18 (IL-18), thereby amplifying the inflammatory response.

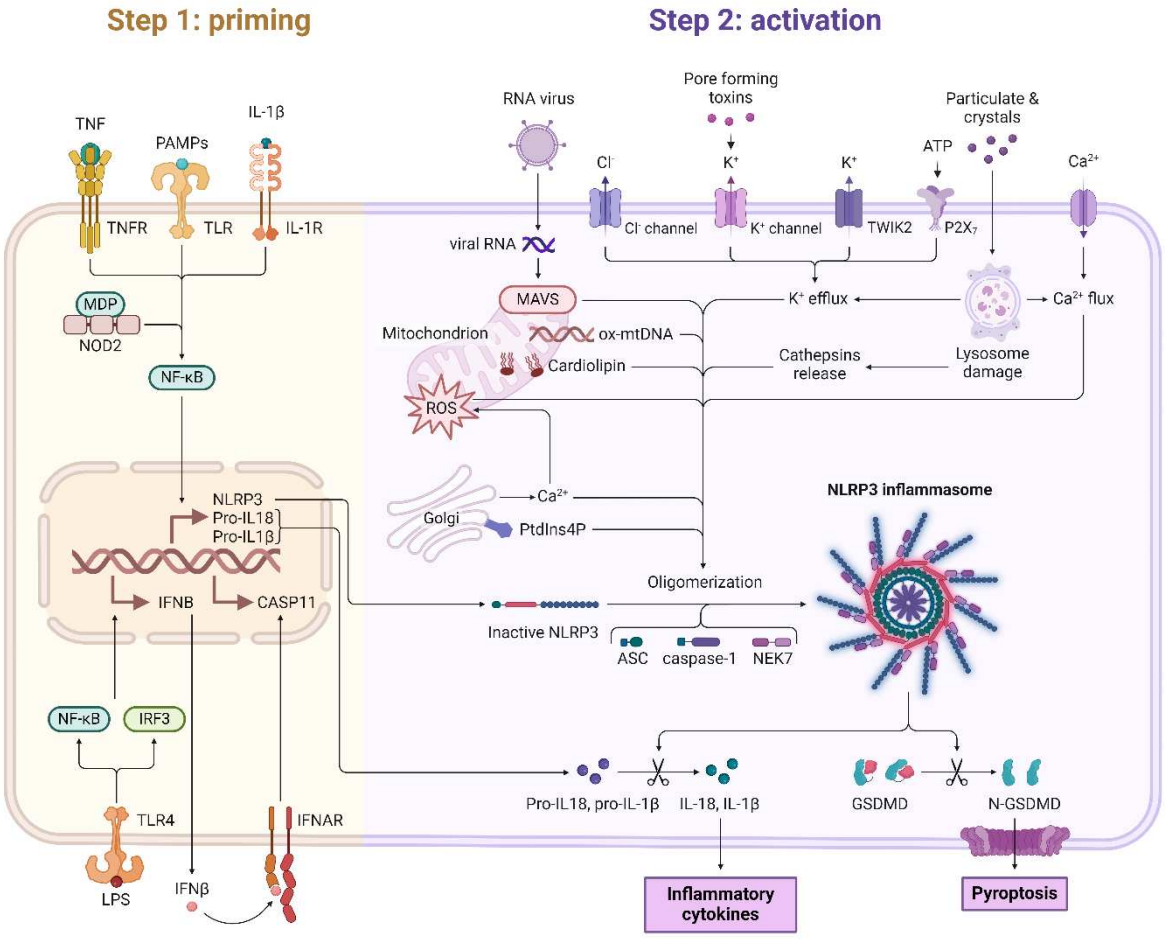

Figure 7: Priming and activation of the NLRP3 inflammasome occur in two steps. First, signal 1, or priming, is generated by the activation of cytokines or pathogen-associated molecular patterns (PAMPs), which promote the upregulation of both canonical and non-canonical NLRP3 inflammasome components. The activation signal 2 is then triggered by various PAMPs or damage-associated molecular patterns (DAMPs), such as particles, crystals and ATP. These factors activate a series of signalling events including K^+ efflux, Ca^+ influx, lysosomal rupture and mitochondrial reactive oxygen species (mtROS) production. The translocation of cardiolipin to the outer mitochondrial membrane and release of oxidized mtDNA (Ox-mtDNA) also occur, followed by Cl- influx. RNA viruses activate NLRP3 via the mitochondrial antiviral signalling protein (MAVS). The formation of the inflammasome results in the activation of caspase 1, which cleaves pro-IL-1β and pro-IL-18. Additionally, gasdermin D (GSDMD) is also cleaved, leading to the formation of membrane pores and the induction of pyroptosis. Upon detection of cytosolic lipopolysaccharide (LPS), caspases 4, 5 and 11 are activated, leading to cleavage of GSDMD and induction of pyroptosis[131–136]. Image created: Maritan, M., BioRender (2024) - NLRP3 Inflammasome Activation.

6.1 Inflammasome Stimulation.

The inflammasome, a key component of the immune response, is activated in response to a variety of danger signals, including molecules released during tissue damage, microbial infection and oxidative stress. These signals are recognized by specific sensor proteins within

the inflammasome complex, initiating a cascade of molecular events that culminates in its activation (Fig. 7). Key triggers of inflammasome activation include:

(1) DAMPs: such as extracellular ATP, uric acid, DNA and hyaluronic acid released during tissue damage. These molecules are recognized by inflammasome sensors such as the purine receptor P2X7 and NLRP3, leading to inflammasome activation [137].

(2) PAMPs: which are components of pathogenic microorganisms such as bacteria, viruses and fungi, are recognized by the immune system as indicators of infection. Inflammasome sensors recognize these PAMPs and initiate an inflammatory response to combat the invading pathogens [138].

(3) Oxidative stress, characterized by the increased production of ROS can also trigger inflammasome activation. ROS, including the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), act as danger signals, stimulating inflammasome activation and subsequent inflammation [139].

The NLRP-3 inflammasome is a critical component of the innate immune response, acting as a sensor of danger signals. It is composed of a molecular sensor that recognizes pathogen-associated patterns (PAMPs) or danger (DAMPs), an adaptor protein known as apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain (ASC) and an effector enzyme, usually CASP-1 [140]. Activation of the inflammasome and CASP action results in the cleavage of precursors of pro-inflammatory cytokines, such as pro-IL-1β and pro-IL-18, leading to the release of their active forms, which amplify the inflammatory response [141] [142].

Activation of NLRP-3 occurs in two main stages: the first involves primary signalling that leads to the expression of NLRP-3 and production of pro-cytokines, while the second stage is characterized by the oligomerization of NLRP-3 and the activation of CASP-1, through the canonical pathway, resulting in the cleavage of pro-cytokines. CASP-11, along with its human counterparts CASP-4 and CASP-5, is a component of the non-canonical inflammasome [143]. This non-canonical inflammasome is activated by the recognition of LPS from Gram-negative bacteria.

Caspase-11 regulates inflammatory responses independent of the cleavage of IL-1β and IL-18 and is critical for defense against bacterial infections, but is not directly involved in the processing of these cytokines. In the context of oxidative stress, excessive activation of CASPs, particularly CASP-1, can dysregulate cellular homeostasis, contributing to cell death and excessive inflammation [144]. This dysregulation can hinder proper healing and contribute to the development of chronic inflammatory pathologies.

6.2 Inhibition of the inflammasome: exploration of regulatory mechanisms

Several mechanisms have been identified to inhibit or modulate inflammasome activity, involving a variety of proteins, cytokines and other factors:

(1) Cell death inhibitory proteins: Proteins such as CASP-8 and FLICE-like inhibitory protein (FLIP) have been identified as critical regulators capable of inhibiting CASP-1 activation and the subsequent production of pro-inflammatory cytokines. By blocking inflammasome activation, these proteins play a key role in regulating the inflammatory response and mitigating excessive tissue damage [145].

(2) Anti-inflammatory cytokines: Cytokines such as IL-10 and interleukin-1 receptor antagonist (IL-1Ra) have the ability to neutralize the effects of pro-inflammatory cytokines such as IL-1β and IL-18. By neutralizing these pro-inflammatory mediators, anti-inflammatory cytokines contribute to the attenuation of the inflammatory response and promote the resolution of inflammation [146].

(3) Small molecule inhibitors: Targeting the inflammasome, these inhibitors have emerged as promising therapeutic agents for the treatment of inflammatory conditions. Compounds such as parthenolide, Bay 11-7082 and 3,4-methylenedioxy-β-nitrostyrene have been identified as direct inhibitors of the inflammasome, offering potential avenues for the development of effective anti-inflammatory therapies [147].

(4) Melatonin and carnosol: Melatonin has been shown to attenuate inflammasome-induced pyroptosis by inhibiting NF-κB/GSDMD signaling, suggesting a novel therapeutic approach to prevent and treat inflammatory conditions [148]. Similarly, Carnosol has been shown to inhibit inflammasome activation by directly targeting heat shock protein 90 (HSP90), providing a potential strategy for the treatment of inflammatory conditions.

7. THE CRITICAL ROLE OF MITOCHONDRIA IN INFLAMMATION AND OXIDATIVE STRESS

Mitochondria are a major source of ROS generated during cellular respiration. The mitochondrial electron transport chain generates ROS such as superoxide anion, hydrogen peroxide and hydroxyl radicals as part of normal cellular metabolism. However, excessive production of ROS can lead to oxidative stress, causing cell damage and contributing to the development of various diseases [149].

The respiratory burst, carried out mainly by immune cells such as neutrophils and macrophages, involves the rapid release of ROS to fight invading pathogens. During the

respiratory burst, mitochondria play a critical role in providing the energy needed to generate ROS, thereby contributing to the oxidative microenvironment that aids in pathogen clearance [150,151]. Oxidative stress generated by mitochondria can activate cell signaling pathways, such as MAP kinases (e.g. MAPKs), which regulate the inflammatory response and cell survival. Activation of these pathways can lead to expression of pro-inflammatory genes and regulation of intracellular redox balance.

Mitochondria are involved in the activation of the transcription factor NF-κβ, which regulates the expression of genes involved in the inflammatory response. Mitochondrial signals can trigger the activation of NF-κβ, promoting the transcription of pro-inflammatory cytokines and exacerbating inflammation [152]. Mitochondrial dysfunction is closely associated with several diseases related to oxidative stress and inflammation [153]. On the other hand, antioxidant enzymes present in mitochondria, such as SOD and glutathione peroxidase, help to neutralize excess of ROS [154,155]. Another important process is mitophagy, a specialized form of autophagy that selectively targets damaged mitochondria, playing a vital role in maintaining mitochondrial quality control and ensuring cellular homeostasis. Thus, mitophagy helps to remove damaged mitochondria, thereby mitigating oxidative stress and suppressing inflammatory responses [156]. In summary, the interaction between CASPs, the inflammasome and oxidative stress plays a critical role in maintaining cellular homeostasis and influencing disease pathogenesis.

8. THE POTENTIAL OF PLANT EXTRACTS IN THE MODULATION OF OXINFLAMMATION

Plant extracts play an important role in different phases of wound healing due to their antioxidant, anti-inflammatory and cell growth-promoting properties. Several therapeutic approaches have been investigated to identify the mechanism of action of natural compounds present in plant extracts to optimize the remodeling phase and improve the final quality of skin healing. For instance, the alcoholic extract of *Ixora coccinea* flowers has been associated with increased granulation, tensile strength, and collagen deposition in skin wounds [157]. Similarly, papain, in addition to its role as a debridement agent, has an anti-inflammatory action and contributes to the contraction and joining of wound edges, making it useful in various phases of healing [158]. Other studies emphasize the importance of phenolic compounds, specifically tannins, extracted from barbatim bark *(Stryphnodendron adstringens)*, which stimulate collagen fibers production and promote the proper orientation of these fibers during the remodeling phase of healing [159,160].

The growing global interest in phytochemical and botanical research with therapeutic approaches has focused on their potential for treating inflammation and oxidative stress, due to their antioxidant and anti-inflammatory properties. Many plants offer antioxidant properties, containing compounds such as polyphenols, flavonoids, and carotenoids that neutralize free radicals and reduce cellular oxidative stress. In addition, some plant extracts have demonstrated anti-inflammatory activity by inhibiting the production of pro-inflammatory mediators such as cytokines and prostaglandins. This ability to reduce chronic inflammation has been implicated in conditions such as cardiovascular disease, diabetes, and autoimmune diseases. Additionally, immune response modulation is a key function of certain plant extracts, which help to balance the inflammatory response and reduce excessive inflammation, often by enhancing the activity of regulatory immune cells. Beyond their antioxidant and anti-inflammatory properties, these extracts protect cells from oxidative damage and inflammation by promoting the expression of endogenous antioxidant enzymes and DNA repair proteins, thereby reducing the risk of oxidative stress-related diseases, including cancer and cardiovascular diseases. Furthermore, plant extracts are sources of essential nutrients, vitamins and minerals that contribute to overall health and can be incorporated into the diet to promote wellness and health.

A screening of antioxidant properties and inhibition of inflammation-related enzymes conducted by Mainka, (2021) on eleven plant species revealed that 70% exhibited high antioxidant activity, significantly neutralizing free radicals and inhibiting the activity of inflammation-associated enzymes, such as hyaluronidase and lipoxygenase. This effect was attributed to the main active components identified in the extracts, such as flavonoids (luteolin, apigenin, kaempferol, quercetin derivatives) and caffeoylquinic acids, caffeic acid and their conjugates [161].

Plant extracts rich in tannins, derived from leaves and stem bark, exhibit significant anti-inflammatory and antioxidant properties, which contribute to addressing this issue. Studies suggest that tannins, phenolic compounds found in various plants, play a crucial role in modulating the inflammatory response. For instance, sorghum extract (*Sorghum sp*.) was evaluated for its ability to inhibit the production of nitric oxide, IL-6 and ROS in LPS-induced RAW 264.7 macrophages, demonstrating potent anti-inflammatory activity [162]. Moreover, the presence of flavonoids and triterpenes in extracts of *Saraca asoca* and *Portulaca oleracea* has been associated with the reduction of lipid peroxidation and the promotion of vascularization, both of which are crucial for wound healing [163]. The antioxidant activity of tannin-rich extracts is often attributed to their ability to scavenge free radicals, which not only protects cells from oxidative stress, but also facilitates healing by promoting wound contraction and angiogenesis

[164] . Studies with *Terminalia chebula* bark extracts have shown that the tannins produced accelerate healing in animal models, possibly by modulating the expression of growth factors such as VEGF, which is crucial for the formation of new blood vessels during the healing process[165]. Furthermore, research on the anti-inflammatory activity of *Arthropteris orientalis* extracts has shown that compounds such as glycosides and flavonoids, along with tannins, inhibit the proliferative phases of inflammation, thereby confirming the efficacy of these extracts in promoting healing [166]. Additionally, research on a tannin fraction from pistachio shell extract revealed antioxidant properties and inhibitory activity against pancreatic lipase, indicating that tannins not only modulate inflammation but also play a role in regulating lipid metabolism [167].

A comprehensive systematic review of preclinical in vitro and in vivo evidence on the *Brassicaceae* family highlighted its potential anti-inflammatory, antioxidant and regenerative properties. The secondary metabolites present in Brassicaceae, such as polyphenols, terpenes/carotenoids, and glucosinolates, are responsible for these effects. Moreover, these plants have shown positive effects on regulating skin inflammation, accelerating the wound healing process and inhibiting the development of edema. They also have effects on the modulation of inflammatory pathways, including the nuclear factor kappa β (NF-κβ) pathway [168].

Recent studies [174]show that natural compounds present in plant extracts can alleviate inflammatory symptoms, regulate the composition of the intestinal microbiota, and exert therapeutic effects on diseases such as obesity, peripheral neuropathy, diabetes, and cancer [169]. Furthermore, the antioxidant capacity of polyphenols can be used to prevent or treat various oxidative stress-related diseases. Plant extracts have also been demonstrated antimicrobial and anti-aging properties. These findings indicate a great therapeutic potential of plant extracts and emphasize the need for further research in this area.

9. ANTIOXIDANTS AND HOW THEY ACT

Antioxidants are molecules that can neutralize or inhibit the damaging effects of ROS and RNS by donating electrons or scavenging free radicals (Fig. 8). When an antioxidant donates an electron to a free radical, it becomes a radical. However, unlike harmful free radicals, many antioxidants form radicals that are stable and unreactive. This stability is due to resonance, where the negative charge resulting from the electron donation is distributed throughout the molecular structure of the antioxidant, stabilizing the radical formed [170,171]. For instance, phenolic compounds such as catechin can form phenoxide radicals that are stabilized by

resonance, preventing these radicals from becoming highly reactive [170,171] . In addition, the chemical structure of antioxidants, including the presence of hydroxyl groups, can increase their stabilizing capacity after electron donation [172,173] . Thus, while free radicals are often unstable and reactive, radicals formed from antioxidants can be relatively inert, allowing these compounds to fulfill their protective functions without contributing to the formation of more free radicals [99,100,174–177].

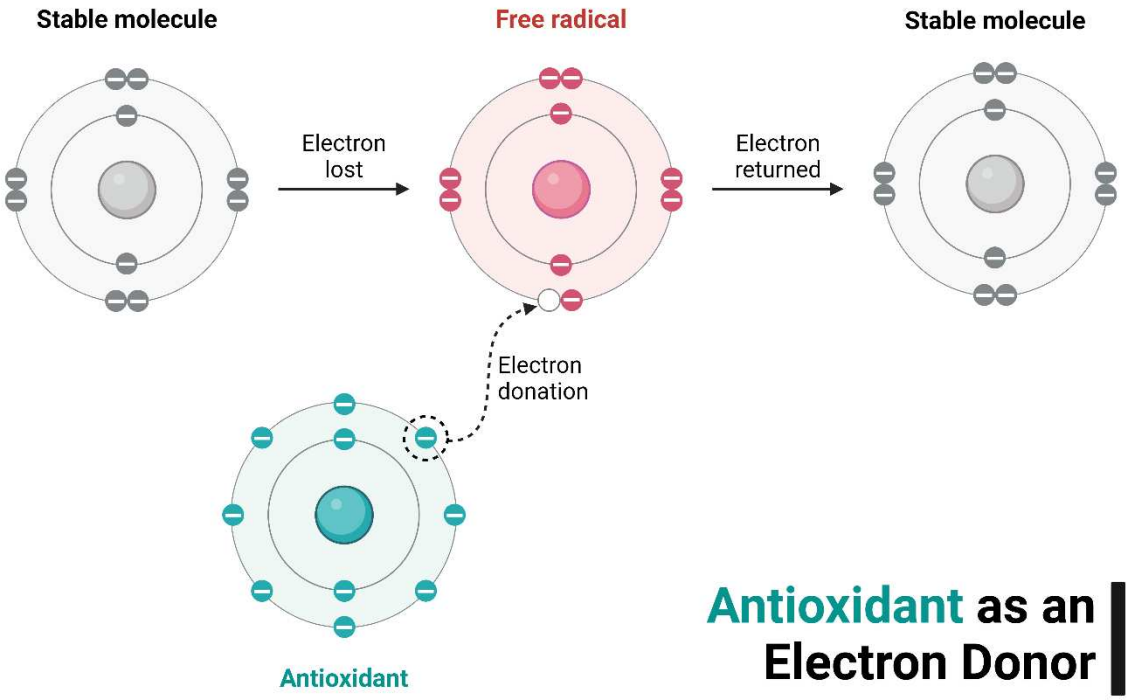

Figure 8: An antioxidant is a molecule that is stable enough to donate an electron to a highly reactive free radical, neutralizing it and reducing its potential to cause damage. These compounds function by delaying or inhibiting cell damage, mainly through their ability to scavenge free radicals[178]. Image created: Jiang, W., BioRender (2023) - Antioxidant as an Electron Donor.

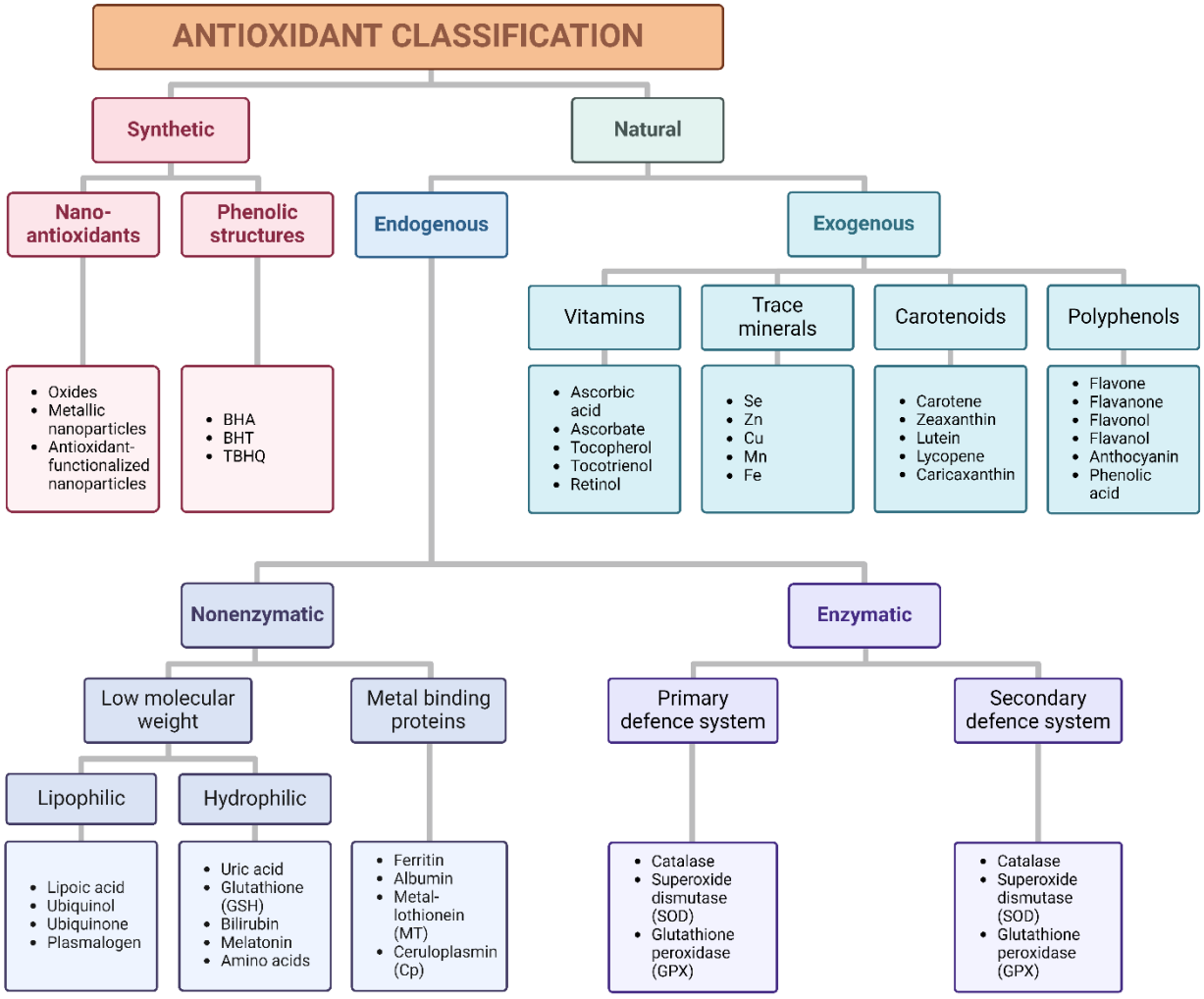

Figure 9: Classification of antioxidants: - Synthetic: nano-antioxidants and phenolic structures. - Natural: endogenous (non-enzymatic and enzymatic) and exogenous (vitamins, minerals, carotenoids and polyphenols) followed by their main actions in the body. Image: Jiang, W., BioRender (2023) - Classification of antioxidants.

Antioxidants can be divided into two main categories: synthetic and natural (Fig. 9). Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxyanisole (BHT), are widely used due to their efficacy and stability, but have raised safety concerns as they have been associated with adverse health effects, including carcinogenicity [179–182]. On the other hand, natural antioxidants are divided into endogenous, which includes both non-enzymatic and enzymatic, and exogenous, which includes vitamins, minerals, carotenoids and polyphenols [183,184]. The mechanisms of action of antioxidants in the body include the donation of electrons or protons that neutralise free radicals, thereby preventing oxidative stress and the oxidation of lipids and proteins [185,186]. In addition, natural antioxidants, such as polyphenols found in fruits and vegetables, have shown potential to improve health by reducing inflammation and the risk of chronic diseases [180,187]. The choice between synthetic and natural

antioxidants is often influenced by safety and efficacy considerations, with a growing trend towards the use of natural antioxidants due to their acceptability and health benefits [182,188,189].

9.1 Types of endogenous antioxidants

Enzymatic: Enzymatic antioxidants are proteins that catalyze ROS and RNS degradation. Examples include superoxide dismutase (SOD), catalase and glutathione peroxidase [99,101]. These enzymes work in concert to convert harmful free radicals into less reactive molecules, protecting cells from oxidative damage. SOD: converts the superoxide radical (O_2^{--}) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), reducing the oxidative potential of the cell; - Catalase (CAT): is responsible for metabolism hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2), protecting cells from damage caused by (H_2O_2); - Glutathione peroxidase (GPx): uses reduced glutathione (GSH) to reduce (H_2O_2) and other organic peroxides, forming water and oxidizing glutathione to oxidized glutathione (GSSG) [190–192].

Non-enzymatic: Non-enzymatic antioxidants are non-protein molecules that can neutralize ROS and RNS by donating electrons or removing reactive species [193], as well as playing a role in regulating gene expression, cell cycle progression and inflammation [101]. The most important non-enzymatic antioxidants include - Vitamin C (ascorbic acid), a powerful water-soluble antioxidant that neutralizes free radicals such as the hydroxyl radical (^{-}OH) and the peroxyl radical (ROO^{-}) and regenerates the reduced form of vitamin E; it acts as an enzymatic cofactor in the hydroxylation reactions of proline and lysine, stabilizing collagen fibers and its absence results in fragile tissue, as observed in scurvy [194,195]; - Vitamin E (tocopherol and tocotrienol), a family of fat-soluble compounds that protect cell membranes from oxidative damage by scavenging lipophilic radicals and preventing lipid peroxidation; - Glutathione (GSH), is a tripeptide composed of three amino acids (cysteine, glycine and glutamic acid) and plays a central role in cellular antioxidant defense, neutralizing ROS and participating in the regeneration of other antioxidants such as vitamin C and vitamin E; - Flavonoids, which are polyphenolic compounds found in fruits, vegetables and teas, which have significant antioxidant properties and protect cells against oxidative damage and inflammation [99,101,193,196].

9.2 Antioxidant mechanisms of action

The mechanism of action of enzymatic antioxidants is to catalyze the breakdown of ROS into harmless by-products, thereby reducing oxidative stress. They scavenge free radicals,

regenerate other antioxidants, chelate metal ions and induce the activity of antioxidant enzymes [93].

Highly reactive and unstable free radicals tend to react with other molecules in search of stability. These reactions can cause oxidative damage to biomolecules such as lipids, proteins and DNA, contributing to oxidative stress and cellular aging [174,197]. Antioxidants can act as electron or hydrogen donors to free radicals, converting them into stable, non-reactive forms. For example, vitamin C (ascorbic acid) is a water-soluble antioxidant that can donate an electron to a hydroxyl free radical (OH^-), converting it to water (H_2O) and the ascorbic acid radical (ascorbate). Similarly, vitamin E (tocopherol) is a fat-soluble antioxidant that can donate hydrogen to a peroxyl radical (ROO^-), converting it to a stable tocopheroxyl radical [198–200]. Some antioxidants can form stable complexes with free radicals, preventing them from interacting with other molecules and thus neutralizing their oxidative activity. For example, uric acid [201], an endogenous antioxidant present in blood plasma, can form stable complexes with the peroxyl radical (ROO^-), inhibiting its ability to oxidize lipids and proteins [175,202]. Similarly, melatonin [203], a hormone produced by the pineal gland, can form complexes with hydroxyl radicals (OH^-), neutralizing their reactivity and protecting cells from oxidative damage [204,205].

9.3 Antioxidants in oxidative damage repair and wound healing.

Antioxidants are present from the initial inflammatory phase to the final remodeling of scar tissue. They stimulate angiogenesis and extracellular matrix synthesis, which are essential for tissue closure and integrity, but increased metabolic activity can generate ROS and RNS. To neutralise free radicals, vitamin E, for example, can prevent lipid peroxidation, thereby protecting cell membranes from structural damage [202]. Similarly, glutathione can bind to oxidation products in proteins, facilitating their removal and degradation by cellular proteolytic pathways [177,202].

Some antioxidants can act as 'quenchants' of reactive species, deactivating their oxidative activity and converting them to less reactive forms. For example, ubiquinone (CoQ10), an essential component of the mitochondrial electron transport chain, can react with the peroxyl radical (ROO^-) to form ubiquinol (CoQH2), a more potent and effective antioxidant in neutralizing free radicals [206]. They are also involved in metal sequestration and can bind to pro-oxidant metal ions such as iron and copper, preventing their ability to generate free radicals via the Fenton and Haber-Weiss reactions [207,208]. Ceruloplasmin is a metalloprotein that binds to free copper in blood plasma, preventing it from participating in pro-oxidant reactions [209] that generate hydroxyl radicals (OH^-). Similarly, phytic acid, a polyphosphate compound found in

plant foods, can bind to pro-oxidant metal ions in the gastrointestinal tract, reducing its bioavailability and ability to cause oxidative damage to cells [210,211].

During the inflammatory phase, vitamins C and E, glutathione and associated zinc and copper particles act as reducing agents, providing electrons or hydrogen to free radicals, preventing their harmful reactivity. In addition, they can modulate the inflammatory response by reducing the production of the pro-inflammatory cytokines IL-1α, IL-2, NO and TNF-α and facilitating resolution of inflammation [96,212–214]. Next, antioxidants, such as ascorbic acid (vitamin C), are fundamental in protecting fibroblasts, ensuring adequate production of collagen and other matrix proteins that provide support and resilience to scar tissue formation, thus promoting tissue regeneration and wound healing [101,202]. They also help to re-epithelialize the wound by protecting keratinocytes from lipid oxidation and promoting the formation of new epidermis [215–217]. At the end of the remodeling process, antioxidants continue to play an important role in regulating the oxidative microenvironment and extracellular matrix. They can attenuate the formation of hypertrophic or keloid scars, preventing long-term complications and promoting tissue regeneration. In addition, antioxidants facilitate vascular regression and reorganization of the extracellular matrix, such as the gradual replacement of type III collagen with type I collagen, thus helping the scar to mature and restoring tissue integrity [218–220].

Antioxidants can modulate the cell signaling pathways involved in the response to oxidative stress. The nuclear transcription factor Nrf-2 (nuclear factor erythroid 2-related factor 2) regulates the expression of genes involved in antioxidant defense and DNA repair. Normally inactive in the cytoplasm, nuclear factor erythroid 2-related factor 2 (Nrf-2) dissociates from Keap1 in response to oxidative stress, translocate to the nucleus and induces the expression of antioxidant genes such as SOD, CAT and GPx, thereby increasing the antioxidant capacity of the cell [221,222]. Curcumin and resveratrol have shown inhibitory effects on pro-oxidant enzymes such as NADPH oxidase and xanthine oxidase, thereby reducing ROS production [223,224].

The nervous system, with its high metabolic oxygen demand, lipid content and limited regenerative capacity, is particularly susceptible to oxidative stress. Research suggests that antioxidants such as alpha-lipoic acid, coenzyme Q10 and flavonoids offer protection against neurodegenerative diseases such as Alzheimer's, Parkinson's and multiple sclerosis [225–229]. Oxidative stress can impair immune responses, while antioxidants can enhance the body's defense against pathogens and boost immune function. Studies have shown that supplementation with antioxidants such as vitamin C and vitamin E can boost the immune system [101,225,226].

10. OXINFLAMMATION IN WOUND HEALING: PRO- AND ANTI-INFLAMMATORY PATHWAYS

In the complex process of wound healing, the interaction between oxidative stress and inflammation significantly influences the outcome of tissue repair. OxInflammation, characterized by the interaction between oxidative stress and inflammatory responses, is critical in regulating immune responses during wound healing.

Pro-inflammatory pathways are essential for initiating the wound healing process, facilitating the recruitment of immune cells and fighting pathogens at the wound site. Key pathways such as NF-κβ, TGF-β, IFN-γ, TLR-4, COX-2, NLRP3 and IL-6 are fundamental in promoting the inflammatory response and tissue repair [230].

On the other hand, anti-inflammatory pathways are essential for resolving inflammation, promoting tissue regeneration and preventing excessive scarring. Pathways such as IL-10, Nrf-2, heme oxygenase 1 (HO-1) and hypoxia-inducible factor-1 (HIF-1) play a role in suppressing the inflammatory response, supporting tissue healing and maintaining tissue homeostasis [216].

OxInflammation, characterized by the interaction between oxidative stress and inflammatory responses in skin wounds, is a fundamental process in the regulation of immune responses during the healing process. Macrophages, in particular, exhibit a remarkable plasticity that allows them to adapt to variations in the microenvironment, leading to a variety of activations such as classical (M1) or alternative (M2) macrophage activation during the progression of infection and cell damage.

Initially, the Inositol 1,4,5-triphosphate (IP3) pathway is stimulated by proinflammatory cytokines such as TNF-α and IL-1, which act directly on the smooth endoplasmic reticulum, promoting the release of intracellular Ca^{2+} and enabling the release of adhesion molecules (selectins) present in Weibel-Palade vesicles, facilitating diapedesis. During this process, neutrophils are the first cells to arrive at the site of injury, followed by macrophages. In addition to the IP3 pathway, pro-inflammatory cytokines such as TNF-α or IL-1 activate the NF-κβ and Iκκ kinase (IKK) (survival) pathways via membrane receptors, inducing the expression of other proinflammatory genes. At sites of inflammation, activated inflammatory cells release free radicals and enzymes, causing molecular damage and oxidative stress that must be cleared.

To trigger a pro-inflammatory response, TLR's are activated by PAMPs, initiating transcription factors, expression of pro-inflammatory genes and biosynthesis of ROS. One of the key events for efficient tissue repair is the elimination of apoptotic cells by phagocytes in the injured tissue. The numerous infiltrating neutrophils represent a large reservoir of short-lived inflammatory cells programmed to undergo apoptosis. Another example linking oxidative

stress and apoptosis is the release of ROS from mitochondria, which are readily recognized by immune cells and stimulate their migration. The production of ROS during the inflammatory response facilitates the clearance of toxins and pathogens by inducing the expression of antioxidant genes.

The balanced production of pro- and anti-inflammatory cytokines, such as IL-1, TNF-α and IL-6, activates signaling pathways that increase the production of ROS, including superoxide and hydrogen peroxide, during inflammatory responses. This imbalance can lead to pyroptosis. Interleukin IFN-γ plays an important role in the acute inflammatory phase by recruiting leukocytes to the site of infection and stimulating the polarization of M1 macrophages, which produce proinflammatory cytokines. Excessive ROS production can lead to oxidative stress and tissue damage, negatively affecting the healing process. Careful regulation of the redox balance is essential to ensure proper resolution of inflammation and progression to the tissue repair phase.

Plant extracts, rich in antioxidants and anti-inflammatory compounds such as polyphenols and flavonoids, have shown promise in modulating OxInflammation and promoting wound healing [215]. These compounds can neutralize ROS, modulate inflammatory pathways and stimulate tissue regeneration, offering potential therapeutic benefits for wound management [231]. However, further research is needed to understand the mechanisms of action and to optimize the clinical use of plant extracts in wound healing. In summary, the interplay between oxidative stress and inflammation in OxInflammation significantly affects all phases of wound healing, from inflammation to tissue remodeling. Understanding and targeting this interaction through plant extracts and other therapeutic strategies holds promise for improving wound healing outcomes and addressing the challenges of treating chronic and acute wounds.

11. CONCLUSION

The healing process is a complex and multifaceted biological orchestration that is essential for tissue recovery after injury (Fig. 10). It begins with an immediate inflammatory response, which is critical for fighting infection and setting the stage for tissue repair. This phase triggers the activation of immune and connective tissue cells, which work together to clear debris and begin to rebuild the extracellular matrix.

This is followed by the proliferative phase, a period of intense cellular activity in which fibroblasts and endothelial cells promote the formation of granulation tissue and new blood vessels respectively, while keratinocytes work to restore the skin barrier. The final remodeling of the scar involves a reorganization of the extracellular matrix, with a transition from type III

to type I collagen, resulting in a more resistant scar tissue, although never identical to the original.

At the same time, redox balance and the inflammatory response play a crucial role in the effectiveness of healing. A careful balance between reactive oxygen species and antioxidant systems is necessary to prevent cell damage and promote proper healing. Similarly, precise modulation of inflammation is required to prevent wound chronicity and facilitate efficient healing.

Chemical mediators play a fundamental role in regulating the tissue repair process by modulating the inflammatory response, cell proliferation and extracellular matrix deposition. For example, cytokines such as TNF-α and TGF-β, which are modulated by the NF-κβ pathway, have proinflammatory and pro-fibrotic effects, respectively, that influence wound healing. Specific inhibitors of chemical mediators can be used therapeutically to modulate the repair process and promote more effective healing.

In summary, healing is a dynamic process that depends on the harmonious interaction between inflammation, cell proliferation and tissue remodeling, as well as the precise regulation of chemical mediators at all stages of repair. An in-depth understanding of these mechanisms not only paves the way for advanced healing therapies, but also highlights the incredible ability of the body to regenerate itself, balancing destruction and construction, damage and repair, in a continuous cycle of renewal. The dynamic phases of the repair process involve a coordinated series of cellular and molecular events aimed at restoring the integrity and functionality of damaged tissue, and it is essential to recognize the interrelationship and influence of chemical mediators at all stages of repair in order to develop effective therapies to promote wound healing and prevent complications associated with tissue repair disorders.

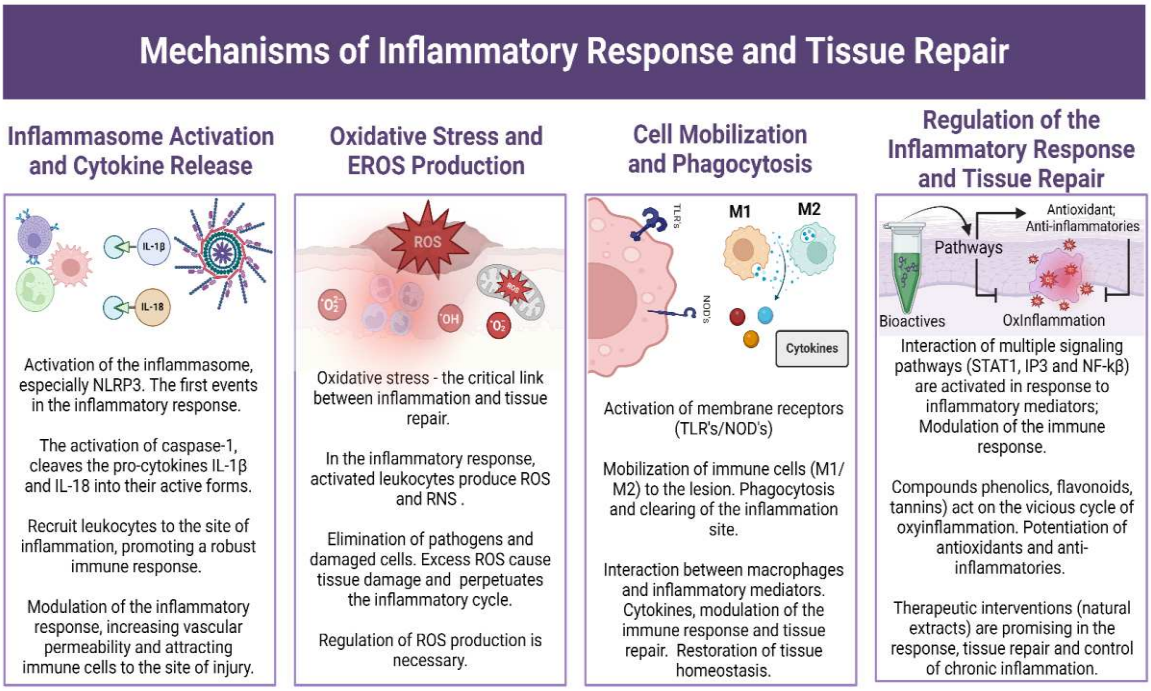

Figure 10: This infographic summarizes the critical events that occur during the inflammatory response and tissue repair, highlighting the importance of inflammasome activation, reactive species production, cell recruitment and regulation of the inflammatory response. Understanding these mechanisms is fundamental to the development of effective therapeutic approaches. Authorial image, Biorender (2024), Mechanisms of Inflammatory Response and Tissue Repair.

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CHAPTER 2

OxInflammatory responses in the wound healing process: A Systematic review.

Accepted in the *Journal Antioxidants*, date 31 May 2024.

https://doi.org/10.3390/antiox13070823

LOPES, Fernanda Barbosa, D.Sc., Federal University of Viçosa, October 2024. **OxInflammatory Responses in the Wound Healing Process: A Systematic Review.** Advisor: Reggiani Vilela Gonçalves. Co-advisor: Mariáurea Matia Sarandy de Souza; Manoela Maciel dos Santos Dias.

Abstract: Significant sums are spent every year to find effective treatments to control inflammation and speed up the repair of damaged skin. This study investigated the main mechanisms involved in the skin wound cure. Consequently, it offered guidance to develop new therapies to control OxInflammation and infection and decrease functional loss and cost issues. This systematic review was conducted using the PRISMA guidelines, with a structured search in the MEDLINE (PubMed), Scopus, and Web of Science databases, analyzing 23 original studies. Bias analysis and study quality were assessed using the SYRCLE tool (Prospero number is CRD262 936). Our results highlight the activation of membrane receptors (IFN-δ, TNF-α, toll-like) in phagocytes, especially macrophages, during early wound healing. The STAT1, IP3, and NF-kβ pathways are positively regulated, while Ca^{2+} mobilization correlates with ROS production and NLRP3 inflammasome activation. This pathway activation leads to the proteolytic cleavage of caspase-1, releasing IL-1β and IL-18, which are responsible for immune modulation and vasodilation. Mediators such as IL-1, iNOS, TNF-α, and TGF-β are released, influencing pro- and anti-inflammatory cascades, increasing ROS levels, and inducing the oxidation of lipids, proteins, and DNA. During healing, the respiratory burst depletes antioxidant defenses (SOD, CAT, GST), creating a pro-oxidative environment. The IFN-δ pathway, ROS production, and inflammatory markers establish a positive feedback loop, recruiting more polymorphonuclear cells and reinforcing the positive interaction between oxidative stress and inflammation. This process is crucial because, in the immune system, the vicious positive cycle between ROS, the oxidative environment, and, above all, the activation of the NLRP3 inflammasome inappropriately triggers hypoxia, increases ROS levels, activates pro-inflammatory cytokines and inhibits the antioxidant action and resolution of anti-inflammatory cytokines, contributing to the evolution of chronic inflammation and tissue damage.

Keywords: oxidative stress; inflammation; wound closure; inflammasome

1. Introduction

Skin wounds are a global public health challenge that imposes high costs on healthcare systems. Health organizations estimate that billions of dollars are spent each year to purchase

preventative materials and treat wound complications [1,2]. Complex interactions between extracellular matrix molecules, soluble mediators, multiple resident cells, and infiltrating leukocyte subtypes surround the dynamic process of wound healing [3,4]. As the immediate goal of repair is to achieve tissue integrity and homeostasis, the first step is the inflammatory reaction. In this phase, vascular phenomena are characterized by hemostasis and coagulation, followed by cellular exudation [5]. During the proliferative phase, fibroblasts, keratinocytes, and endothelial cells proliferate to synthesize extracellular matrix (ECM) components, such as elastic fibers and collagen. During this phase, angiogenesis is stimulated to oxygenate and nourish the newly formed tissue, known as granulation tissue [3,4,6]. Granulation tissue is gradually replaced in the third phase (remodeling), and type III is replaced by type I collagen, increasing tissue resistance. Therefore, the third phase is characterized by fibroblasts, endothelial cell apoptosis, and progressive collagen accumulation (mainly type I fibers) [7-10].

The differences between acute inflammation and chronic inflammation are not only the duration but also the intensity of the cellular response. A low-intensity cellular response characterizes acute inflammation, whereas a high-intensity response characterizes chronic inflammation. When the damaged tissue is repaired and the infection is cleared, there is what is known as the ‘resolution of inflammation [11-13]. In the middle of acute and chronic inflammation is an OxInflammation process. To understand the relationship between oxidative stress and inflammation during the wound healing process, it is necessary to highlight cells as neutrophils, being responsible for producing immunological effectors such as interferon-γ (IFN-δ) and interleukins (IL), which will recruit macrophages to the wound area [14-18]. Through NF-kB and IL-12 stimulation, macrophages produce pro-inflammatory cytokines and growth factors, such as TNF-α, IL-6, and IL-8 [19,20], essential to resolve the inflammatory progression. In this process, Caspase-1 is autocatalytically activated upon inflammasome recruitment. Two key pro-inflammatory cytokines, pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18), which are initially inactive, have their activation facilitated by caspase-1 [21,22]. IL-1β induces the expression of genes that control vasodilatation, cell migration, and endothelial cell responses that facilitate immune cell infiltration into the injured tissue. IL-18 is required for interferon-gamma (IFN-δ) production and is a co-stimulatory cytokine involved with adaptive immunity [23]. However, these compounds produced and released by macrophages can contribute to tissue damage. The clinical consequences are persistent, low-grade, increased inflammatory response, impaired epithelialization, and granulation tissue formation. Therefore, it is necessary to have a

perfect balance between pro-inflammatory and anti-inflammatory mechanisms, including anti-inflammatory cytokines and prostaglandin biosynthesis, to avoid poor tissue repair [24].

At the same time, phagocytes also produce reactive oxygen species (ROS) and nitrogen species (RNS), increasing their microbicide and tumoricidal activity. ROS and RNS are produced by macrophages in the process known as respiratory burst. These reactive species act as signaling molecules promoting the up-regulation of pro-inflammatory cytokines and down-regulation of anti-inflammatory cytokines, resulting in chronic inflammation. Although inflammation is essential to the healing process, an imbalance in the inflammatory process caused by oxidative stress can prolong the healing process because it is associated with an increase in ROS by activated immune cells, a deficit in angiogenesis and migration, and impaired cell proliferation [25-27]. In chronic inflammation, the tissue characteristically shows an infiltrate composed mainly of mononuclear cells (monocytes, macrophages, and lymphocytes), signs of angiogenesis and fibrosis [28]. Therefore, oxidative stress balance plays a fundamental role in resolving inflammation and healing skin wounds [29,30].

As part of the immune system, ROS are needed to kill bacteria and other microorganisms, consequently reducing inflammation [27]. In wound healing, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase reduces molecular oxygen and generates ROS excess in macrophages. Excessive ROS production inhibits cell migration and proliferation, affecting the expression and function of anti-inflammatory mediators. This effect enhances the inflammatory process, showing positive feedback among inflammatory and oxidative pathways [29]. Normal wound healing promotes the expression of many antioxidant genes [27,30], such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), which from the antioxidant defense network in living systems and act at different levels [31,32], including limiting the excessive production of ROS, inhibiting the expression and activity of pro-inflammatory mediators such as COX-2 and iNOS, and attenuating the production of ROS [29,33]. However, the negative regulation of these antioxidant genes results in prolonged inflammation, and a delay in the healing process [34-36], and the excess ROS generated during inflammation can lead to cell damage, such as membrane rupture, DNA damage, and protein oxidation. By altering cellular functions, oxidative stress is induced, thus inhibiting cell migration and proliferation and affecting the expression and function of inflammatory mediators [37]. Without sufficient antioxidant activity, wound healing can be delayed, severe tissue damage can occur, and chronic inflammation can persist [38,39].

It is well known that inflammatory and oxidative markers play an essential role in skin wound closure. However, little is known about the relationship between inflammatory and oxidative effectors during different phases of the wound-healing process. A comprehensive analysis of signaling pathways and the relation of mechanisms involved in oxidative damage with its physiological response has not been systematically evaluated. Therefore, this study will help understand the mechanism underlying wound healing and guide decision-makers in developing new products and treatments that can accelerate skin wound closure. Consequently, it offered guidance to develop new therapies to control OxInflammation and infection and decrease functional loss and cost issues. The methodological bias analysis allows us to assess the strength of current evidence, findings, and research limitations in this field.

2. Material and Methods

2.1. Guiding Question

What is the central cellular mechanism's relationship between inflammation and oxidative stress in a murine model of the skin wound healing process? What are the primary inflammatory and oxidative effectors involved with skin wound healing? Finally, what are the main inflammatory and oxidative mechanisms activated during skin wound healing?

2.2. Literary Research

This systematic review was conducted in accordance with the PRISMA guidelines (Figure 1) [40], ensuring thorough data selection, extraction, and analysis. A detailed literature search was conducted using the PubMed/Medline, Scopus, and Web of Science databases. On 13 April 2021, an advanced search was carried out on these platforms. The search strategy was designed to include two main approaches: direct searches in electronic databases and indirect screening of the reference lists of identified studies.

The search filters were built around four key terms: wound healing, oxidative stress, inflammation, and animal studies. For PubMed/Medline, the search filter used standardized descriptors from the MeSH hierarchical thesaurus, the MeSH, and TIAB commands to extract indexed articles and citations. These descriptors were adapted to meet the search requirements of the Web of Science (TS = descriptor) and Scopus (TITLE-ABS-KEY [descriptor]) databases. No chronological limits were imposed, and all original full-text studies published up to 2021 were included in the review. Two reviewers, FBL and MMS, carried out the initial literature search, removed duplicate articles, and selected titles and abstracts based on predefined eligibility criteria detailed in Table S1.

The full-text articles of potentially relevant studies were then independently assessed for eligibility by three reviewers: FBL, MMS, and RVG. Agreement between reviewers during data selection and extraction was measured using the kappa test, resulting in a kappa value of 0.937. Any inconsistencies were resolved through consultation between the reviewers.

2.3. Selection of Studies

In order to ensure the integrity and reliability of our review of the wound healing process, we implemented a rigorous selection and analysis methodology. Initially, two reviewers, FBL and MMS, undertook the data extraction independently in order to eliminate any potential bias. This independent analysis was of the utmost importance in maintaining objectivity, particularly during the data collection and selection phases. The focus of our review was on original experimental studies conducted in vivo, specifically using animal models, and published in English. Furthermore, only studies with full texts available were included. In order to further refine our selection process, we established clear eligibility criteria. Firstly, studies that directly investigated the healing of skin wounds. Secondly, studies that analyzed oxidative and inflammatory markers which are key in assessing the wound healing process. Finally, studies that involved excisional wounds in animal models ensure a consistent and relevant research context. Exclusion criteria were: (i) articles without full-text available; (ii) secondary studies (i.e., literature reviews, commentary, letters to the editor, and editorials); and (iii) studies not peer-reviewed or formally published in indexed journals.

2.4. Data Extraction

Three independent reviewers (FBL, MMS, and RVG) extracted the essential data, which were categorized into five descriptive levels: characteristics of the publication (author, year, and country), characteristics of the animal model (species, sex, age, and weight), intervention (control group, dose, frequency, route and time of intervention), main outcomes observed after treatment and secondary outcomes. In case of disagreement on the extracted data, two additional reviewers (RVG and RDN) participated in the discussion to resolve the issue.

2.5. Bias Analysis

The quality of the studies was assessed using the SYRCLE's (Systematic Review Center for Experimentation with Laboratory Animals) risk of bias (RoB) tool [41], examining various methodological domains. The protocol for this systematic review has been registered in PROSPERO (262 936) and is available in full on the NIHR HTA program website

(https://www.crd.york.ac.uk/prospero/, Accessed on 05 jul. 2021). Selection bias assessed the generation of the random sequence, baseline characteristics, and allocation concealment to ensure equivalent groups at baseline. Performance bias focused on random assignment and blinding of caregivers and researchers to minimize conscious or subconscious bias. Detection bias analyzed the random assessment of outcomes and blinding to ensure objective and consistent measurement of outcomes. Attrition bias involves evaluating incomplete outcome data since high dropout rates can lead to biased results. Communication bias checked the selective communication of results to ensure that all planned results were communicated. Other biases considered ethics, the appropriateness of statistical methods, and work safety measures, increasing the credibility and replicability of the study. We used Cochrane's Review Manager 5.3 (RoB 2.0) program to create a figure that visually presented the risk of bias in all the included studies. The items in the RoB tool were categorized and scored as "yes" (low risk of bias), "no" (high risk of bias), or "unclear" (not reported, making the risk uncertain). This exhaustive approach ensures that conclusions are based on solid and reliable data, contributing to more effective and ethical scientific research.

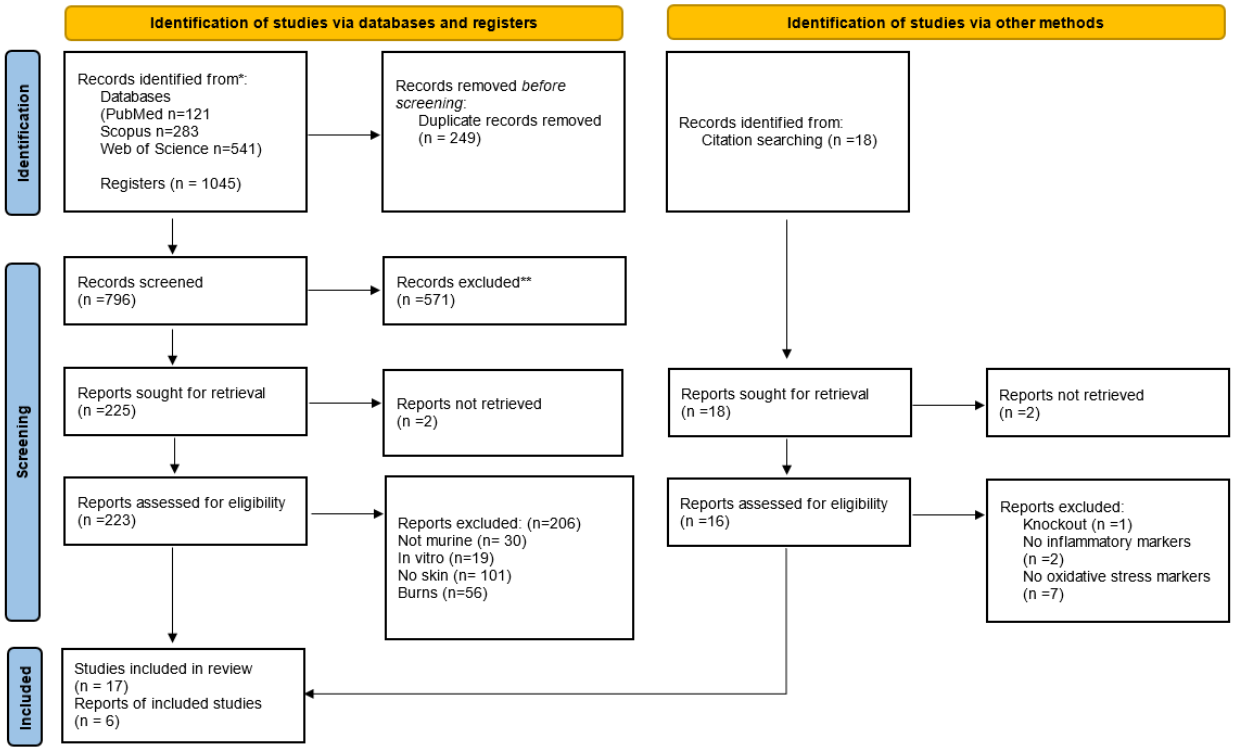

Figure 1. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow diagram. The flowchart indicates the research records obtained at all standardized stages of the search process required to develop systematic reviews and meta-analyses. Based on the PRISMA statement (http://www.prisma-statement.org). *Consider, if feasible to do so, 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.

3. Results

3.1. Publication Characteristics

The initial search resulted in 121 studies in PubMed/Medline, 283 in Scopus, and 541 in the Web of Science database, totaling 1045 studies, of which 249 duplicates were excluded. After reading the titles and abstracts, another 571 irrelevant studies were excluded, and 225 studies were read in full. Only 23 studies fully met the inclusion criteria and were included in the systematic review (Figure 1). The studies were performed in India (39%); Brazil, United States of America, and Korea (13% each); Tunisia, Taiwan, Malaysia, Japan, and Indonesia (4.35% each). The ethical approval for the use of animals in the experimental design was specified in all studies.

3.2. Characteristics of the Animal Models

Considering the twenty-three studies in this review, 16 (69.6%) of them were performed with rats and 7 (30.4%) with mice. All studies used non-knockout mice as animal models and an untreated control group (100%). Animal sex was reported in 78% of the studies (52% used males, 17% used females, and 9% used males and females). Age was presented in 78% of the studies, ranging between 4 and 24 weeks, and 21.7% ignored this data. Animal weight was reported in 61% (n = 14) of the studies (Table S2). Rats' weight ranged between 150 g and 310 g among the Wistar (43.5%), Charles-Foster albino, and Sprague–Dawley strains (13% each). Mice weight ranged between 18 g and 30 g among the C57BL/6 mice and ICR mice (8.7% each); Swiss albino, BALB/c, hairless SKH-1, CrljBgi, and CD-1 (4.37% each) (Table S2). The data evaluated in this review were extracted from control animals only to investigate the relationship between oxidative stress and inflammation in the normal healing process.

3.3. Excisional Wound Characteristics

Dorsal skin wounds were performed in 22 studies, with 61% of the studies with excision wounds and 39% with excision and incision wounds (Table S3). The methodology and specification of hygiene and asepsis before performing the wounds were neglected in 69.6% of the studies, and 30.4% (n = 7) reported the use of alcohol or saline solution or betadine as an antiseptic. Of the studies performed in rats (n = 16, 69.6%), four of them showed wounds with 500 mm^2, three of them showed wounds between 50 and 300 mm^2, four of them showed wounds

between 10 and 20 mm^2, and four of them showed 2–6 mm^2 wounds. Only one study with rats neglected this information. On the other hand, in studies using mice (n = 7, 30.4%), three reported 10 mm^2 wounds, and four showed a wound between 3.5 and 8 mm^2. Only one study in mice neglected this information.

The experimental period for monitoring wound healing in rats was 8 to 15 days (n = 5, 21.7%) and 16 to 22 days (n = 9, 56.2%). Only two studies (n = 2, 8.7%) did not report this period in rats. In mice, the experimental period ranged from 3 to 7 days (n = 4, 17.4%) and from 8 to 14 days (n = 3, 13%), according to Table S3.

3.4. Main Biological Results

3.4.1. Oxidative Stress

Oxidative stress was analyzed in all the studies included in this review. The level of antioxidant enzymes, such as catalase (CAT), glutathione (GSH) and superoxide dismutase (SOD), was analyzed in 74% of the studies. The pro-oxidant markers evaluated were malondialdehyde (30.4%; n = 7), lipid peroxidation (13%; n = 3), 3-nitrotyrosine (4.3%, n = 1), DNA damage (4.3%, n = 1), and 4-hydroxynonenal (4.3%, n = 1). In addition, the protein carbonyl (PCN) was analyzed in 8.7% of the studies. Among the oxidative stress mediators, hydrogen peroxide (H_2O_2) was investigated in 8.7% (n = 2) of the studies. Other markers, such as nitric oxide (NO) and myeloperoxidase (MPO), were analyzed in 17.4% (n = 4) and 30.4% (n = 7) of the studies, respectively (Figure 2).

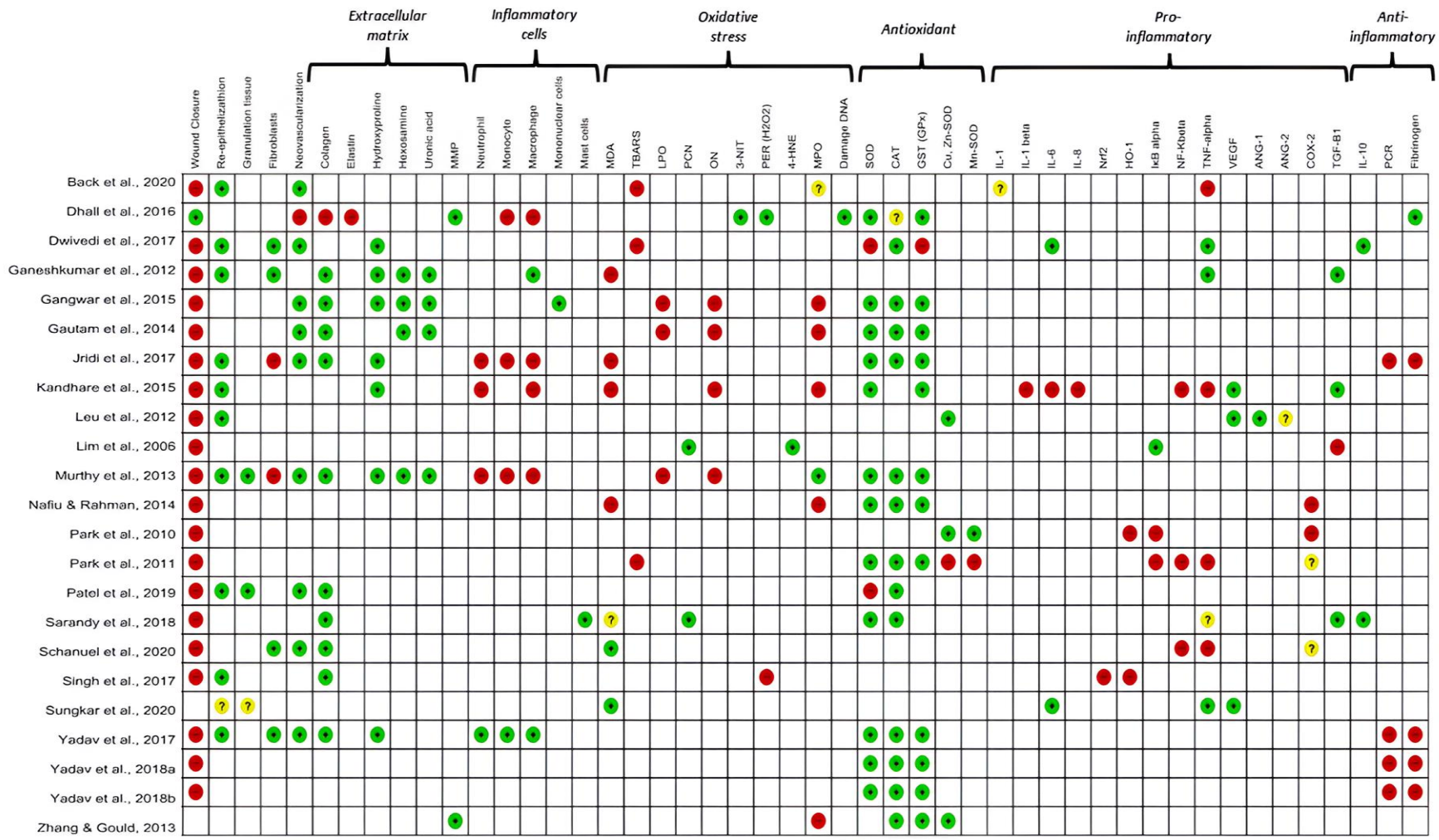
Extracellular matrix
Inflammatory cells
Oxidative stress
Antioxidant
Pro-inflammatory
Anti-inflammatory
Wound Closure
Re-epithelialization
Granulation tissue
Fibroblasts
Neovascularization
Collagen
Elastin
Hydroxyproline
Hexosamine
Uronic acid
MMP
Neutrophil
Monocyte
Macrophage
Mononuclear cells
Mast cells
MDA
TBARS
LPO
PCN
ON
3-NIT
PER (H2O2)
4-HNE
MPO
Damage DNA
SOD
CAT
GST (GPx)
Cu, Zn-SOD
Mn-SOD
IL-1
IL-1 beta
IL-6
IL-8
Nrf2
HO-1
IκB alpha
NF-Kbeta
TNF-alpha
VEGF
ANG-1
ANG-2
COX-2
TGF-B1
IL-10
PCR
Fibrinogen
Back et al., 2020
Dhall et al., 2016
Dwivedi et al., 2017
Ganeshkumar et al., 2012
Gangwar et al., 2015
Gautam et al., 2014
Jridi et al., 2017
Kandhare et al., 2015
Leu et al., 2012
Lim et al., 2006
Murthy et al., 2013
Nafiu & Rahman, 2014
Park et al., 2010
Park et al., 2011
Patel et al., 2019
Sarandy et al., 2018
Schanuel et al., 2020
Singh et al., 2017
Sungkar et al., 2020
Yadav et al., 2017
Yadav et al., 2018a
Yadav et al., 2018b
Zhang & Gould, 2013

Figure 2. Results of the primary and secondary outcomes of the individual studies analyzed. The colour green: increased; red: decreased; yellow: undetermined and white: not analysed, indicate the results measured between the studies. MMP: matrix metalloproteinase; MDA: malondialdehyde; TBARS: Thiobarbituric acid reactive substances; LPO: lipid peroxidation; PCN: carbonylated protein; ON: nitric oxide; 3-NT: 3-nitrotyrosine; H2O2: hydrogen peroxide; 4HNE: 4-hydroxynonenal; MPO: metalloproteinase; SOD: superoxide dismutase; CAT: catalase; GST: glutathione transferase; *CuZnSOD:* copper–zinc superoxide dismutase; MnSOD: manganese superoxide dismutase; IL-1: interleukin-1; IL-1β: interleukin-1-beta; IL-6: interleukin-6; IL-8: interleukin-8; NRF2: nuclear factor erythroid factor 2; HO-1: the inducible isoform of HO; IKβ: Ikappaβ kinase; NF-kβ: nuclear factor kappa β; TNF-α: Tumor Necrosis Factor receptor alpha; VEGF: vascular endothelial growth factor; ANG1 and 2: angiotensin (1-2), COX-2: cyclooxygenase-2; TGF-β: transforming growth factor beta; IL-10: interleukin-10; CRP: C-reactive protein; FIB: fibrinogen. References of the articles in the figure: Back et al., 2020 [42]; Dhall et al., 2016 [43]; Dwivedi et al., 2017 [44]; Ganeshkumar et al., 2012 [45]; Gangwar et al., 2015 [46]; Gautam et al., 2014 [47]; Jridi et al., 2017 [48]; Kandhare et al., 2015 [49]; Leu et al., 2012 [50]; Lim et al., 2006 [51]; Murthy et al., 2013 [52]; Nafiu &Rahman, 2014 [53]; Park et al., 2010 [54]; Park et al., 2011 [55]; Patel et al., 2019 [56]; Sarandy et al., 2018 [57]; Schanuel et al., 2020 [58]; Singh et al., 2017 [59]; Sungkar et al., 2020 [60]; Yadav et al., 2017 [61]; Yadav et al., 2018a [62]; Yadav et al., 2018b [63]; Zhang & Gould, 2013 [64].

3.4.2. Inflammation

Most studies (78.3%) attempted to explain their results by quantifying different mediators and investigating inflammatory pathways activated during wound repair. The pro-inflammatory mediators evaluated were tumor necrosis factor-alpha (TNF-α) (n = 8, 30.8%), cyclooxygenase-2 (COX-2), transforming growth factor β (TGF-β) (n = 4, 14. 4%), interleukin 6 (IL-6), vascular endothelial growth factor (VEGF), nuclear factor- kβ (NF-κβ), IκB kinase (IKβ), and heme oxygenase 1 (HO-1) (13.4%, n = 3 each). Interleukin 1β (IL-1β), interleukin 8 (IL-8), NR-F2 related to the factor 2 (Nrf2), angiopoietin-1 and angiopoietin-2 (4.3%, n = 1 each) were additionally evaluated. Anti-inflammatory mediators such as interleukin-10 (IL-10) and C-reactive protein (CRP) were analyzed in 8.7% (n = 2) and 17.4% (n = 4) of the studies, respectively. Fibrinogen, a marker of the acute phase of inflammation, was analyzed in 22% (n = 6) of the studies (Figure 2).

3.4.3. Relationship between Oxidative Stress and Inflammation

The oxidative markers investigated in this review were produced by polymorphonuclear cells (n = 4, 17.4%) and mononuclear cells (n = 7, 30%) during the inflammatory and proliferative phases of the skin wound healing process. There was a predominance of monocytes, neutrophils, and macrophages in the initial phase of inflammation up to 10 days after excision. There was a predominance of macrophages and neutrophils (n = 2, 28.5% each) in the initial phase of inflammation from 3 to 7 days, (n = 3, 43% each) from 10 to 12 days and (n = 2, 28.5%

each) from 18 to 20 days after excision in the seven studies that evaluated cellularity (Table S3). We observed considerable evidence that macrophages are functionally polarised in response to pathogen-associated patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Predominantly pro-inflammatory Macrophages (M1-type phenotypes) have a prolonged polarisation that may lead to tissue damage and contribute to pathogenesis. However, with an anti-inflammatory action (M2-type phenotypes), they play a critical role in resolving inflammation by producing anti-inflammatory mediators (Figures 2 and 3).

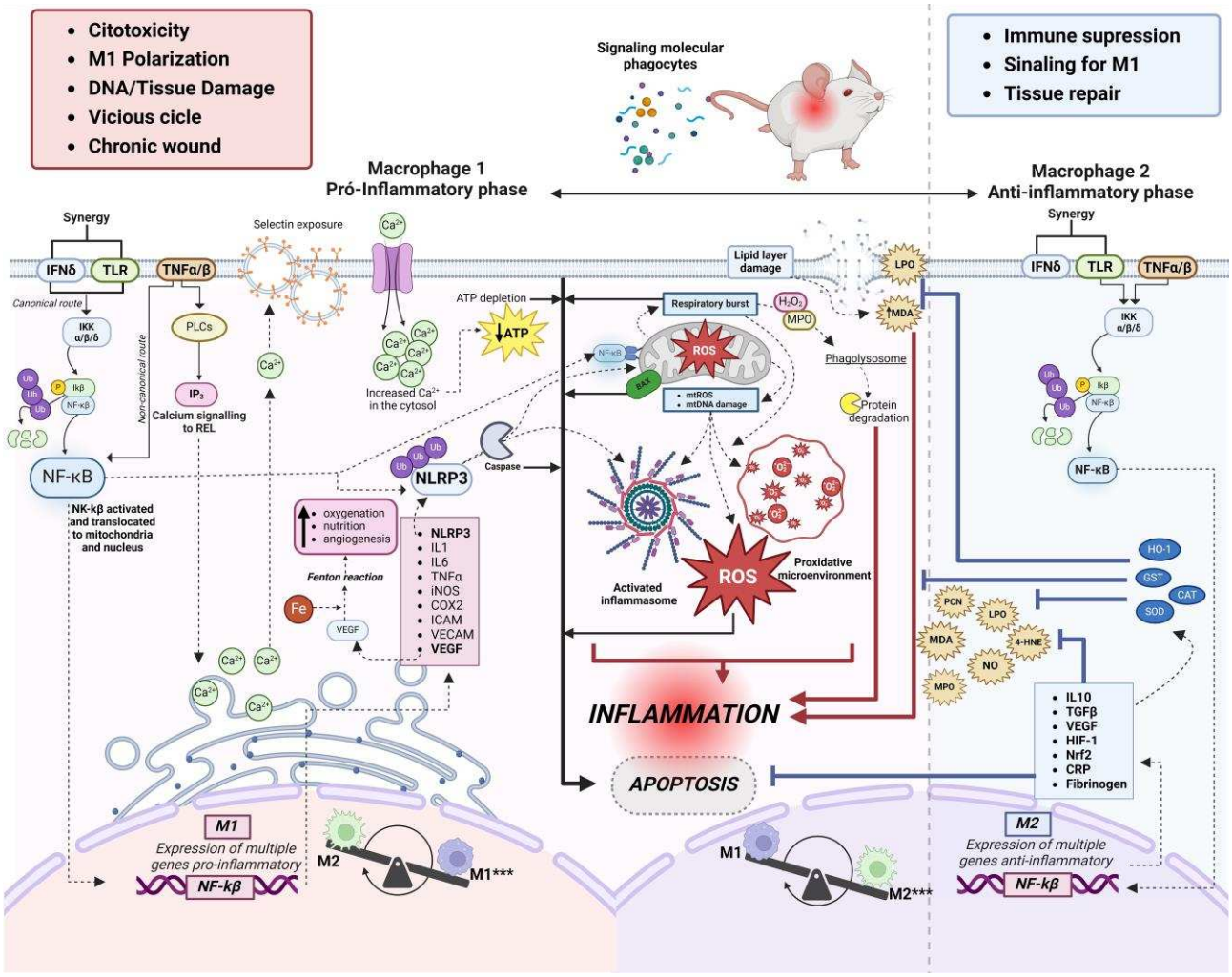

Figure 3. Overview of the interrelationship of major pathways and coexisting inflammatory mediators between oxidative stress and inflammatory process in excisional skin wound healing. ***Phenotypic plasticity of macrophages; presence of M1 (pro-inflammatory phase) and presence of M2 (anti-inflammatory phase). 4HNE: 4-hidroxinonenal; Ca^{2+}: ion calcium; CAT: catalase; COX-2: cyclooxygenase-2; Fe^{+}: ion iron; GST: glutathione transferase; H_2O_2: hydrogen peroxide; HIF-1: Hypoxia-inducible factor 1; ICAM: intercellular adhesion molecule; IFN-δ: Interferon-gamma receptor; IKK: inhibitor complex nuclear factor-κβ kinase; IKβ: IkappaB kinase; IL-1: interleukin 1; IL-6: interleukin-6; IL-10: interleukin 10; iNOS: Inducible nitric oxide synthase; IP3: IP3 signaling pathway; LPO: lipid peroxidation; MPO: metalloproteinase; NF-kβ: nuclear factor kappa β; NLRP3: inflammasome NLRP3; ON: nitric oxide; PCN: carboniled protein; PCR: C-reactive protein; ROS: reactive oxygen species; SOD: superoxide dis-

mutase; TGF-β: transforming growth factor beta; TLR: toll-like receptor; TNF-α: Tumor Necrosis Factor receptor alpha; VECAM: vascular adhesion molecule; VEGF: vascular endothelial growth factor. Figure created on BioRender.com.

3.4.4. Classical Macrophages (M1)

Individual studies in this review describe the activation of IFN-δ and Toll-like receptors, which act synergistically and simultaneously with the TNFα/β pathway, initiating a pro-inflammatory cascade within the cytosol upon exposure to DAMPS and PAMPs. NF-κB activation occurs mainly through the phosphorylation of inhibitory molecules mediated by IκB (kinase (IKK) (26%), including IκBα [22,30-34]. Once activated, NF-kβ can translocate to the nucleus and contribute to the transcription of pro-inflammatory cytokines. In addition, it can also directly interact with the mitochondria and collaborate with NLRP3 (as described below). Our results showed that, during the inflammatory phase, the transcription of pro-inflammatory molecules such as IL-1 (4.3%) [42] and IL-6 (13.4%) [44,49,50,60] is responsible for activating cell migration and upregulates cytokines release such as VEGF (13.4%) [45,50,60], which promote tissue oxygenation. In addition, free iron availability increases, creating a pro-oxidative microenvironment and indirectly inducing angiogenesis. Furthermore, the studies analyzed showed increased levels of the B-cell inhibitor alpha gene, nuclear factor kappa light polypeptide enhancer (IκBα), and TNF-α in the inflammatory phase in 7 studies (34%) [42,44,45,49,55,58,60].

In parallel, the high expression of NLRP3 leads to the proteolytic cleavage of caspase-1, which is responsible for IL-1b and IL-18 maturation produced in response to TLR/NF-kβ pathway activation. The NLRP3 component of the inflammasome, now activated, goes on to be translocated for interaction with mitochondria, but its main role is the activation of Caspase1. The release of mitochondrial factors into the cytosol (mitochondrial ROS and DNA), together with NF-kβ, increases the activation of inflammasomes, the generation of ROS, and the formation of a pro-oxidant microenvironment. Another mitochondrial event is the respiratory burst, which generates oxidation products (H_2O_2 and MPO) and acidification of the phagolysosome, which are rapidly marked in polymorphonuclear cells and completely degrade cytosolic proteins, culminating in an inflammatory process. Our results showed that in one study [49] (4.3%), by quantifying polymerase gamma (pol-γ) and the BAX protein associated with Bcl-2, it occurs through apoptosis stimulation and inhibition of collagen-1 expression during NLRP3 inflammasome activation. All these events have positive feedback, further increasing inflammation and apoptosis. In addition, the studies included in this review indicated that a TNF-α-stimulated pro-inflammatory environment activates IP3 (13.4%) [44,45,61] and phospholipases (PLCs) pathways, which signal the release of calcium from the smooth endoplasmic reticulum

(SER). IP3 triggers several stimuli, including calcium influx, mitochondrial ROS generation, and NLRP3 inflammasome activation. As a result, caspase-1, IL-1β, and IL-18 can be activated.

3.4.5. Alternative Macrophages (M2)

Compensatory plasticity for alternatively activated macrophages (M2) occurs in response to inflammatory events. The response is induced by the synergistic activation of IFN-δ and Toll-like receptors and concomitant TNFα/β, which act in the same way as classical macrophages (M1), but now with a genetic expression of anti-inflammatory cytokines such as transforming growth factor-β1 (TGF-β1) (14.4%) [45,49,51,57], and IL-10 in 3 studies (13%). In addition, C-reactive protein and fibrinogen down-regulation was observed, indicating the end of the acute phase of inflammation in studies [48,61-63] (17.4%).

In two studies, metalloproteinase increased (8.7%) in the initial phase of inflammation and decreased rapidly after 7–10 days. This result may have occurred because tissue matrix metalloproteinase (TIMP) inhibitors play a crucial role in maintaining the balance of active and inactive MMPs during the late phase of the wound-healing process. Oxidation and the formation of carbonylated proteins are often associated with inflammatory processes. Metalloproteinases may regulate inflammation, and carbonylated proteins may result from the oxidative stress associated with inflammation. Only two studies [51,57] (8.7%) used the oxidation of carbonylated proteins as a marker of oxidative stress, which showed high levels, indicating a relevant antioxidant role.

Dynamic balance is demonstrated with reduced levels of TNF-alpha, IL-1beta, IL-6, IL-8, HO-1, Nrf2, IκBα, and NF-κβ in 6 studies [42,49,54,55,58,59]. These results show the final part of the activation of survival pathways, such as the NF-kβ pathway, which induces the expression of genes and other pro-inflammatory molecules critical to initiating the inflammatory process. Physiologically, the NF-κβ pathway stimulates the anti-inflammatory defenses of cells and reduces the harmful effects of oxidative stress by preventing premature and excessive activation of the NLRP3 inflammasome in macrophages [65]. In addition, it promotes autophagy [66], negatively regulates the activation of the NLRP3 inflammasome [67-69], and mitophagy, when associated with the p62 protein, provides an essential regulatory cycle through which NF-κβ orchestrates a reparative inflammatory response and avoids excessive collateral damage [70]. These results were observed over an average of up to 22 days of wound healing (Table S3). Most of the studies included in this review described an increase in the antioxidant enzymes CAT (55.9%) [44,46-48,52,53,55-57,61-64] and GST (51.6%) [43,46-49,52,53,55,61-64] to quench H_2O_2 (8.6%) [43,59] and prevent

ROS accumulation in the injury site. As a result, anti-apoptotic signaling pathways are activated. This stimulates cells' survival and proliferation.

Inflammatory cells (neutrophils, macrophages, and mast cells) are described in Figure 2., whose primary function is to eliminate potential microorganisms [71] and to induce tissue repair [43,45,46,48,49,52,57,61], were assessed in 35% of the studies. With the regional presence of mononuclear cells [45,46,57,61] and the production and release of chemical mediators produced by them, fibroblast migration and activation are intensified. Fibroblasts are the main constituents of granulation tissue. Under the influence of growth factors, they are activated and migrate from the edges to the wound's center.

As a result, morphological changes occur in the scar tissue. The increase in re-epithelialization (43.5%) [42,44,45,48-50,52,56,59,61], granulation tissue rich in fibroblasts (17.4%) [44,45,58,61], blood vessels (39%) [42,44,46-48,52,56,58,61] and extracellular matrix components such as collagen (43.5%) [48-50,52,53,57,64-67], hydroxyproline (30.4%) [44-46,48,49,52,61], hexosamine and uronic acid (7.4% each) [45-47,52] were observed in association with increased antioxidant enzymes activity and progression of resolutive inflammatory phase.

At the end of the inflammatory process, there is a gradual decrease in pro-inflammatory and an increase in anti-inflammatory mechanisms. The reduced inflammation may be related to the reduction in NF-kβ activation and Ca^{2+} mobilization, which inactivates the NLRP3 inflammasome. The events that occur in the anti-inflammatory phase lead to inhibition of apoptosis, immunosuppression, M1 Signaling, inflammation resolution, and tissue repair. All relevant results, including histological data and oxidative stress markers, antioxidants, and cytokines, are described in Figure 3.

3.4.6. Clinical Perspectives

The treatment of skin wounds is influenced by various clinical factors, including the size, depth, and location of the injury. A detailed understanding of the mechanisms activated during wound healing is crucial in designing appropriate treatment strategies to minimize the risk of scarring and infection. Despite the availability of several treatment methods, such as the physical removal of debris and biofilm and the application of systemic and topical antimicrobials, substantial progress is still required. One promising area of research involves the controlled release of antimicrobial agents through tissue-engineered scaffolds. However, progress in this field is hampered by a limited understanding of the primary mechanisms activated during tissue recovery. These mechanisms are complex and involve a series of biological processes that need to be thoroughly understood to enhance treatment efficacy. The reconstruction of wounds using

auto- and allografts is a common practice for tissue replacement. However, these methods have significant limitations and often demonstrate low clinical value in cases of delayed skin wound healing. One reason for this fragility is that most research in this area has been restricted to in vitro tests, which do not replicate the complexities of living organisms. In this context, this review allows an understanding of the OxInflammation process during the wound healing process based on the studies that used in vivo models. It was possible to observe that many therapies are in preliminary stages and need to be understood more about the mechanisms before being translated into the human context. Therefore, OxInflammation is an important mechanism that should be controlled to solve clinical problems associated with skin wound healing.

3.5. Risk of Bias and Methodological Quality Assessments

The risk of bias analyzed from SYRCLE's tool is described in Figure 4 and 5. The results obtained from individual studies are reported in Figure 3. None of the trials met all the methodological criteria investigated. However, the number of studies with an overall high risk of bias was low (4.3%) [41]. Only two studies provided clear information on the generation of the random sequence [58,61], allocation concealment and blinding of participants were not reported in 100% of the studies, and blinding of results was only described in one study [42]. However, a low risk of bias was found when assessing whether the studies had incomplete data on outcomes (78.3%) [42-49,52,53,55,56,58-63], wound closure (91.3%) [42,44-59,61-63], intervention (95.6%) [42-53,55-59,61-64], selective reporting, ethical approval, validation tool and statistical methods (100% each) in studies, applicability (95.6%) [42-53,55-59,61-64] and other sources of bias (65.2%) [42,44,46,48-51,53,55,57-60,62,63]. In addition, the current evidence is reliable because the bias analysis showed a low risk of bias. Some methodological quality indicators showed an unclear and limited risk of bias due to underreporting and guideline adherence. A single study was classified as high risk of bias because it did not report wound closure and intervention, essential indicators in studies involved in wound healing.

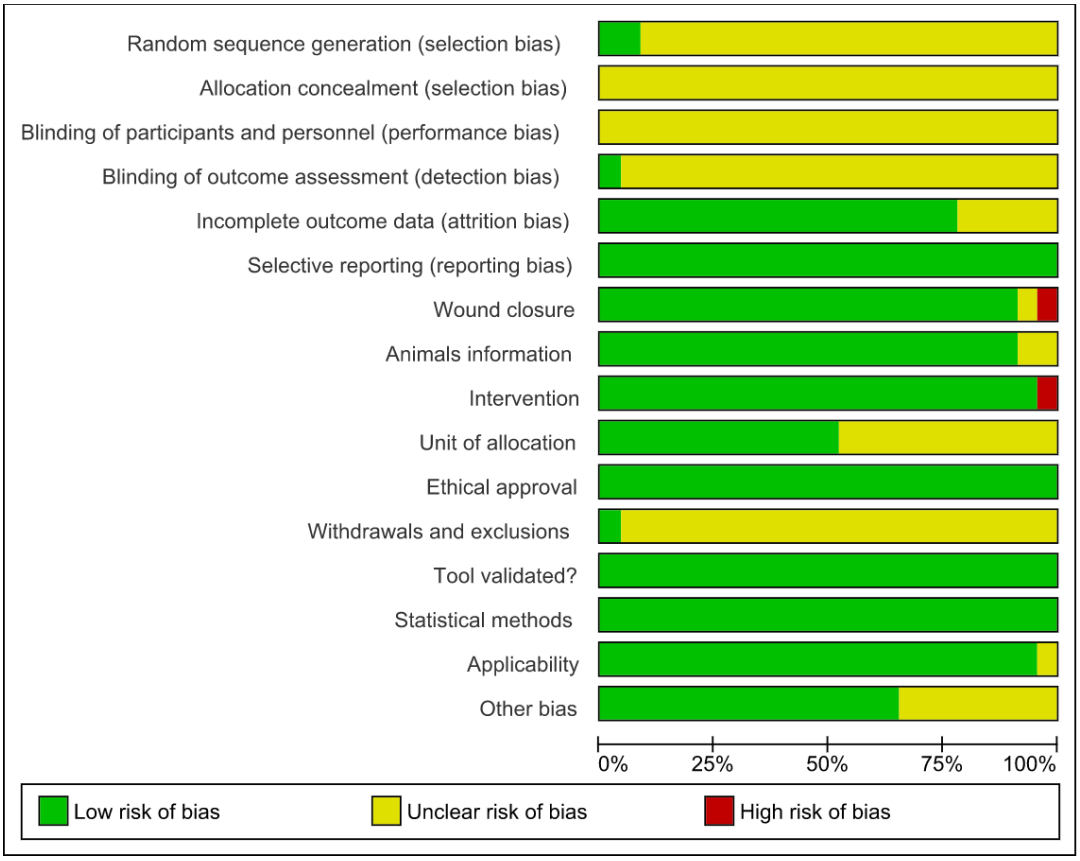

Figure 4. Risk of bias and methodological quality indicators for all studies included in the systematic review that assessed inflammation and oxidative stress during skin wound healing.

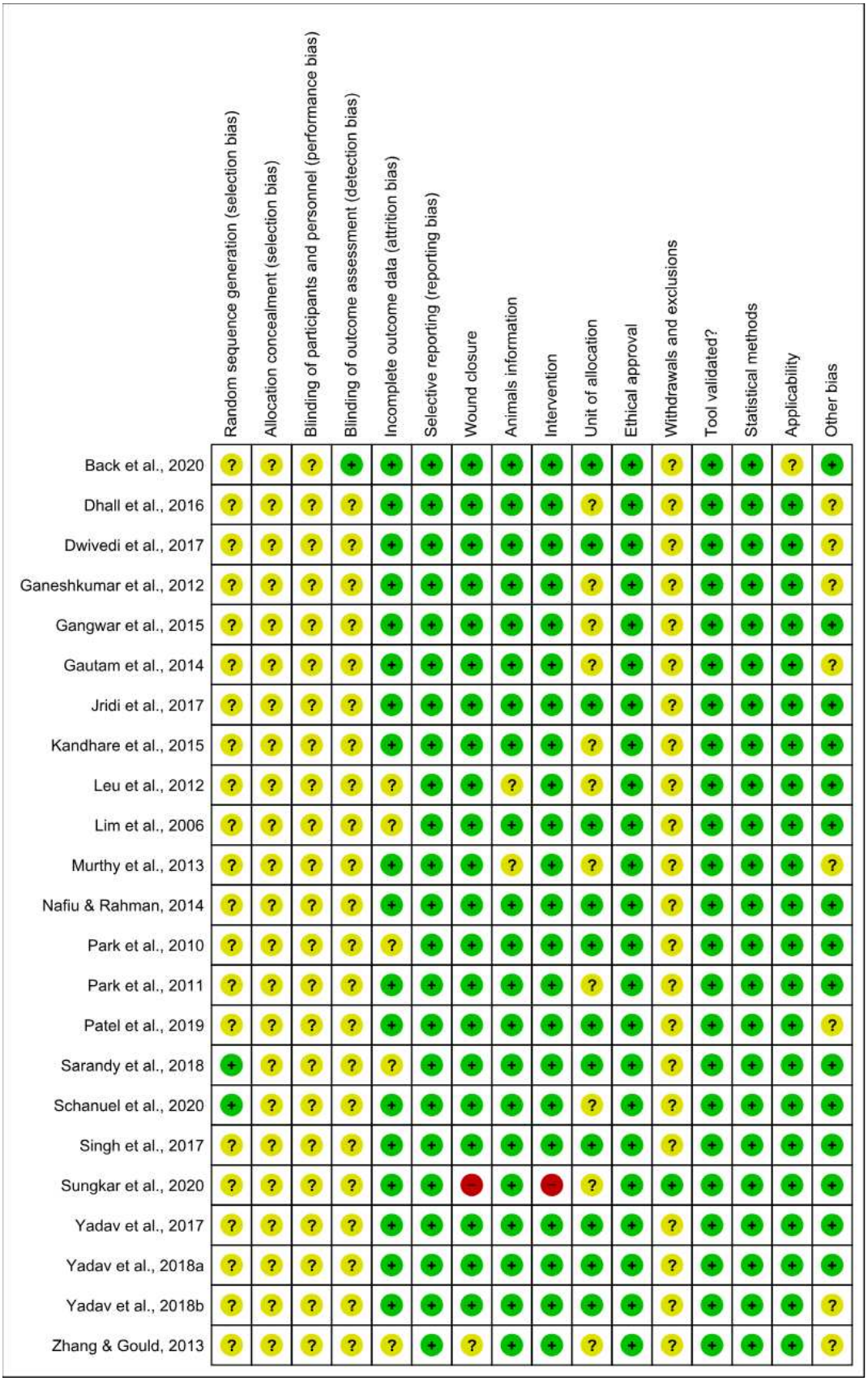

Figure 5. Risk of bias summary: review authors' judgments about the risk of bias items for each included study. Green: low risk of bias; Yellow: unclear risk of bias; and Red: high risk of bias.

References of the articles in the figure: References of the articles in the figure: Back et al., 2020 [42]; Dhall et al., 2016 [43]; Dwivedi et al., 2017 [44]; Ganeshkumar et al., 2012 [45]; Gangwar et al., 2015 [46]; Gautam et al., 2014 [47]; Jridi et al., 2017 [48]; Kandhare et al., 2015 [49]; Leu et al., 2012 [50]; Lim et al., 2006 [51]; Murthy et al., 2013 [52]; Nafiu &Rahman, 2014 [53]; Park et al., 2010 [54]; Park et al., 2011 [55]; Patel et al., 2019 [56]; Sarandy et al., 2018 [57]; Schanuel et al., 2020 [58]; Singh et al., 2017 [59]; Sungkar et al., 2020 [60]; Yadav et al., 2017 [61]; Yadav et al., 2018a [62]; Yadav et al., 2018b [63]; Zhang & Gould, 2013 [64].

4. Discussion

4.1. General Characteristics of the Studies

In this study, we conducted a systematic review to investigate the relationship between inflammation and oxidative stress in the skin wound healing process in a mouse model. Our results provided strong evidence that inflammation and oxidative stress are coexisting processes with a clear overlap in pathways and mechanisms. Furthermore, the morphological changes observed in the skin repair were mainly associated with antioxidant, vascular, chemotactic, and survival activation effectors such as NF-Kβ, IKβ, IP3, and IFN-δ. Mice and rats were the most commonly used animal models, possibly due to their greater availability, low cost, and ease of use. These characteristics can justify their primary choice in half of the studies distributed in different continents analyzed, with India being the country that presented the most studies. Indian traditional medicine is one of the oldest medical sciences in the world [72], being the largest producer of medicinal plants. In addition, about 70% of the rural population depends on the traditional system based on herbal medicine [73,74].

In addition, prolonged wound healing can result in more damaged cells, chronic inflammation, and high levels of ROS, which can compromise the effectiveness of the repair process and the overall health of the affected area [43,58]. It is, therefore, important to promote effective and rapid healing whenever possible to minimize these adverse effects [56].

4.2. Relationship between Inflammation and Oxidative Stress

It is already known that inflammation and oxidative stress coexist [75-79] in a mechanism defined as “OxInflammation” [13], which contributes to prolonged inflammation in diabetes, obesity, and burns [80-83]. However, the different pathways, mechanisms, and molecules that link these processes have not been studied together (Figure 3).

In particular, macrophages have a plasticity that allows them to respond to changes in their microenvironment. Accordingly, these cells are able to change their activation phenotype, leading to the broad classification of classical (M1) or alternative (M2) macrophage activation, which is present as infection progresses and cells are damaged [84]. Initially, the IP3 pathway is

stimulated by pro-inflammatory cytokines such as TNF-α and IL-1 [85,86], which act directly on the smooth endoplasmic reticulum, stimulating intracellular Ca^{2+} release and enabling the release of adhesion molecules (selectins) present in Weibel-Palade vesicles. These adhesion molecules facilitate diapedesis [85]. During this process, neutrophils are the first cell type to arrive at the injured site, followed by macrophages [84]. In addition to the IP3 pathway, pro-inflammatory cytokines (TNF-α or IL-1) activate the NF-Kβ and IKK (survival) pathways via membrane receptors and induce the expression of other pro-inflammatory genes (IL-1, IL-18, TNF-α, iNOS, COX-2) [50,86-88]. At inflammation sites, activated inflammatory cells release free radicals [77], as well as enzymes and chemical mediators, resulting in molecular damage (DNA, proteins, and lipids) and oxidative stress that must be eliminated. Toll-like receptors are activated by PAMPs to trigger a pro-inflammatory response, triggering transcription factors, pro-inflammatory gene expression, and ROS biosynthesis [78]. Next, one of the key events required for efficient tissue repair is apoptotic cell elimination by phagocytes in the injured tissue [59]. The abundant infiltrating neutrophils represent a large reservoir of short-lived inflammatory cells programmed to undergo apoptosis [56]. Another example related to the link between oxidative stress and apoptosis is represented by the release from the mitochondria of ROS mediators that are quickly recognized by immune cells and promote their migration [4,28]. ROS (pro-oxidative microenvironment, mtROS, ROS, and inflammasomes) produced as part of the inflammatory response facilitate the clearance of toxins and pathogens and induce antioxidant gene expression. However, prolonged cytokines production can stimulate oxidative stress and chronic inflammation-related diseases and eventually lead to piroptosis [28,75,76]. In this oxidative environment, the overexpression of TGF-β has been associated with an increase in the deposition of extracellular matrix and the activation of fibroblasts [89,90]. Another important cytokine in this phase of acute inflammation is IFN-δ. In synergy with tool-like receptors or membrane lipopolysaccharide (LPS), IFN-δ can induce NF-κB activation and M1 macrophages-related immunological effectors such as IL-6, TNF-α, IL-1β and nitric oxide (NO) [76]. The imbalance in cytokine production, such as IL-1, TNF-α, and IL-6, activates signaling pathways that increase ROS production, including superoxide and hydrogen peroxide [91,92], during inflammatory responses and activates the IFN-δ pathway, which will synergistically increase ROS production via mitochondrial ROS and ROS via NADPH oxidase [92,93]. The main function of this pro-inflammatory cytokine is to recruit leukocytes to an infection site and stimulate M1 macrophage polarization, which produces pro-inflammatory cytokines such as IL-1 and IL-12 [85]. Interestingly, IFN-δ increases NO

production by macrophages [45,88]. During the inflammatory process, NO is produced by inducible NO synthase (iNOS) in a calcium-independent process [94-96]. It is also known that a mitochondrial form of NOS (mtNOS) exists, although detailed information about it is not yet fully understood [79,96]. NO is produced at low concentrations, has a short lifetime, and can generate reactive nitrogen species (RNS) such as nitrous acid (HNO_2), nitrogen dioxide (NO_2), and peroxynitrite ($ONOO^-$), which results from NO reaction with superoxide ion (O_2), an important oxidative stress initiator. In addition, Ahmed and Ismail [97] and Wani et al. [98] reported that the membrane receptors for IFN-δ trigger pathways that act in the cytosol of the macrophage, signaling the mitogen-activated protein kinase (MAPK)/c-Jun amino-terminal kinase (JNK) and producing inflammatory and apoptotic mediators that amplify the production of ROS and the inflammatory response. Carbonylated proteins are formed as a result of free radical attack on amino acid side chains [61]. Free radicals have a dual function: they positively stimulate macrophages to generate excessive oxidative stress to eliminate pathogens, induce cell death through caspase activation, and create an imbalance in glutathione levels [99]. Antagonistically, oxidative stress is activated in the presence of inflammation. The balance between ROS production and antioxidant defenses is important for the resolution of inflammatory diseases, as well as for efficient tissue repair [37]. In response to tissue injury, the body initiates a chemical signaling cascade (PRRs, DAMPs, PAMPs) that stimulates responses aimed at healing the injured tissue [18]. Low ROS levels activate signaling pathways to initiate physiological processes, whereas high levels damage biomolecules [37]. Signaling centers in cellular physiology, such as mitochondria, play a role in inflammatory diseases. Studies suggest that ATP synthase inhibition also directs a tissue homeostatic response by activating the NF-Kβ pathway (a physiological regulator of mitochondrial respiration)—through specific NF-Kβ receptors on the mitochondrial surface [100]. This process leads to the concomitant production of mtROS [101-104], which regulates cellular and tissue processes during healing [105]. This product activates the inflammasome NLRP3, which triggers pro-inflammatory interleukins such as IL-1β and IL-18 [21,106,107], an essential step in the innate immune response. Furthermore, under hypoxic conditions, NO can also be produced during the respiratory chain reaction [96,98,108]. The latter reactive nitrogen species (RNS) can further lead to ROS production, such as reactive aldehydes, malondialdehyde, and 4-hydroxynonenal [86,96]. During injury, there is a reduction in oxygen and ATP and an increase in ROS through the superoxide radical (O^{2-}), hydrogen peroxide (H_2O_2), singlet oxygen [(O_2 (1 Δg)], ozone (O_3), nitric oxide (NO) and peroxynitrite anion ($ONOO^-$) produced by the

cell during the respiratory process (respiratory burst). Although they are physiologically produced by normal metabolic pathways, ROS are amplified during the inflammatory process, causing increased oxidative stress [29,109,110]. In addition, they induce the release of latent TGF-β, and chronic inflammation perpetuates its activation, creating a vicious cycle that leads to abnormal healing and the formation of hypertrophic scars [111-113]. The resulting cellular damage can be summarized in three ways: lipid peroxidation, protein degradation, and DNA damage [114]. The lipid membrane is highly susceptible to oxidative damage, setting off a chain reaction that not only impairs intramembrane transport but also generates toxic by-products. In addition, lipid peroxidation stimulates the production and action of pro-inflammatory mediators that increase ROS biosynthesis, leading to antioxidant depletion [115]. In this context, anti-inflammatory mediators have been observed to play an important role in redox signaling during skin wound healing, in addition to resolving the inflammatory process. Various endogenous and/or exogenous antioxidant defenses (CAT, SOD, GSH, and vitamin E) are present in tissues to minimize the toxicity of oxygen metabolites [78,79]. With redox balance, TGF-β inhibits the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, stimulates IL-10[91], allows the differentiation of fibroblasts into myofibroblasts to help cover wounds [114], inhibits the TGF-β/Smad pathway and consequently reduces fibrosis and keloids [111,112].

Enzymes that protect cells exposed to ROS, such as cyclooxygenases (COX), myeloperoxidases (MPO), uncoupled nitric oxide synthase (NOS), peroxidases and NADPH oxidase (NOX), enhance antioxidant activity by scavenging free radicals and inactivating their reactions [78,115]. This results in accelerated and successful healing with less time for wound closure. Oxidative stress resulting from oxidants and antioxidants imbalance disrupts redox signaling, causing molecular damage and an inability to neutralize and protect against reactive damage. This imbalance, when the ability to counter-regulate the pro-oxidant state is lost, is a central cause of oxidative stress. The successful control of inflammation and oxidative stress requires a joint effort in the pursuit of tissue repair. Thus, inflammation and oxidative stress have a necessary role in the biological healing response. The answer to this question is primarily related to inflammation. Chronically, the higher the production of pro-inflammatory or the lower the production of anti-inflammatory factors, the higher the continuous production of inflammatory products (silent inflammation) and the regulation of other important processes. Regulation can occur by modulating signaling pathways, influencing antioxidant enzyme synthesis, repair and healing processes, apoptosis, and cell proliferation in a continuous and complexly modulated cycle (Figure 6).

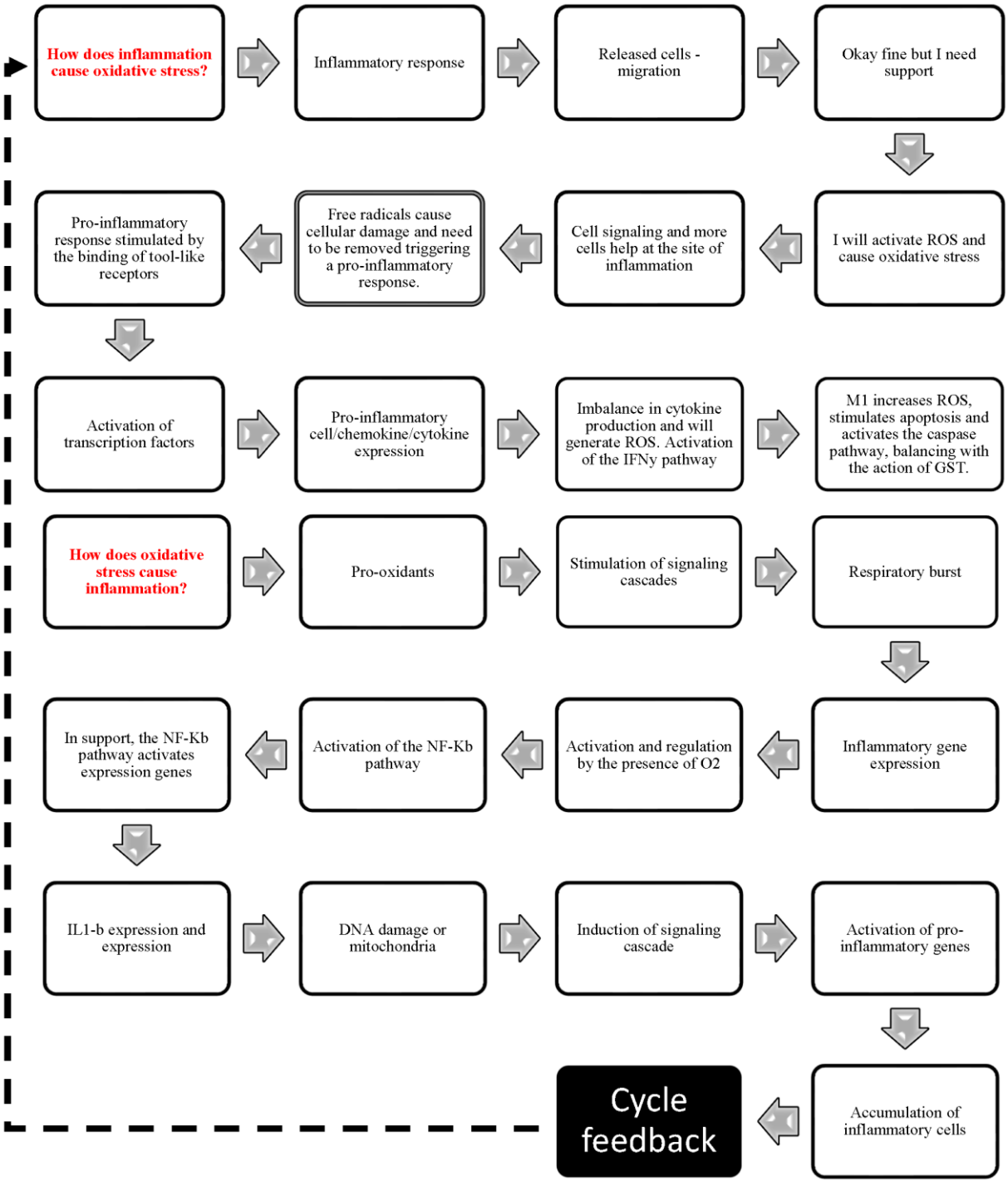

Figure 6. Continuous cycle and interrelating oxidative stress and inflammation, schematizing the major signaling pathways, synthesis of pro- and anti-inflammatory mediators, and antioxidant enzymes involved in the repair of excisional wounds.

4.3. Clinical Resolution of Inflammation and Skin Repair

Obesity, vascular disease, neurodegenerative disease, burns, and diabetes with ulcers [35,39,75,80-82,116-118] are the main factors contributing to chronic wounds, which account for a large proportion of healthcare costs. Complications related to inflammation contribute to delayed wound closure. One of the most important is the hypoxia response pathway [118], particularly in hyperglycemic patients, resulting in impaired neovascularisation and poor wound healing outcomes, as well as impaired cell migration and production of anti-inflammatory agents that control OxInflammation. This process can lead to secondary infections due to the colonization of microorganisms prone to biofilm formation [119,120]. Acceptable complications of delayed healing include increased risk of secondary infection, chronic pain, reduced quality of life, and potential progression to chronic ulcers. However, prompt treatment reduces chronic inflammation, promotes tissue regeneration, and prevents associated complications. This study focused on the mechanisms that influence inflammation, which can affect wound healing in different ways, regardless of wound size or delay in the onset of healing. In this context, we have identified the leading influencers in the control and resolution of inflammation and regulation of skin wound healing (Figure 7). Therefore, a hierarchical design is used to reduce the redox balance, the expression of pro-inflammatory cytokines, the activity of the inflammasome, the bacterial load and the stimulation of tissue regeneration. By identifying the therapeutic targets and levels of resolution involved in regulating the inflammatory response at a clinical level, we point to the action of antioxidants in controlling the inflammatory response over time and improving the quality of regenerated skin. Furthermore, the demonstration of a low risk of bias in the studies and the replication of the results in different experimental contexts have increased confidence in the efficacy of antioxidants as a therapy to accelerate wound healing.

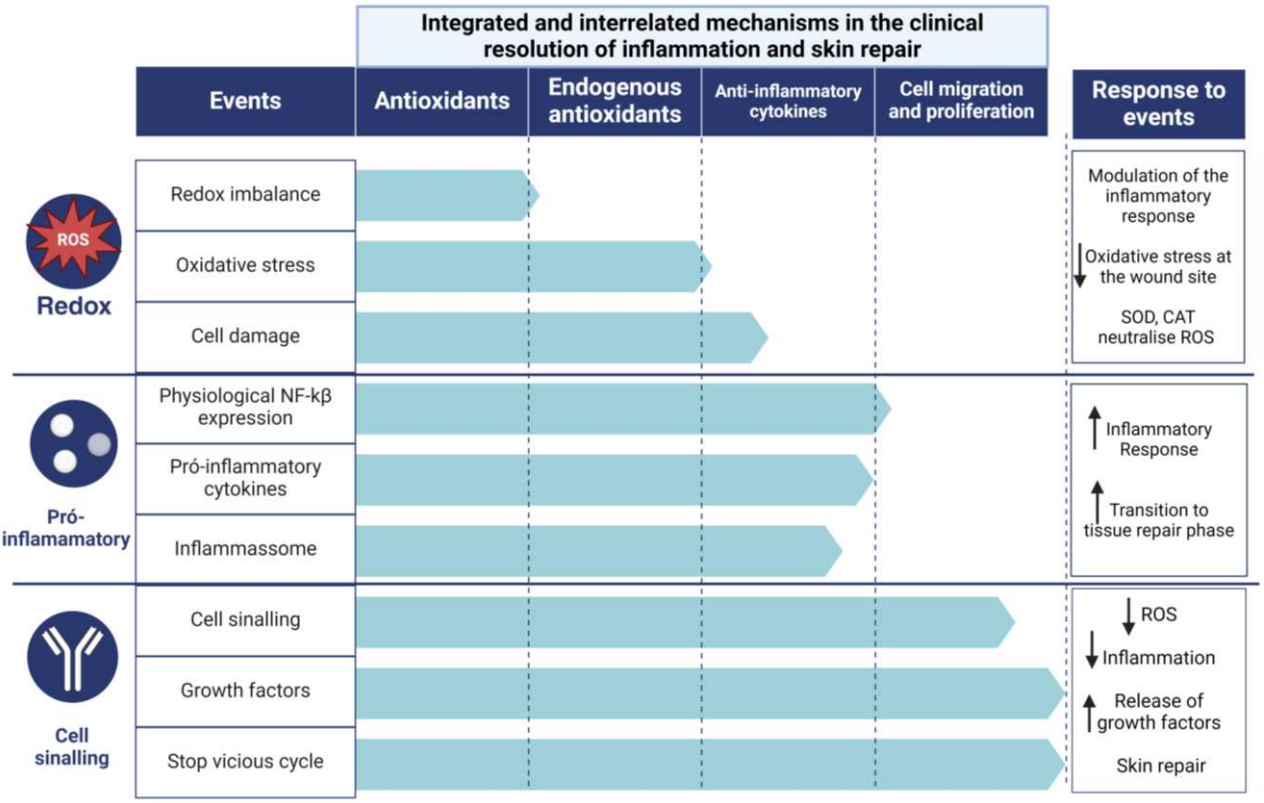

Figure 7. The clinical antioxidant and anti-inflammatory modulation of inflammatory mechanisms and the consequent reduction and control of oxidative inflammation.

4.4. Limitations

Systematic reviews are considered to be high-level studies, depending on the quality of the studies, and allow individual studies to be assessed in a blinded manner (without the influence of researchers), helping with decision-making, identifying gaps in knowledge, and allowing individual studies that can then guide future studies. In this case, the methodological heterogeneity between the studies analyzed was identified mainly from the divergent characteristics of the animal models, such as age, weight, and total number of animals. When assessing the risk of bias and methodological quality, we found that most of the studies did not clearly indicate the main outcomes assessed, and although only two studies (each) omitted information on wound closure and the intervention used, these studies presented a high risk of methodological bias, which prevented the reproducibility of the studies. Using the SYRCLE tool, we identified specific limitations in the research reports, mainly related to the omission of important information, such as the experimental randomization procedures, the procedures for assigning the animals to the experimental groups, the way the animals were housed, the data collection methods and the blinding of the researchers in relation to the experimental groups.

5. Conclusions

Our results confirm that oxidative stress and inflammation are coexisting and interrelated events. These events are involved in feedback mechanisms that ensure the reciprocal stimulation of cytokines and chemokines, membrane receptors, inflammatory signaling pathways, antioxidants, and other growth factors regulated during the inflammatory process in different diseases affecting the population, as these molecules act in different regulatory pathways and exert specific functions at each stage of the healing process, inflammatory resolution, and clinical condition. In this review, we found that IP3, IKK (IK-β and NFK-β), and IFN-δ are the most studied molecular pathways in the healing process of skin wounds. These pathways trigger the production of pro-inflammatory cytokines, such as TNF-α and IL-1, and stimulate signaling and M1 polarization through the activation of membrane receptors or cytosolic pathways. Physiologically, NF-κβ induces NLRP3, which stimulates the production of mtROS, triggering a respiratory burst that amplifies the pro-oxidative microenvironment and oxidative stress. In chronic wounds, this process becomes a vicious cycle of increased inflammation, delayed healing, and/or hypertrophic scarring that threatens resolution. Furthermore, few studies have assessed the total landscape of pro-inflammatory (IL-1, IL-6, IL-8, EGF, and ANG 1-2) and anti-inflammatory (IL-10, CRP, and fibrinogen) cytokines or chemokines, oxidative stress bioproducts (LPO, MDA, PCN, ON, 4-HNE, and DNA damage); which are important tools to understand their role in the complex dynamic equilibrium during wound healing. In general, inflammation is beneficial when acute and detrimental when prolonged. Taking this into account, therapeutic inhibition of oxidative and inflammatory events should progressively lead to inflammatory resolution and tissue regeneration by reducing the redox balance, the expression of pro-inflammatory cytokines, the activity of the inflammasome, and the bacterial load. Therefore, our results provide new insights into the relationship between oxidative stress and inflammation in the inflammatory process of wound healing.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/ 10.3390/antiox13070823/s1, Table S1. Complete search strategy with search filters and a number of studies recovered in the databases PubMed-Medline, Scopus, and Web of Science. Table S2. A description of the main features of the studies included in this systematic review that evaluated oxidative stress and inflammation in excision wound healing is included. Table S3. Description of the main features related to excisional wounds and days of wound healing monitoring.

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: The data can be made available upon request through the email: fernanda.b.lopes@ufv.br.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

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Supplementary Materials:

Supplementary Materials: The following are available online at https://www.mdpi.com/article/ 10.3390/antiox13070823/s1, Table S1. Complete search strategy with search filters and a number of studies recovered in the databases PubMed-Medline, Scopus, and Web of Science. Table S2. A description of the main features of the studies included in this systematic review that evaluated oxidative stress and inflammation in excision wound healing is included. Table S3. Description of the main features related to excisional wounds and days of wound healing monitoring.

Table S1: Filters

Data base	Descriptors	Items Found	Time	Date
PUBMED	#1 Filter wound healing **("wound healing"[MeSH Terms] OR "wound healing"[Title/Abstract])**	169,760	14:42:26	13/04/2021
	#2 Filter antioxidants **("antioxidants"[MeSH Terms] OR "antioxidants"[Title/Abstract] OR "oxidative stresses"[Title/Abstract] OR "antioxidative stress"[Title/Abstract] OR "oxidative stress injury"[Title/Abstract])**	169,099	14:42:42	13/04/2021
	# 3 Filter inflamation **("inflammation"[MeSH Terms] OR "inflammation mediators"[MeSH Terms])**	719,904	14:57:04	13/04/2021
	Total: #1 and #2 and #3 ("wound healing"[MeSH Terms] OR "wound healing"[Title/Abstract]) AND ("antioxidants"[MeSH Terms] OR "antioxidants"[Title/Abstract] OR "oxidative stresses"[Title/Abstract] OR "antioxidative stress"[Title/Abstract] OR "oxidative stress injury"[Title/Abstract]) AND ("inflammation"[MeSH Terms] OR "inflammation mediators"[MeSH Terms])	121	15:05:27	13/04/2021
Data base	**Descriptors**	**Items Found**	**Time**	**Date**
SCOPUS	#1 Filter wound healing (TITLE-ABS-KEY ("wound healing"))	182,548	15:10:02	13/04/2021
	#2 Filter antioxidants (TITLE-ABS-KEY ("antioxidants")) OR (TITLE-ABS-KEY ("oxidative stresses")) OR (TITLE-ABS-KEY ("antioxidative stress")) OR (TITLE-ABS-KEY ("oxidative stress injury"))	638,662	15:11:04	13/04/2021
	#3 Filter inflammation (TITLE-ABS-KEY ("inflammation")) OR (TITLE-ABS-KEY (“inflammation mediators"))	809,911	15:13:03	13/04/2021
	Total: #1 and #2 and #3 ((TITLE-ABS-KEY (“wound healing"))) AND ((TITLE-ABS-KEY ("antioxidants")) OR (TITLE-ABS-KEY ("oxidative stresses")) OR (TITLE-ABS-KEY ("antioxidative stress")) OR (TITLE-ABS-KEY ("oxidative stress injury"))) AND ((TITLE-ABS-KEY ("inflammation")) OR (TITLE-ABS-KEY ("inflammation mediators"))) AND (LIMIT-TO (EXACTKEYWORD, "Animal Model"))	383	15:16:07	13/04/2021
Data base	**Descriptors**	**Items Found**	**Time**	**Date**
WEB of SCI	#1 Filter wound healing (TS=wound healing)	89,305	15:20:08	13/04/2021
	#2 Filter antioxidants (TS=antioxidants OR TS=oxidative stresses OR TS=antioxidative stress OR TS=oxidative stress injury)	649,047	15:22:39	13/04/2021

E N C E	#3 Filter inflamation (TS=inflammation)	623,193	15:24:47	13/04/2021
	#4 Filter animals TS=Mice OR TS=Mouse OR TS=Rat OR TS=Rats OR TS=Dog OR TS=Dogs OR TS=Rabbits OR TS=Murine model OR TS=Guinea pig OR TS=Hamster OR TS=Animal model	4.113,737	15:26:43	13/04/2021
	Total: #1 and #2 and #3 and #4 (TS=wound healing) AND (TS=antioxidants OR TS=oxidative stresses OR TS=antioxidative stress OR TS=oxidative stress injury) and (TS=inflammation) AND (TS=Mice OR TS=Mouse OR TS=Rat OR TS=Rats OR TS=Dog OR TS=Dogs OR TS=Rabbits OR TS=Murine model OR TS=Guinea pig OR TS=Hamster OR TS=Animal model)	541	15:28:35	13/04/2021

Table S2: Animal model – Rat and mice

Reference	*Country*	*Strain*	*Sex*	*Age*	*Weight*	*Control group*	*Ethics Commit-tee*	*Statistical test*
Back et al., 2020	Brazil	Wistar rats	M	2 mo	?	Negative: untreated Positive: Dersani®	Yes	Anova
Dhall et al., 2016	United States of America	C57BL/6 mice	?	24 weeks	?	Negative: untreated	Yes	Anova; Student's t test.
Dwivedi et al., 2017	India	Wistar rats	?	?	160 - 180 g	Negative: DMSO. Positive: Vitamin E	Yes	Anova; hoc Scheffe's test.
Ganeshkumar et al., 2012	India	Wistar rats	M	?	180 - 200 g	Negative: PBS	Yes	Anova
Gangwar et al., 2015	India	Charles Foster albino rats	?	?	150 - 200 g	Negative CMC Positive: Vitamin E	Yes	Anova; Dunnett's test
Gautam et al., 2014	India	Charles-Foster albino rats	M/F	?	180 - 230 g	Negative: CMC Positive: Vitamin E	Yes	Anova; Dunnett's test
Jridi et al., 2017	Tunisia	Wistar rats	?	Youngs	150 - 200 g	Negative: untreated Positive: Cicaflora©	Yes	Anova; Dunnett's test
Kandhare et al., 2015	India	Wistar rats	M	Adults	180-200g	Negative: Did not receive injury; No receive any drug treatment. Positive: framycine sulfate ointment	Yes	Anova; Bonferroni's test
Leu et al., 2012	Taiwan	BALB c/mice	?	8 weeks	?	Negative: untreated	Yes	Student's t-test and Tukey-Kramer
Lim et al., 2006	United States of America	Hairless SKH-1 mice	F	8 weeks 18 months	?	Negative: Exposed to ambient filtered room air	Yes	Anova
Murthy et al., 2013	India	Charles-Foster albino rats	M/F	?	160-180g	Negative: CMC Positive: Vitamin E	Yes	Anova; Dunnett's test

Nafiu & Rahman, 2014	Malaysia	Sprague–Dawley rats	F	Adults	?	Negative: Deionized water Positive: Solcoseryl®	Yes	One way-Anova.
Park et al., 2010	Korea	CrljBgi: CD-1 (ICR) mice	F	4 weeks	?	Negative: Diet no CLA isomers	Yes	Anova; Dunnett's test
Park et al., 2011	Korea	ICR mice	F	4 weeks	?	Negative: untreated	Yes	Duncan's - SPSS Statistics
Patel et al., 2019	Japan	Sprague–Dawley rats	M	9 weeks	290-310g	Negative: blank hydrogels (without drugs)	Yes	Anova
Sarandy et al., 2018	Brazil	Wistar rats (Rattus norvegicus)	M	5 weeks	198,25 ± 26,11g	Negative: saline Positive: silver sulfadiazine	Yes	D'Agostino-Pearson test; Kruskal-Wallis
Schanuel et al., 2020	Brazil	C57bl/6 mice	M	12 weeks	?	Negative: standard (10% energy from fat) chow	Yes	Kolmogorov–Smirnov normality test and The parametric Welch-corrected unpaired t-test and A nonparametric Mann– Whitney test.
Singh et al., 2017	Korea	ICR mice	M	8 weeks	30g	Negative: untreated Positive: Vanillyl alcohol	Yes	Anova
Sungkar et al., 2020	Indonesia	Whistar rats	M	3-4 mo	150-300g	Negative: ?	Yes	Normality test (Shapiro-Wilk test) and One Way Anova.
Yadav et al., 2017	India	Albino Wistar rats	M	Adults	180-200g	Negative: untreated Positive: povidone iodine ointment	Yes	Anova; Dunnett's test
Yadav et al., 2018a	India	Albino Wistar rats	M	Adults	180 ± 20 g	Negative: untreated Positive: povidone iodine ointment	Yes	Anova; Dunnett's test
Yadav et al., 2018b	India	Albino Wistar rats	M	Adults	180-200g	Negative: untreated Positive: povidone iodine ointment	Yes	Anova; Dunnett's test
Zhang & Gould, 2013	United States of America	Sprague–Dawley rats	M	8 weeks	?	Negative: normoxia at sea level	Yes	nQuery Advisor 7.0; Tukey's Test

Table S3: Cutaneous wounds

Reference	*Lesion*	*Site*	*Antisepsis*	*Anesthesia*	*Analgesia*	*Inst. For Biopsy*	*Wound Nº/size*	*WH (days)*
Back et al., 2020	Excision	Dorsum	?	Xylazine (90 mg/kg) and ketamine (13 mg/kg)	cetoprofen (0.5 mg/kg)	Biopsy punch	2/50mm2	0, 2, 7, and 12
Dhall et al., 2016	Excision	Dorsum	?	?	?	Biopsy punch	?/10 mm2	0, 1, 3, 5, 7, 9, 12, 14
Dwivedi et al., 2017	Excision/Incision	Dorsum	?	?	?	?	?/500 mm2	1, 4, 7, 10, 13, 16, 19,21
Ganeshkumar et al., 2012	Excision/Incision	Dorsum	?	?	?	?	1/20 mm2	0, 4, 8, 12
Gangwar et al., 2015	Excision/Incision	Dorsum	?	Pentobarbitone (35 mg/kg); Ether	?	?	?/~500 mm2	0, 4, 8, 12, 14, 16, 18, 20, 22
Gautam et al., 2014	Excision/Incision	Dorsum	?	ketamine hydrochloride (50mg/kg)	?	?	1/≈500 mm2	0, 4, 8, 12, 14, 16, 18, 20, 22, 24
Jridi et al., 2017	Excision	Thoracic region	?	ketamine (100 mg/kg body weight)	?	?	1/~150 mm2	0, 2, 4, 6, 8, 10, 12
Kandhare et al., 2015	Excision	Dorsum	?	80 mg/kg dose of ketamine	?	?	1/300mm2	0, 4, 8, 12, 16, 20
Leu et al., 2012	Excision	Dorsum	Betadine	2 to 2.5% vaporized inhaled isoflurane	?	Scissors and a scalpel	2/ 10 mm2	0, 1, 3, 5, 7
Lim et al., 2006	Excision	Dorsum	?	Isoflurane	?	Biopsy punch	2/ 3.5mm	0, 1, 2, 3, 4, 5, 6, 7, 8, 9
Murthy et al., 2013	Excision/Incision	Dorsum	?	ketamine (30mg/kg, ip)	?	?	1/ ≈500mm2	0, 4, 8, 12, 14, 16, 18, 20, 22
Nafiu & Rahman, 2014	Excision	Dorsum	?	Ethyl ether	?	Biopsy punch	?/6mm	0, 1, 3, 5, 7, 9, 11
Park et al., 2010	Excision	Dorsum	Alcohol	Isoflurane	?	Biopsy punch	2/4mm	0, 1, 2, 3
Park et al., 2011	Excision	Dorsum	?	Isoflurane	?	Biopsy punch	2/4mm	0, 1, 2, 3
Patel et al., 2019	Excision	Dorsum	Saline and ethanol	Isoflurane	?	?	1/20 x 20mm	0, 4, 8
Sarandy et al., 2018	Excision	Dorsum	?	ketamine (60 mg/kg) and xylazine (10 mg/kg)	Pentobarbital (30mg)	Biopsy punch	3/ 12 mm	0, 7, 14, 21
Schanuel et al., 2020	Excision	Dorsum	?	ketamine (150 mg/kg) and xylazine (15 mg/kg)	?	?	1/10 mm2	0, 6, 10
Singh et al., 2017	Excision	Dorsum	?	?	?	?	4/ 8mm2	3, 5, 7
Sungkar et al., 2020	Excision	Dorsum	Alcohol	?	?	?	1/ 20 mm2	?
Yadav et al., 2017	Excision/Incision	Dorsum	Saline	Ethyl ether	?	Scissors and a scalpel	1/ 500 mm2	3, 6, 9, 12, 15, 18
Yadav et al., 2018a	Excision/Incision	Dorsum	Saline	Ethyl ether	?	Scissors and a scalpel	1/ 500 mm2	0, 3, 6, 9, 12, 15, 17
Yadav et al., 2018b	Excision/Incision	Dorsum	Saline	Ethyl ether	?	Scissors and a scalpel	1/ 500 mm2	0, 3, 6, 9, 12, 16
Zhang & Gould, 2013	Excision	?	?	?	?	?	?	?

CHAPTER 03

Commiphora leptophloeos leaves and bark control the OxInflammation by the modulation of Toll-like 4/ NFK-β/ NFR2 pathways

LOPES, Fernanda Barbosa, D.Sc., Federal University of Viçosa, June 2024. ***Commiphora leptophloeos*** **leaves and stem bark extracts control the OxInflammation by the modulation of Toll-like 4/ NFK-β/ NFR2 pathways.** Advisor: Reggiani Vilela Gonçalves. Co-advisor: Mariáurea Matia Sarandy de Souza; Manoela Maciel dos Santos Dias.

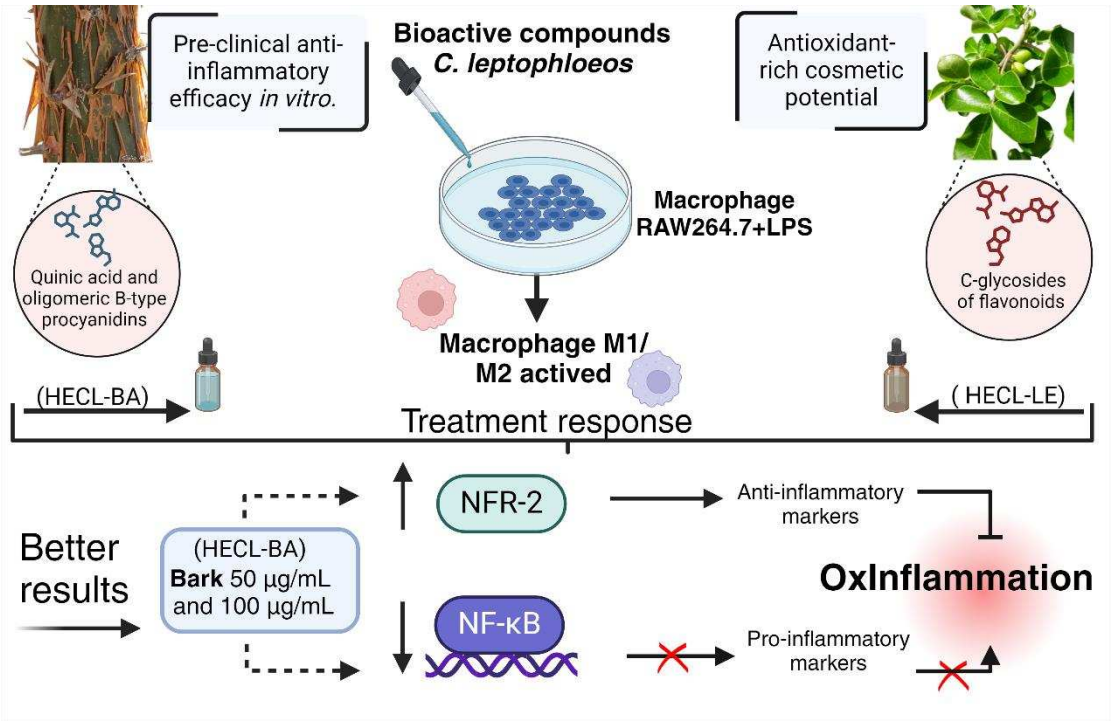
ABSTRACT

Commiphora leptophloeos (Mart.) J.B. Gillet (Burseraceae) is a plant of Brazilian biodiversity, widely known as 'imburana'. Its stem bark and leaves are rich in bioactive phenolic compounds with anti-inflammatory and antioxidant properties. In this study, we investigated whether the hydroethanolic extract of *C. leptophloeos* (HECL) can control oxidative stress and the inflammatory process. (OxInflammation) after in vitro exposure to lipopolysaccharide (LPS) and hydrogen peroxide (H_2O_2) in RAW264.7 (macrophages), and its potential mechanism involved. The chromatographic profile of *C. leptophloeos* leaf extract (HECL-LE) showed the presence of six flavonoids (orientin, isoorientin, vitexin, isovitexin, isoquercitrin and quercetrin. In addition, the stem bark extract (HECL-BA) showed the presence of quinic acid tannins and type B oligomeric procyanidins. The antioxidant potential was confirmed by more than 80% inhibition of the 1,1-difenil-2-picrilhidrazil radical (DPPH) in a dose-dependent manner in stem bark and for all leaf concentrations. Our stem bark extracts and leaf extracts are non-hemolytic and was able to protect against protein denaturation with up to 20% protection ($p<0.05$) in certain concentrations (50, 100 and 200 µg/ml for stem bark, and 200 µg/ml for leaf) compared to the positive control f. There was an increase in cell viability and proliferation using the bark (50 and 100µg/ml) and leaf (350µg/ml). Nitric oxide production was reduced by the bark (100, 200, and 300 µg/ml) and leaf (500, 750, and 1000 µg/ml) extracts after 3h LPS exposure. Only the 350 µg/mL leaf extract increased catalase activity. In addition, 50 µg/mL bark extract increased cell migration capacity (21%) compared to untreated cells. There was a reduction in the Toll-like 4 expression and reduction in NF-κβ expression after extracts exposure. Therefore, the pro-inflammatory cytokines such as BAX, IL-6, TNF-α, and COX2 were down-regulated. In addition, there was an increase in the NRF-2 expression controlling the oxidative stress, and contributing to the resolution of acute inflammation. Therefore, we observed OxInflammation was controlled TLR4/ NF-κβ down-regulation and NRF-2 upregulation, showing a dose-response profile. The next step was to understand if the oxidative stress control by NRF-2 was related to heme-oxygenase-1 (HO-1) activation by the heme group degradation or by the hypoxia-inducible factors (HIFs). Our results showed that there was a decrease in the HO-1, and HIF-1 expression, possibly due to cross-regulation between NF-κβ, HO-1 and HIF-1. We observed that the *C. leptophloeos* extracts showed therapeutic potential

in vitro in reducing OxInflammation and restoring cellular homeostasis, probably due to the action of condensed tannins, demonstrating their efficacy *in vitro*. Due to its high concentration of flavonoid C-glycosides, the leaf extract has been shown to be a potent antioxidant with cosmetic potential protecting the skin against premature ageing and damage caused by free radicals, UV rays and pollution, highlighting the need for further studies to better understand the mechanisms of action.

KEYWORDS: Plants extracts; "Imburuna"; “Umburuna”; Antioxidant; Inflammation; Oxidative stress; Oxinflammation.

Graphical abstract



1. INTRODUCTION

The Brazilian plant *Commiphora leptophloeos* stands out as a promising candidate. This species, known as "imburana" or "umburana", is native to the caatinga, an exclusively Brazilian biome. The literature describes the use of various parts of the plant (roots, latex, flowers, fruits and seeds) for colds, coughs, bronchitis, colic, diarrhea, pain and inflammation, while the stem bark is used topically for wounds, inflammation and gynecological infections [1–9]. However, in

folk medicine, the leaves and stem bark are the most commonly used parts, and their preparation varies between teas, syrups, decoctions, and macerations, with oral and topical administration [4,5,7,10]. Empirically, it is used in treating respiratory diseases, edema, diabetic lesions, poisoning, gastritis, and ulcers, as an antiemetic and tonic, among many others therapeutic applications [5,7,9–11]. It is known for its preclinical topical anti-inflammatory medicinal properties, including the treatment of inflammatory diseases in *in vivo* models of carrageenan-induced paw edema and Zymosan-induced air pouch [12,13], beyond its antioxidant [12–14], and antifungal properties [15–17].

The expansion of botanical studies has been exploited to treat diseases associated with inflammation and oxidative stress, owing to the antioxidant and anti-inflammatory properties of plant extracts[18–20]. Notably, OxInflammation refers to a continuous and positive interaction between oxidative stress and inflammation, that can lead to long-term tissue damage [21]. Furthermore, it describes a pro-oxidative state that creates a feedback loop, where oxidative stress results from an imbalance between reactive oxygen species (ROS) production and the body's ability to counteract them [18,22]. Chronic oxidative stress can trigger inflammation, a tissue response involving cell activation and mediator secretion [21]. The relationship between oxidative stress and inflammation is bidirectional, with ROS promoting inflammation and inflammation, in turn, increasing ROS production, thus perpetuating both processes [23–26] .

Nuclear factor-κβ (NF-κβ) plays a central role in regulating inflammation-related pathways by controlling the expression of genes involved in immune responses and inflammation [27]. Activation of the NF-κβ survival pathway leads to the expression of pro-inflammatory cytokines (e.g., IL-6), cyclooxygenase-2 (COX-2), and the pro-apoptotic protein BAX, while simultaneously promoting anti-inflammatory and cytoprotective factors such as NRF-2, HIF-1, and HO-1. IL-6 contributes to immune regulation and the acute phase response, while COX-2 enhances prostaglandin production, worsening inflammation and pain [28]. NF-κβ also regulates IL-10, which suppresses excessive immune responses [27,29]. Additionally, NF-κβ signaling interacts with NRF-2, HIF-1, and HO-1 to modulate antioxidant defenses and cellular adaptation to hypoxia and inflammation [30–33].

Antioxidant activity and the ability to scavenge free radicals are crucial for reducing the oxidative stress, which contributes to inflammation [34]. Protecting cells from hydrogen peroxide-induced oxidative stress is essential for assessing cell viability in inflammatory conditions, which impacts tissue regeneration [35–37]. Nitric oxide (NO) production plays a key role in

inflammation, and studying modulation by antioxidant helps understand their effects [38,39]. Protecting against protein denaturation and hemolysis is important for maintaining cell and tissue integrity during OxInflammation [40,41]. Additionally, analyzing genes expression related to inflammation and antioxidant response provide insights into how plant extracts modulate molecular pathways involved in these processes [42–44].

Although the therapeutic potential of different parts of *Commiphora leptophloeos* is increasingly recognized, there remains a gap in understanding the key pathways and cellular mechanisms activated after its exposure. By exploring the specific properties of the leaves and stem bark of *C. leptophloeos* and identifying the in vitro cellular mechanisms of inflammation and oxidative stress *in vitro*, we offer a new, innovative, and promising perspective for developing effective and economically viable treatments. Therefore, this study aims to investigate the potential of the hydroethanolic extract of *C. leptophloeos* (HECL) to modulate OxInflammation after in vitro exposure to lipopolysaccharide (LPS) and hydrogen peroxide (H_2O_2) in RAW264.7 macrophages, and the underlying mechanisms involved.

2. MATERIALS AND METHODS

2.1 Plant Material

Commiphora leptophloeos (Mart.) J.B.Gillett stem bark and leaf were collected in the community of Carao, Altinho, Pernambuco, Brazil, coordinates 8° 29′32″S and 36°03′ 03″W, in July 2019. The vegetal material was identified in the Geraldo Mariz Herbarium of the Federal University of Pernambuco (UFPE) (number 46.191). Permission to collect the material for this study (process number 35017) was issued by the Brazilian Biodiversity Authorization and Information System (SISBIO). The National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) authorized scientific research (process number A618873). The accepted name plant was checked at http://www.theplantlist.org (accessed on August 27, 2024), possessing as synonyms *Burcera leptophloeos Mart., Bursera martiana Engl. and Bursera orinocensis Engl.*

2.2 Preparation of Hydroethanolic Extracts

The sample extraction procedure was applied for stem bark and leaves. Raw material was dried in a circulating air oven (temperature below 45°C), and then ground in a knife mill. An amount of 200 g of each powder material was extracted with ethanol: water (70:30, v/v) by maceration for 48 h in a plant/solvent proportion of 1:10 (w/v) (extract 1). The extracts were filtered through

Whatman™ paper nº1 and the same vegetal material was extracted (remaceration) under the same conditions previously described (extract 2). Extracts 1 and 2 were combined and concentrated under reduced pressure using a rotary evaporator (temperature below 35º C) (Buchi-Model V-700, Altendorfer Str. 3, Essen, Germany) to remove the organic solvent. After that, they were frozen and lyophilized at 200 mT for 72h at – 20ºC (Model 101, LiotopR, São Carlos, São Paulo, Brazil). The yield of the crude leaf extract and bark stem extract were 13.0 and 25.6 g, respectively.

2.3 Phytochemical Characterization

The chromatographic profile of leaf and stem bark extract was performed through UPLC-ESI-IT-MS and HPLC-PDA/ELSD analyses. Details about the methodology were previously described by Dantas-Medeiros et al. (2021)[13] and Dantas-Medeiros et al. (2021)[17].

2.4 Preparation of the samples

The concentrations of the stem bark (25, 50, 100, and 200µg/ml) and leaf extract (100, 200, 350, 500, 750, and 1000µg/ml) extracts from *Commiphora leptophloeos*, based on the description of previous studies, were prepared to carry out the in vitro tests. The extract was abbreviated as HECL (Hydroethanolic Extract of *Commiphora leptophloeos*) for stem bark (HECL-BA) and for leaves (HECL-LE) throughout the paper.

2.5 DPPH Assays

Antioxidant activity was evaluated by the reduction of the 1,1-difenil-2-picrilhidrazil radical (DPPH), which forms diphenylpicrylhydrazine, a yellow-colored compound, in a reaction that usually stabilizes after 30 minutes from initiation [45]. HECL-BA (25, 50, 100, and 200µg/mL) and HECL-LE (100, 200, 350, 500, 750, and 1000µg/ml) concentrations diluted in methanol were tested. Ascorbic acid at 12.5 µg/mL methanol was used as a standard of comparison since it can inhibit 75% of DPPH radical. For each 50 µL extract concentration and the 50 µL of ascorbic acid concentration, 250 µL of DPPH (0,1mM/L) was added. After 30 minutes of reaction in the dark, the samples were analyzed in a microplate reader at 517 nm. The ability of the extracts to reduce the DPPH radical was calculated as follows:

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

2.6 Denaturation Assay

A solution of bovine serum albumin (BSA) was prepared with a final concentration of 2% (w/v) in a Tris hydroxymethyl-aminomethane buffer (Tris-HCl, 0.1 M, pH = 7.4). In microtubes, 300µL of this solution was combined with 200µL of various concentrations of HECL-BA (50, 100 and 200µg/mL) and HECL-LE (200, 350 and 500µg/mL) and incubated at 70°C, (for 30 min) in a water bath. An amount of 200 µL Tris-HCl buffer and 300 µL of BSA without heating were used as a negative control, and the same conditions with heating were used as a positive control. Once at room temperature, each reaction, performed in triplicate, had the absorbance measured at 660nm. The percentage of protein denaturation inhibition was calculated using the equation:

$$\% \text{ of Denaturation Inhibition} = (Abs_{positive\ control} - Abs_{sample}) \times 100 / Abs_{positive\ control}$$

2.7 Preparation of Red Blood cells

To evaluate the membrane stabilization and hemolytic activity of the extracts, 5 mL of blood was first collected from a healthy volunteer. In a Falcon tube with heparin, 5 mL of blood was diluted in 10 mL of 5 mM phosphate buffered saline (PBS) at pH 7.4 and centrifuged for 15 min at 100g. After centrifugation, the plasma was removed and PBS was added for washing. Three successive centrifugations were performed to obtain the red blood cell concentrate, and the layer of white blood cells formed after centrifugation was removed. This concentrate was kept refrigerated at 4°C and then diluted to a final concentration of 2%.

2.7.1 Membrane Stabilization Assay

The experiment was performed in triplicate, 100 µL of PBS + 400 µL of erythrocytes as negative control and 100 µL of different HECL-BA (50, 100, 200 µg/mL) and HECL-LE (200, 350, 500 µg/mL) concentrations in 400 µL of erythrocytes for the test. They were then incubated in a water bath at 56°C for 20 minutes to promote erythrocyte lysis. After cooling to room temperature, the samples were centrifuged at 1400 ×g for 10 minutes. A volume of 200 µL of the supernatant was used for reading into a 96-well plate at 540 nm.

2.7.2 Hemolytic Assay

In a 96-well Elisa plate, 25 µL of PBS was used as the negative control, 25 µL of a PBS - Triton X-100 (1%) solution was used as the positive control, and 25 µL of different HECL-BA (50, 100 and 200 µg/mL) and HECL-LE (200, 350 and 500 µg/mL) concentrations were used in

triplicate. In a microplate, 175µL of erythrocyte solution (2%) was added in all wells and incubated for 24 hours at 37°C. After this period, the absorbance was measured at 540nm.

2.8 Protease Inhibitory Activity

To evaluate protease inhibitory activity, 25 µL trypsin (10 mg/mL) and 125 µL PBS were used as positive control and 150 µL PBS were used as negative control. For the test samples, 25µL of trypsin (10mg/mL) + 125µL HECL-BA (50, 100 and 200µg/mL) and HECL-LE (200, 350 and 500µg/mL) concentrations were added and incubated for 10 minutes at room temperature. A volume of 500µL of 2% BSA was added into each tube and incubated for 1 hour at 30°C in a water bath. To stop the reaction, 300µL of 20% trichloroacetic acid was used. The samples were centrifuged at 1400 ×g for 10 minutes and the absorbance of the supernatant was measured at 220 nm into a 96-well plate.

2.9 Cell Viability

Cell viability of RAW 264.7 macrophages was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described [46,47]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37°C.

To assess the effect of HECL (stem bark and leaf), all concentrations were tested and analyzed for cell viability over 24 hours. RAW 264.7 macrophages were seeded in 96-well plates at 1×10^5 cells/well in 200µL medium. After 24 hours, different HECL-BA (50 and 100 µg/mL) and HECL-LE (350 and 500 µg/mL) concentrations were added, and the incubation continued at 37°C and 5% CO_2 for the next 24 hours. The negative control (100% growth) was performed with cells cultured in a medium without any treatment. An amount of 50 µL of MTT solution (0.5 mg/mL) was added to each well and the cells were incubated for 3 hours at 37°C. The formazan crystals formed during the incubation were dissolved in 100 µL DMSO, and the absorbance was measured at 570 nm. For each sample, the result was expressed as the percentage of absorbance relative to the control group.

2.10 Protective capacity and Cell Viability after induction of oxidative stress with Hydrogen Peroxide.

A priori, RAW 264.7 macrophages were subjected to oxidative stress induced by hydrogen peroxide at different concentrations (0.5, 0.75, 1.0 and 2.0 mM) and after 24 hours the cell viability was analyzed. A concentration curve was constructed to determine the dose that

resulted in an 80% reduction in cell viability. With the damaging concentration of H_2O_2 defined, other RAW264.7 macrophages were incubated (37°C and 5% CO_2) for 24 hours and treated with extracts at different concentrations of the stem bark and leaves (HECL-BA and HECL-LE). After incubation, extract-treated cells were exposed to the chosen toxic concentration, which was set at 0.75 mM H_2O_2. The protective effect of the extract after H_2O_2 exposure was assessed by a cell viability test using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test, at 3h of incubation with H_2O_2.

2.11 Nitric Oxide Determination

For *in vitro* quantification of nitric oxide (NO) metabolites, RAW 264.7 macrophages were cultured in a 96-well plate (1x 10^5 cells/well) for 24 hours. Cultures were subjected to the following conditions:

(1) negative control: non-inflamed cells (not exposed to lipopolysaccharide -LPS) and non-treated cells;

(2) positive control: inflamed cells (exposed to 10 µg/mL LPS for 4h);

(3) standard for comparison: cells treated with dexamethasone 100 µg/mL and exposed to LPS for 4h:

(4) experimental groups: cells pretreated with extracts (50 and 100µg/mL HECL-BA and 350 and 500µg/mL HECL-LE) for 24h and exposed to LPS for 4h.

After LPS exposure, 50 µL of supernatant was then collected and transferred to a 96-well plate and 100 µL of a 1:1 solution of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride (each diluted 1 g in 100 mL of 2.5% phosphoric acid) was added and incubated for 10 minutes at room temperature in the absence of light. A standard curve of nitrite (0-140mM) in DMEM medium was carried out and incubated at the same conditions. The absorbance was determined at 540 nm and the results expressed in µM nitrite.

2.12 Catalase activity

RAW 264.7 macrophages were incubated for 24 hours for cell growth, treated with HECL-BA and HECL-LE in different concentrations for 24h, and then exposed to 0,75 mM H_2O_2 for 4 hours. The treatments were divided in two groups:

Group 1:

- Non-stressed control (Negative control, CRTL-): cells treated only with DMEM culture medium for 24 hours;

- Non-stressed cells treatment: cells treated only with DMEM culture medium for 24 hours and treated with 50 and 100µg/mL HECL-BA and 350 and 500µg/mL HECL-LE for 24 hours, labeled as follows: BA50; BA100; LE350 and LE500.
- Group 2: Non-stressed control (Negative control, CRTL-): cells treated only with DMEM culture medium for 24 hours;
- Stressed control (Positive Control, CRTL+): cells treated with DMEM culture medium for 24 hours, followed by exposure to a predetermined concentration of H_2O_2 for 3h;
- Stressed cells treatment: cells treated only with DMEM culture medium for 24 hours, following treatment with 50 and 100µg/mL HECL-BA and350 and 500µg/mL HECL-LE for 24 hours, followed by exposure to a predetermined concentration of H_2O_2 for 3 hours, labeled as follows: BA50+ H_2O_2; BA100+ H_2O_2; LE350+ H_2O_2 and LE500+ H_2O_2.

After incubation, the culture medium was removed, and the cells were washed with PBS and resuspended in the culture medium. The contents of three wells from the same treatment culture plate were collected and pooled. The samples were centrifuged (732 g, 10 min, at 4°C). The pellets were resuspended in 3 mL lysis buffer (50 mM potassium phosphate buffer pH 7.0, 0.25% Triton X-100, 1 mM EDTA) and homogenized in an Ultra-Turrax® (5 s, 2×). Following that, a 30 µL sample were added to a 96 UV well plate, and 270 µL H_2O_2 solution was also added. The absorbance was measured at 240 nm[48]. The results were expressed in U catalase/mg protein.

2.13 Cyclooxygenase (COX) Assay

Cyclooxygenase (COX) analysis was done using a specific Elisa kit for mice (COX Activity Assay Kit - Caymam Chemical, MI/USA). The COX standard wells, background wells, and sample wells were then prepared on a plate. In addition, wells with specific COX-1 and COX-2 inhibitors were also prepared. The plate was then incubated, and the colorimetric substrate and arachidonic acid was added. After incubation, the absorbance was read at 590 nm. Subsequently, calculations were made to determine the total COX activity in each sample and the percentage of inhibition.

2.14 Gene expression analysis

Gene expression of pro- and anti-inflammatory cytokines was determined by quantitative reverse transcription-PCR (qRT-PCR) analysis. Macrophages (2.5×10^5 cells/well) were seeded

in a 6-well plate and treated with DMEM medium and fetal bovine serum (FBS 10%) and with HECL-BA concentrations (50 and 100 µg/mL) and HECL-LE (350 and 500 µg/mL) followed by incubation at 37°C and 5% CO_2 for 24 hours. To observe the anti-inflammatory effect, after treatment with the HECL extracts (stem bark and leaf), the cells were stimulated with 10 µg/mL of lipopolysaccharide (LPS) for 4 hours, were harvested for total RNA extraction by adding TRI Reagent® (Sigma-Aldrich) and 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 on a µDrop Duo plate using a Multiskan SkyHigh spectrophotometer (ThermoFisher Scientific). The extracted RNA (1000 ng) was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). RT-qPCR was performed using PowerTrack™ SYBR™ Green Master Mix (ThermoFisher Scientific) on a QuantStudio™ 3 Real-Time PCR System (ThermoFisher Scientific). The beta-actin was used as a housekeeping gene and the relative standard curve method was used for quantitative data analysis. Negative control (CRTL-): mRNA from non-stimulated macrophages cells; positive control (CRTL+): mRNA from macrophages stimulated with LPS. The primers used are listed in **Table 1**, obtained from the mouse genome bank (Rat Genome Database – https://rgd.mcw.edu/).

TABLE 1 – Primers Sequence

Gene		Forward	Reverse
Pró-inflammatory	***TLR-4***	5'-CAG GTG GAA TTG TAT CGC CT-3'	5'-CGA GGC TTT TCC ATC CAA TA-3'
	NF-κβ	5'-GCT GCC AAA GAA GGA CAC GAC A-3'	5'-GGC AGG CTA TTG CTC ATC ACA G-3'
	BAX	5'-CCC GAG AGG TCT TCT TCC-3'	5'-GCC TTG AGC ACC AGT TTG-3'
	COX-2	5'-TGC ACT ATG GTT ACA AAA GCT GG-3'	5'-TCA GGA AGC TCC TTA TTT CCC TT-3'
	IL-6	5'-TCC TTC CTA CCC CAA TTT CC-3'	5'-GCC ACT CCT TCT GTG ACT CC-3'
	TNF-α	5'-TAT GGC TCA GGG TCC AAC TC-3'	5'-CCC ATT TGA GTC CTT GAT GG-3'
Anti-inflammatory	***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'
	NRF-2	5'-CGA GGC TTT TCC ATC CAA TA-3'	5'-CGG TGG GTC TCC GTA AAT GG-3'
	HO-1	5'-ATG CCC CAC TCT ACT TCC CTG AGG AGC TG-3'	5'-TAG TGC TGT GTG GCT GGC GTG CAA-3'

Table1: The primer sequences used were as follows: BAX: Pro-apoptotic cytosolic of the Bax family; COX-2: Cyclooxygenase-2; NF-κβ: Nuclear factor-kappa β; IL-6: Interleukin 6; TNF-α: Tumor necrosis factor-a; TLR-4: Toll-like receptors-4; HIF-1: Hypoxia-inducible factor 1-alpha; IL-10: Interleukin 10; NRF-2: Nuclear factor erythroid-derived factor 2; HO-1: Heme oxygenase 1.

2.15 Statistical analysis

Statistical data analysis comparing the effect of Imburana on macrophage events associated with the wound healing process was performed using one-way ANOVA followed by Tukey's *post hoc* test at 5% significance, using GraphPad Prism software, version 8.0 (GraphPad Prism Software Company, 2018, San Diego, CA, USA). Results are expressed as mean ± standard deviation, corresponding to independent 3 replicates. the normality of the data was assessed using the Kolmogorov Smirnov test.

3. RESULTS

3.1 Phytochemical Content of *Commiphora leptophloeos*

The total phenolic and flavonoid contents of the *C. leptophloeos* leaf and stem bark extracts are shown in **Table 2**. The results showed that both extracts contained high levels of phenolic compounds. The total phenolic content of the stem bark extract (349.12 ± 0.04 mg/g) and that of the leaf extract (331.02 ± 0.03 mg/g) were not significantly different. On the other hand, the total flavonoid content of the leaf extract (174.90 ± 0.02 mg/g) was 30.63-fold higher than that of the stem bark extract (5.71 ± 0.06 mg/g), showing a significant difference.

Table 2: Phytochemical content of *Commiphora leptophloeos*

Samples	Total phenolic content (mg of GAE/g of extract)	Total flavonoid content (mg of QE/g of extract)
Leaf extract	331.02 ± 0.03^{a}	174.90 ± 0.02^{a}
Stem bark extract	349.12 ± 0.04^{a}	5.71 ± 0.06^{b}

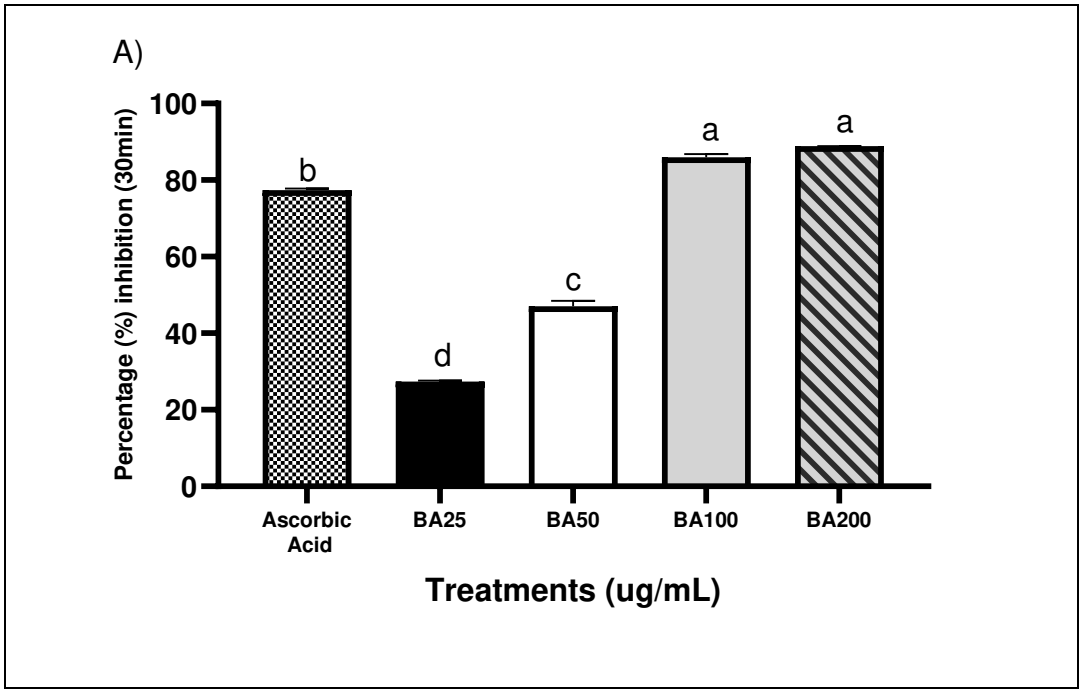
GAE/g: The content of total phenols gallic acid equivalent (GAE) per gram of sample (mg of GAE/g of sample). QE/g: The content of total flavonoids quercetin equivalent (QE) per gram of sample (mg of QE/g of sample). Values are expressed as mean ± standard deviation ($n = 9$). Values with different letters between groups differ statistically by the ANOVA test and Turkey's post-test ($p < 0.05$).

The chromatographic profiles through UPLC-MS allowed the characterization of peaks with mass spectra similar to flavonoid glycoside derivatives from apigenin, luteolin, and quercetin. Six major flavonoids of leaf extract chromatogram were identified as orientin, isoorientin, vitexin, isovitexin, isoquercetrin and quercetrin. The phytochemical characterization of *C. leptophloeos* leaf extract was shown in detail in our previous article published by Dantas-de-Medeiros et al., 2021[13]. On the other hand, the phytochemical profile of stem bark extract

showed a different chromatographic profile. Characterization through mass spectrometry indicated the presence of quinic acid and oligomeric procyanidins derived exclusively from flavan-3-ol. The main precursor ions at m/z 289, 577, 865, 1,153, 1,441, and 1,729 allowed the identification of the oligomeric series composed of one to six units of flavan-3-ols (monomer, dimer, trimer, tetramer, pentamer, and hexamer, respectively), thus confirming the presence of B-type oligomeric procyanidins. The main compound was identified as B-type dimeric procyanidin. The phytochemical characterization of *C. leptophloeos* stem bark extract was shown in detail in our previous article published by Dantas-de-Medeiros et al., 2021 [17].

3.2 Antioxidant Activity Using DPPH Assay

The concentrations of extract analyzed were 25, 50, 100, and 200 µg/mL for HECL-BA and 100, 200, 350, 500, 750, and 1000 µg/mL for HECL-LE. Our results showed an inhibition of at least 80% of the DPPH radical. The concentrations of 100 and 200 µg/mL of bark extract (HECL-BA) showed more than 80% inhibition of the DPPH radical after 30 minutes (Fig. 1A), and the antioxidant potential increased in a dose-dependent manner ($p<0.05$) for HECL-BA. Concentrations of 350, 500, 750, and 1000 µg/mL of the HECL-LE extract showed more than 80% inhibition of DPPH- (Fig. 1B), with no significant difference in inhibition after 30 min ($p>0.05$). No dose-dependent profile was observed for HECL-LE.



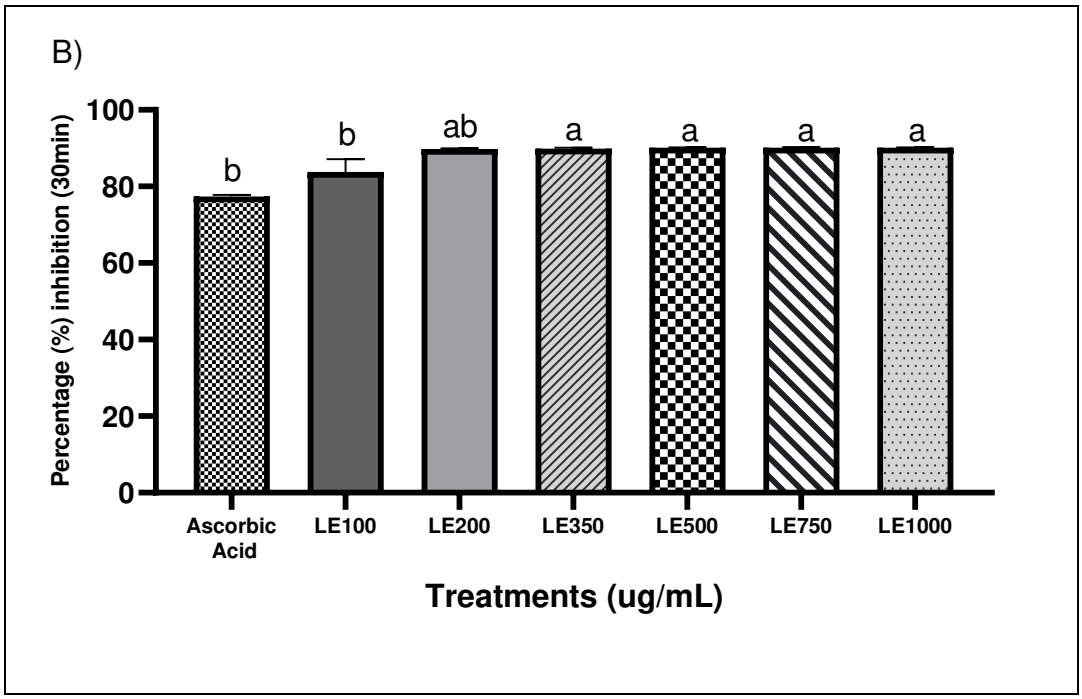
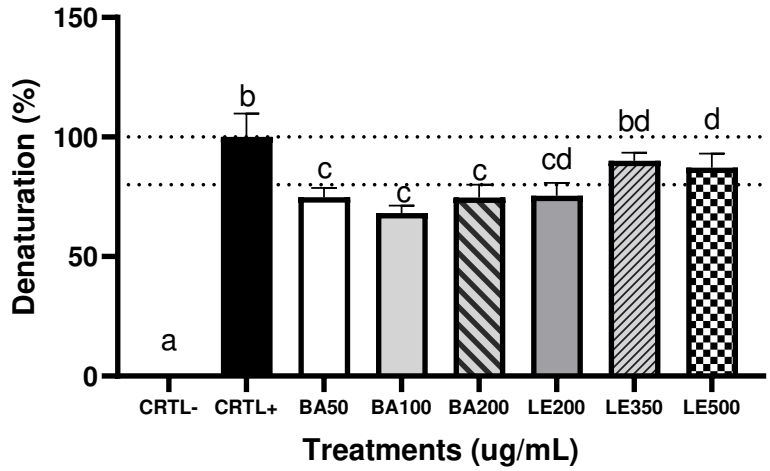


Figure 1. DPPH radical scavenging activity of Hydroethanolic Extract of *Commiphora leptophloeos* - - HECL (Bark – BA, and Leaf – LE). Ascorbic acid (12.5 µg/mL) was used as a reference standard. Different concentrations of HECL-BA (BA25, BA50, BA100, and BA200 µg/mL) and HECL-LE (LE100, LE200, LE350, LE500, LE750, and LE1000 µg/mL) were tested. Percentage inhibition of HECL-BA (A) and HECL-LE (B) concentrations after 30 minutes of reaction with DPPH-. Results in triplicate are expressed as mean ± standard deviation. Means followed by the same letter do not show significant differences $p > 0.05$ (one-way ANOVA with Tukey's *post-hoc* test).

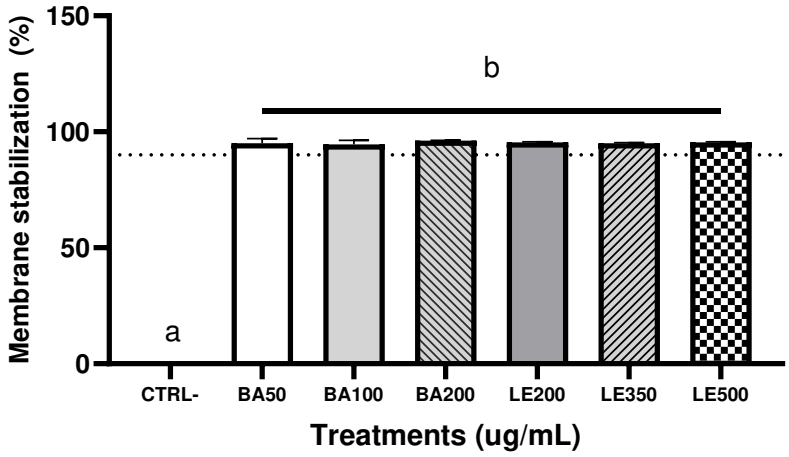
3.3 Denaturation, Hemolytic and Protease Activities.

Considering the tested concentrations, HECL-BA (50, 100, and 200 µg/mL) and HECL-LE (200 µg/mL) were able to protect against protein denaturation with up to 20% protection ($p<0.05$) compared to the positive control (Fig. 2A). However, the HECL-LE (350 and 500 µg/mL), protected less than 20% against protein denaturation compared to the positive control. The results of the membrane stabilization activity showed that all concentrations of the HECL-BA and HECL-LE protected the cells from hemolysis compared to the control (Fig. 2B). None of the HECL-BA and HECL-LE samples promoted hemolysis compared to the positive control (Fig. 2C). Unfortunately, the extract, at the test concentrations, promoted proteolytic activity and did not demonstrate the ability to inhibit the action of the trypsin enzyme when compared to the positive control (Fig. 2D).

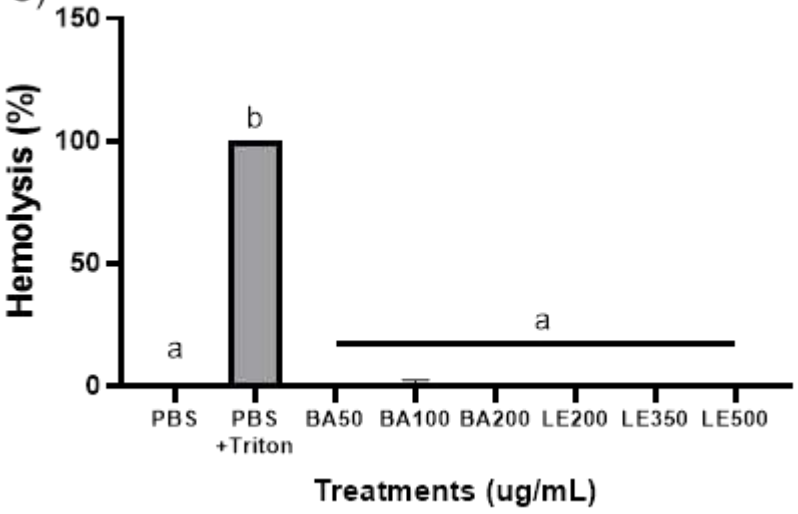
A)


150
100
50
0
Denaturation (%)
a
b
c
c
c
cd
bd
d
CRTL-
CRTL+
BA50
BA100
BA200
LE200
LE350
LE500
Treatments (ug/mL)

B)


150
100
50
0
Membrane stabilization (%)
a
b
CTRL-
BA50
BA100
BA200
LE200
LE350
LE500
Treatments (ug/mL)

C)


150
100
50
0
Hemolysis (%)
a
b
a
PBS
PBS +Triton
BA50
BA100
BA200
LE200
LE350
LE500
Treatments (ug/mL)

D)

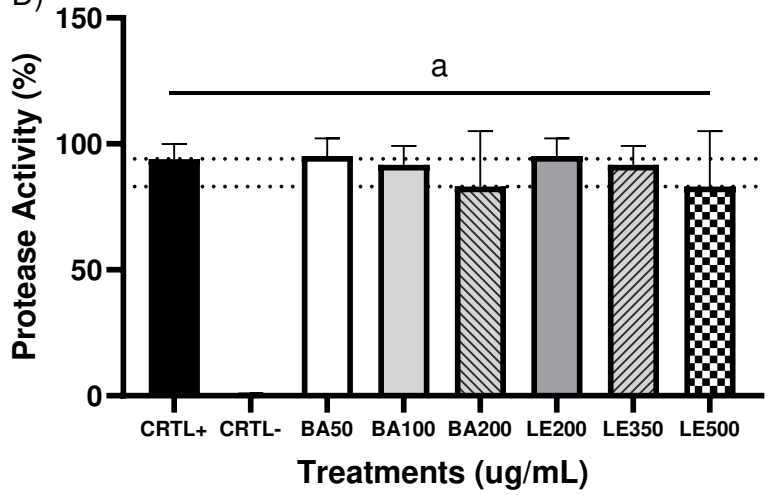
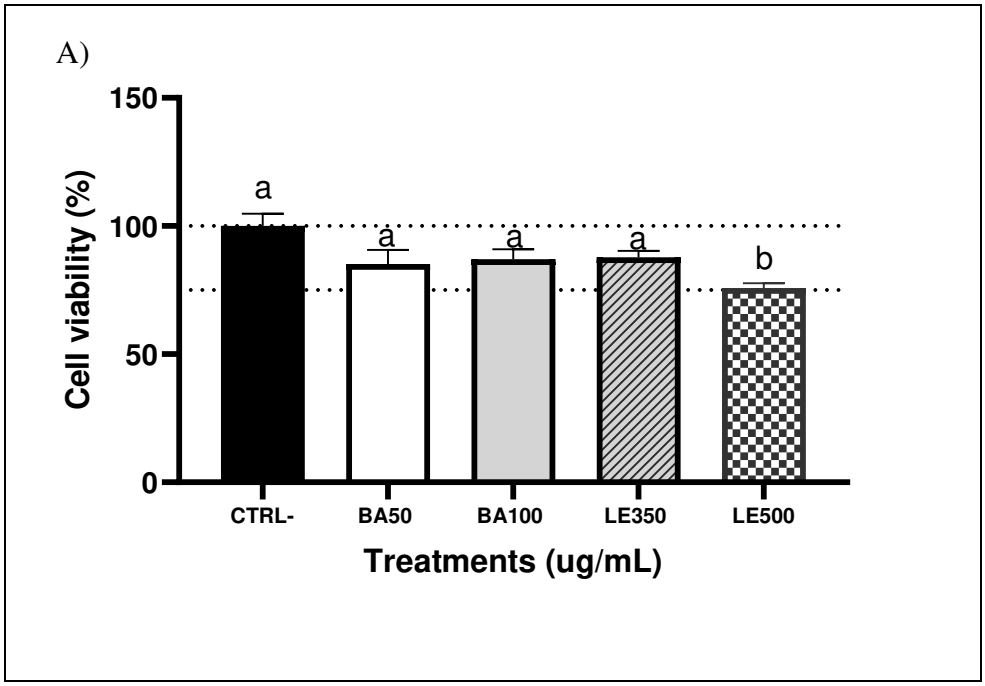

150
100
50
0
Protease Activity (%)
a
CRTL+
CRTL-
BA50
BA100
BA200
LE200
LE350
LE500
Treatments (ug/mL)

Figure 2. Denaturation, hemolytic and protease activities after treatments with Hydroethanolic Extract of *Commiphora leptophloeos* - - HECL (Bark – BA, and Leaf – LE) from the HECL-BA (BA50, BA100, BA200 µg/mL) and the HECL-LE (LE200, LE350, LE500 µg/mL). (A) Potential protection against denaturation: Bovine serum albumin solution in saline solution with the treatments after incubation at 70°C (30 min). Crtl-: Negative control, TRIS buffer. Crtl+: Positive control, TRIS buffer + BSA. (B) Protection against hemolysis: Heparinized erythrocyte suspensions treated and exposed to Triton X-100 (1%) for 24 hours. Ctrl -: Negative control: 100 µL of PBS + 400 µL of erythrocytes. (C) Hemolytic activity: Suspensions of heparinized erythrocytes exposed to the treatments after incubation at 70°C for 20 minutes. Ctrl-: Negative control: PBS buffer; positive control: PBS + Triton X (1%). Crtl+: Positive control, 100 µL of PBS + 400 µL of erythrocytes. (D) Protease activity: Trypsinized protein with shell and leaf concentrations. Crtl-: negative control: PBS buffer; Ctrl+: positive control: PBS buffer + trypsin. Data (n = 3) are expressed as mean and standard deviation. Statistical difference from the control group: Means followed by the same letter are not significantly different $p < 0.05$ (one-way ANOVA with Tukey's *post hoc* test).

3.4 Effect of HECL on RAW264.7 Cell Viability

Among all the concentrations tested, samples from HECL-BA 50 µg/mL (85.2%), 100 µg/mL (87.0%), and HECL-LE 350 µg/mL (87.7%), and 500 µg/mL (70.0%) showed higher or equal 70% of cell viability compared to untreated cells (considered as 100% viability) (Fig. 3A) and then were considered for further analyses.

After exposure of the cells to a stressor agent (0.75mM H_2O_2), we observed that most of the HECL-BA and HECL-LE extracts at the concentrations tested above could also protect against hydrogen peroxide-induced cell damage (Fig. 3B). However, 500 µg/mL HECL-LE did not protect against H_2O_2 damage compared to the positive control (cells stimulated with H_2O_2) (Fig. 3B).



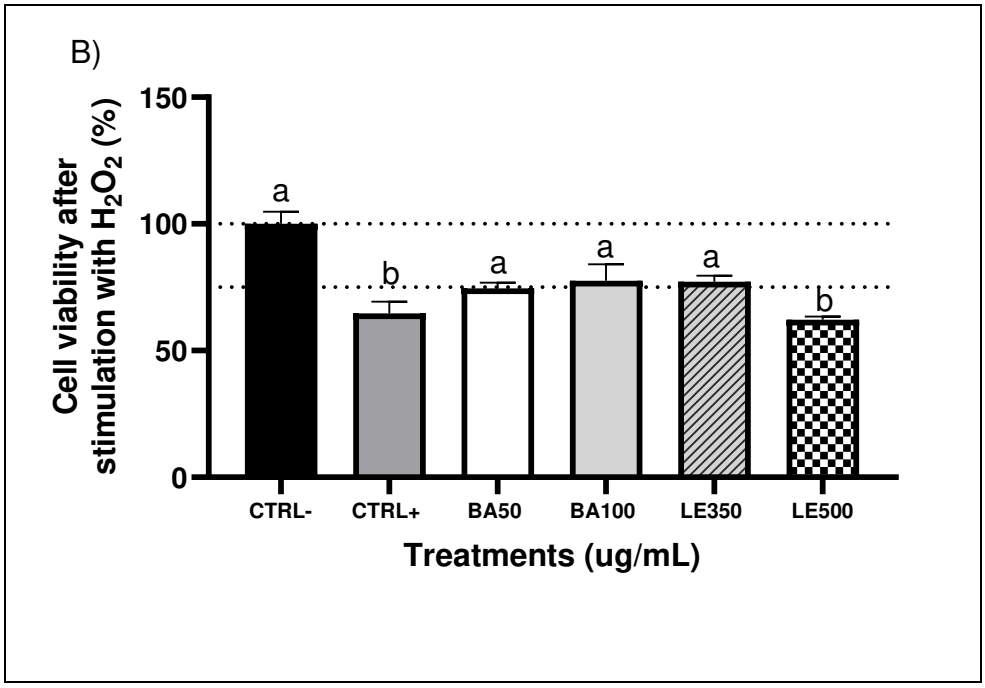


Figure 3. Effect of Hydroethanolic Extract of *Commiphora leptophloeos* - - HECL (Bark – BA, and Leaf – LE) on cell viability. RAW264.7 macrophages were treated separately with DMEM and concentrations of HECL-BA (BA50 µg/mL and BA100 µg/mL) and HECL-LE (LE350 µg/mL and LE500 µg/mL). (A) Cell viability of negative control (cells cultured in DMEM medium without any treatment) and after treatment with HECL-BA and HECL-LE. (B) Cell viability after treatment with HECL-BA and HECL-LE concentrations and subsequent induction of oxidative stress with hydrogen peroxide; Ctrl-: cells cultured in medium without any treatment; Ctrl+: cells treated with DMEM culture medium followed by the exposure to a 0.75mM H_2O_2 for 3h. Means followed by the same letter are not significantly different $p < 0.05$ (one-way ANOVA with Tukey's *post-hoc* test). Data ($n = 3$) are expressed as mean ± standard deviation.

3.5 Effect of HECL on Nitric Oxide Production After Stress induced by LPS.

HECL-BA extract in concentrations of 100, 200, and 300 µg/mL and HECL-LE in concentrations of 500, 750, and 1000 µg/mL were able to reduce LPS-induced stress ($p<0.05$) and consequently NO production (Fig. 4), compared to positive control (cells stimulated with LPS). Furthermore, except for 200 µg/mL HECL-BA, these concentrations did not differ from the 100 µg/mL dexamethasone, usually used as reference standard. The 50 µg/mL HECL-BA and 350 µg/mL HECL-LE did not show a difference from the positive control stimulated with LPS.

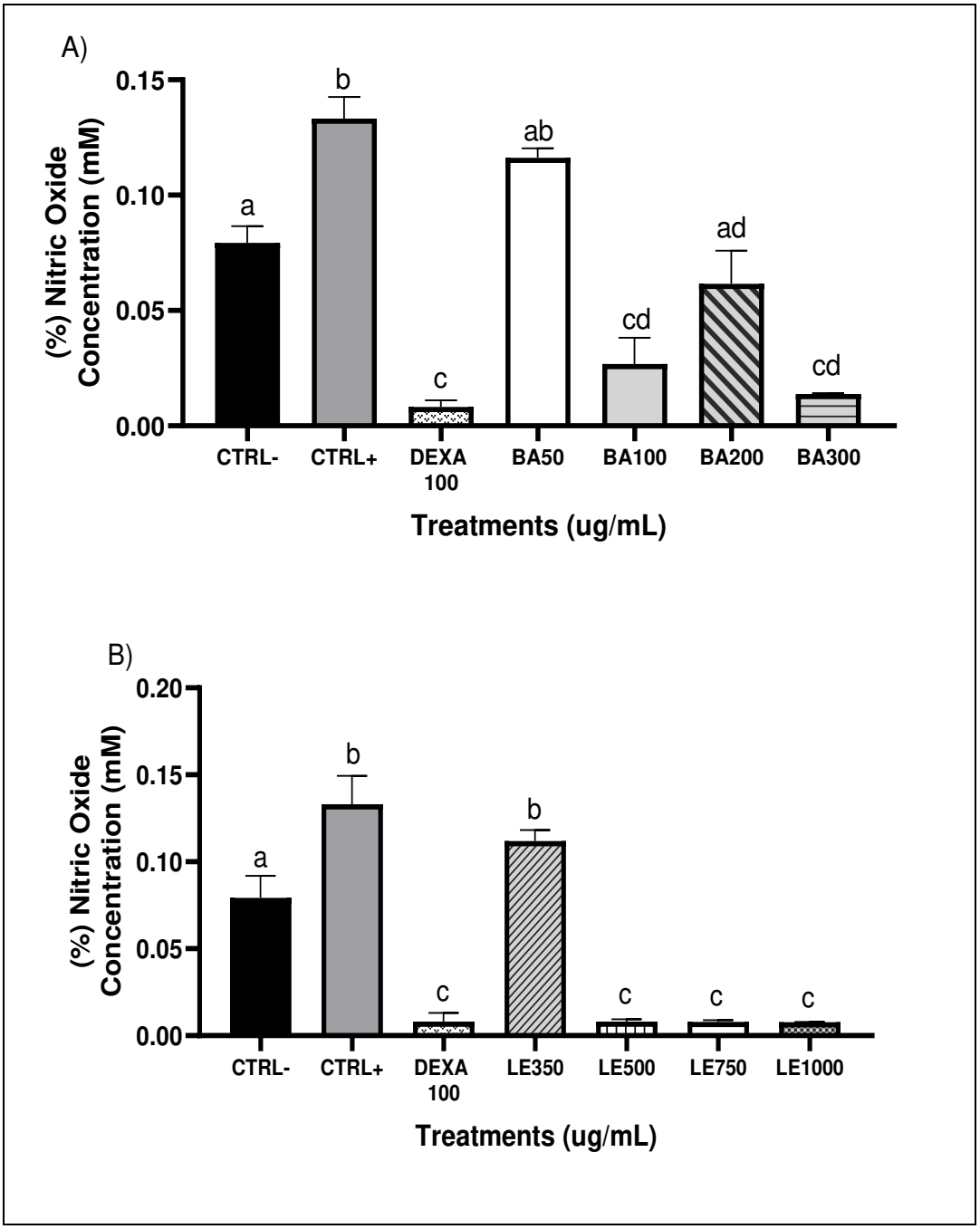


Figure 4. Determination of NO metabolites after lipopolysaccharide (LPS) stimulation. RAW 264.7 macrophages were treated with Hydroethanolic Extract of *Commiphora leptophloeos* - HECL (Bark – BA, and Leaf – LE) extract. Ctrl-: Negative control (cells not exposed to LPS); Ctrl+: Positive control (cells exposed to LPS); DEXA100: reference standard (Cells exposed to LPS and Dexamethasone 100 µg/mL). HECL-BA: BA50, BA100, BA200, and BA300 µg/mL; and HECL-LE: LE350, LE500, LE750, and LE1000 µg/mL (cells treated and exposed to 10 µg/mL LPS for 4 hours). Means followed by the same letter are not significantly different $p < 0.05$ (one-way ANOVA with Tukey's post hoc test). Data (n = 3) are expressed as mean and standard deviation.

3.6 Catalase Activity

The results showed increased catalase activity in the HECL-BA (50 and 100 µg/mL), compared to the positive control stimulated with H_2O_2. No significant difference ($p>0.05$) was observed when the cells were not stimulated with H_2O_2 (Fig.5 A, B). However, for HECL-LE extracts, there was a

reduction of catalase activity using 500 µg/mL concentration, but no difference was observed when using 350 µg/mL compared to the positive control stimulated with H_2O_2. When the cells were not stimulated with H_2O_2, an increase of catalase activity was observed using 350 µg/mL HECL-LE extract (Fig. 5 C, D).

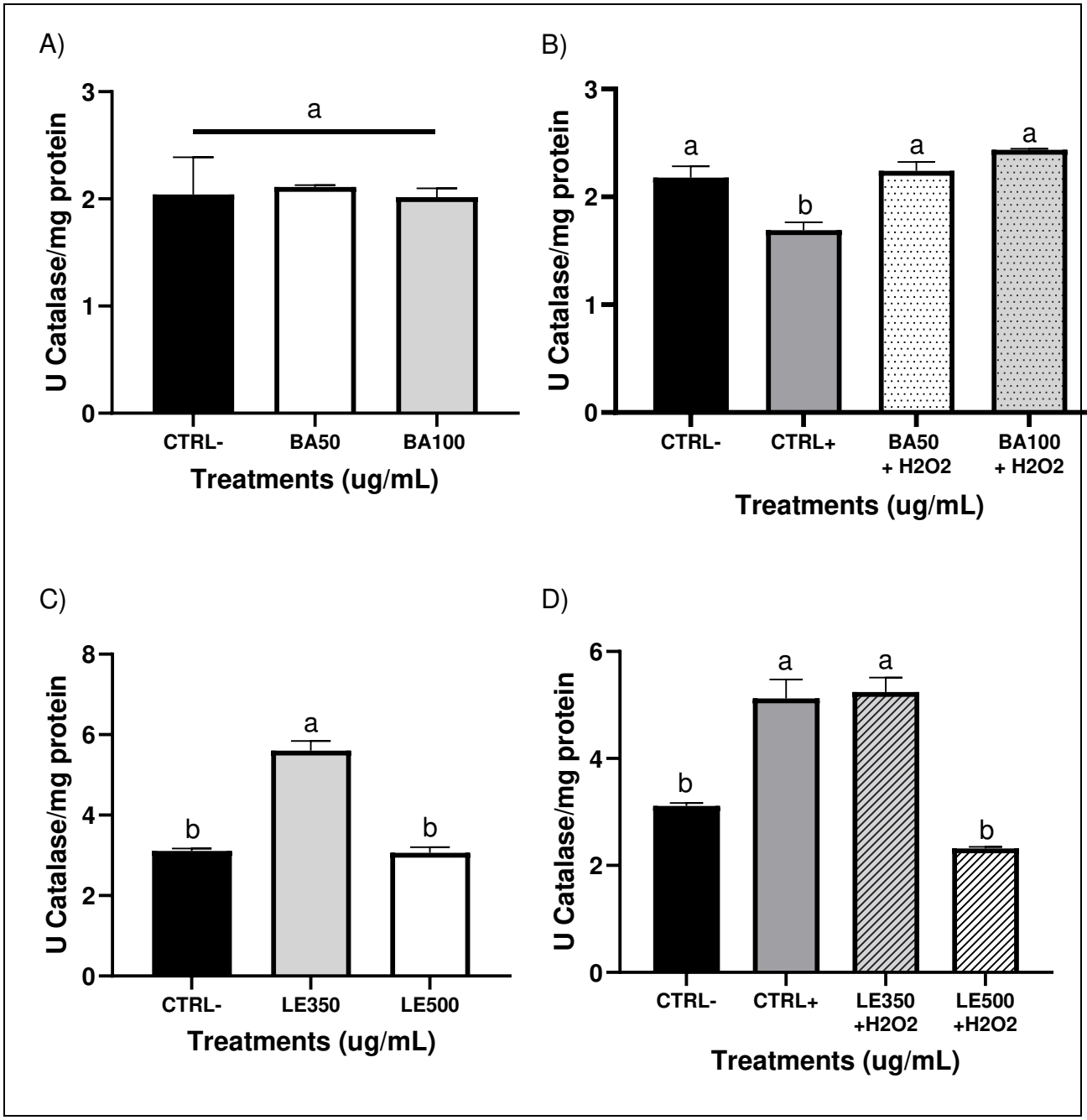


Figure 5. Effect of Hydroethanolic Extract of *Commiphora leptophloeos* - HECL (Bark – BA, and Leaf – LE) on Catalase Activity (U/mg protein). a RAW 264.7 macrophages were treated with concentrations of 50 and 100 µg/mL (HECL-BA) without H_2O_2 stimulation (A) and with H_2O_2 stimulation (B). RAW 264.7 macrophages were treated with concentrations of 350 and 500 µg/mL (HECL-LE) without H_2O_2 stimulation (C) and with H_2O_2 stimulation (D); Negative control (Ctrl-): Cells in DMEM without any treatment and stimulus; Positive control (Ctrl+): Cells stressed with H_2O_2 without treatment. Means followed by the same letter do not have significant difference $p>0.05$ (one-way ANOVA with Tukey *post hoc* test. Data (n = 3) are expressed as mean and standard error.

3.7 Inhibition of COX Activity *in vitro*

Macrophages exposed to the concentrations of B50 ug/mL, B100 ug/mL HECL-BA and L350 ug/mL HECL-LE, showed significant inhibition of total COX activity levels (Fig. 6A) compared to the control (Ctrl).

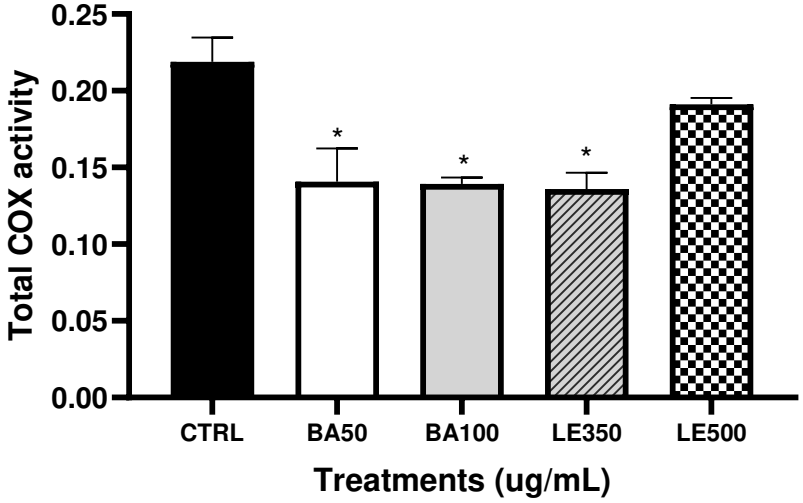


Figure 6. COX inhibitory potential in macrophages exposed to concentrations of HECL (Bark – BA, and Leaf - LE): Comparison of total COX activity (TC) between treatments of BA50 ug/mL, BA100 ug/mL, LE350 ug/mL, LE500 ug/mL and the Total Control (CTRL: Cell+DMEM). Data (n = 3) are expressed as mean and standard error. The significant difference, $p<0.05$ (one-way ANOVA with Tukey's *post hoc* test).

3.8 Expression of Inflammatory Marker Genes

Our results indicate a decrease in *TLR-4* expression in all groups after exposure to HECL, especially in the BA50 and BA100µg/mL groups (Fig. 7A) with no statistical difference between the two dosages. There was a significant reduction in the expression of *NF-κβ* compared to the positive control, especially in the BA50 and BA100 groups (Fig. 7B), while 350 µg/mL of HECL-LE increased the expression of *NF-κβ* compared to the positive control ($p>0.05$). All HECL extract (stem bark and leaf) concentrations reduced *BAX* gene expression compared to positive control (Fig. 7C) and the best result was presented by 100 ug/mL HECL-BA extract. A reduction of *IL-6* expression was observed for all HECL-BA and HECL-LE concentrations compared to the positive control (Fig. 7D). All HECL extracts concentrations showed a reduction of *TNF-α* expression compared to the positive control (Fig. 7E). Regarding *COX-2* gene expression (Fig. 7F), our results showed that all HECL extract (stem bark and leaf) concentrations reduced *COX-2* gene expression compared to positive control, and the HECL-LE extract presented the best results.

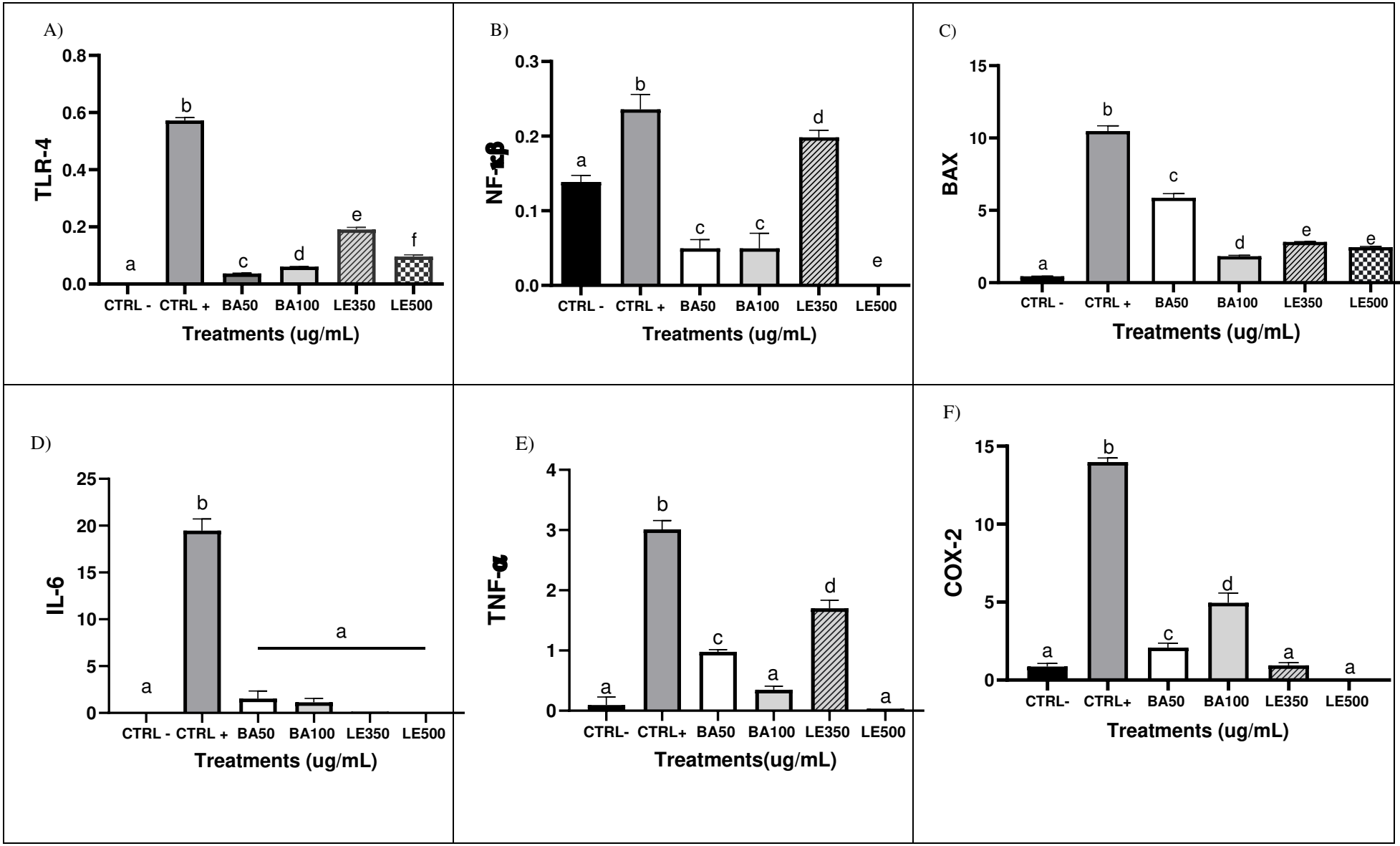

A)
TLR-4
0.8
0.6
0.4
0.2
0.0
a
b
c
d
e
f
CTRL - CTRL + BA50 BA100 LE350 LE500
Treatments (ug/mL)
B)
NF-κβ
0.3
0.2
0.1
0.0
a
b
c
c
d
e
CTRL - CTRL + BA50 BA100 LE350 LE500
Treatments (ug/mL)
C)
BAX
15
10
5
0
a
b
c
d
e
e
CTRL - CTRL + BA50 BA100 LE350 LE500
Treatments (ug/mL)
D)
IL-6
25
20
15
10
5
0
a
b
a
CTRL - CTRL + BA50 BA100 LE350 LE500
Treatments (ug/mL)
E)
TNF-α
4
3
2
1
0
a
b
c
a
d
a
CTRL- CTRL+ BA50 BA100 LE350 LE500
Treatments(ug/mL)
F)
COX-2
15
10
5
0
a
b
c
d
a
a
CTRL- CTRL+ BA50 BA100 LE350 LE500
Treatments (ug/mL)

Figure 7. Relative mRNA levels of pro-inflammatory genes: *TLR-4* (A), *NF-κβ* (B), *BAX* (C), *IL-6* (D), *TNF-α* (E) and *COX-2* (F) after treatments with Hydroethanolic Extract of *Commiphora leptophloeos* (Bark – BA, and Leaf - LE) in different concentrations: (BA50 and BA100) and HECL-LE (LE350 and LE500). CTRL-: Negative control (non-inflamed cells); CTRL+: Positive control (cells inflamed with 10µg/mL LPS). Data (n = 3) are expressed as mean and standard error. Statistical difference from the control group: Means followed by the letter (ns) not significant, p <0.05 (one-way ANOVA with Tukey's post hoc test).

3.9 Expression of anti-inflammatory marker genes

According to our results, all the tested concentrations of HECL extract from stem bark (B 50 and 100 µg/mL) and leaves (L 350 and 500 µg/mL) increased the expression of *NFR-2* (Fig. 8A), with B 50 µg/mL having the highest expression among them, compared to the positive control. However, *HO-1*, *HIF-1*, and *IL-10* (Fig. 8B, C, D) decreased their gene expression compared to the positive control in both parts of the plant.

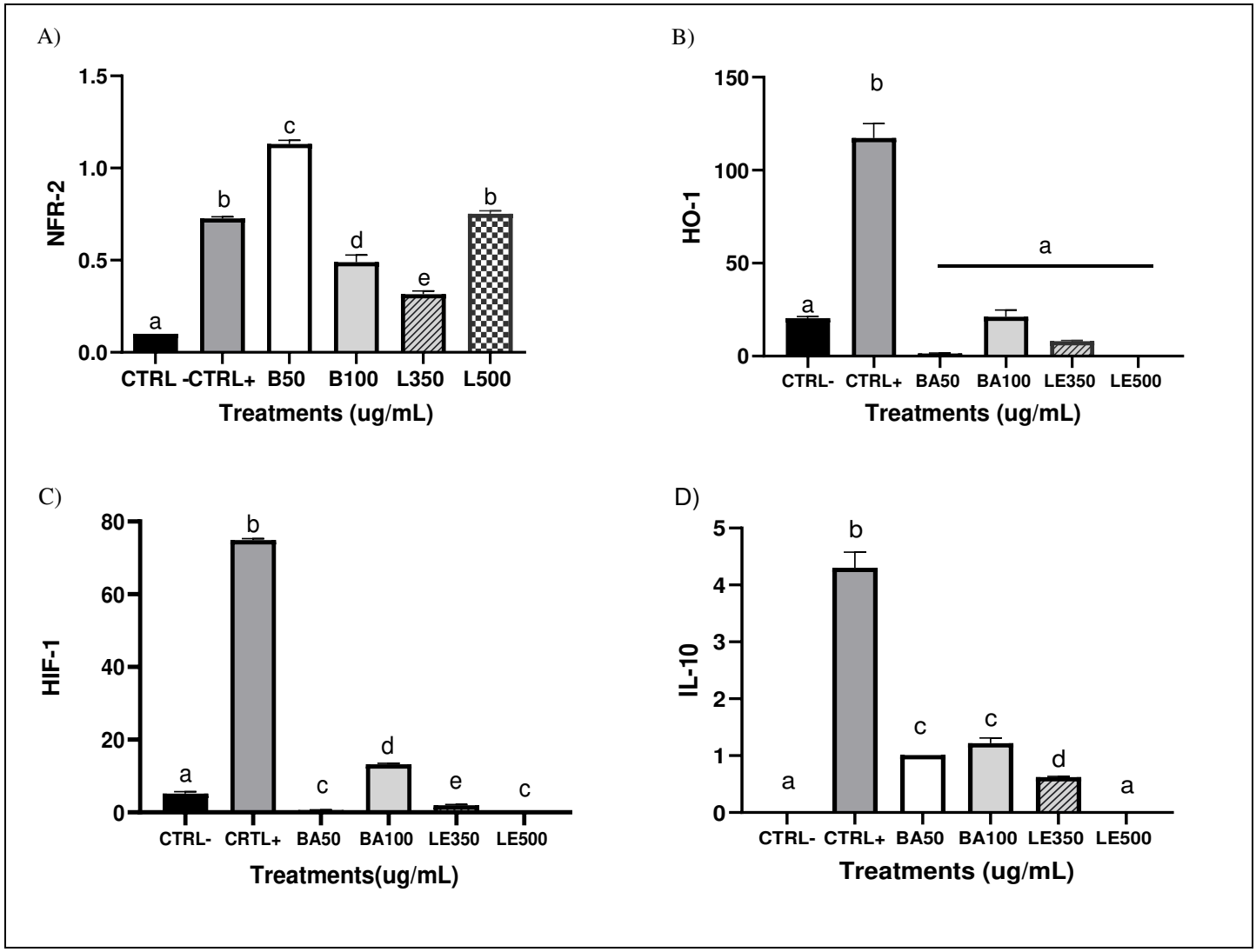


Figure 8. Relative mRNA levels of anti-inflammatory genes after treatments with Hydroethanolic Extract of *Commiphora leptophloeos* (Bark – BA, and Leaf - LE) in different concentrations BA50 and BA100 and LE350 and LE500. *NRF-2* (A), *HO-1* (B), *HIF-1* (C), and *IL-10* (D). Negative control: Non-stimulated cells; Positive control: Cells stimulated with LPS 10µg/mL. Data (n = 3) are expressed

as mean and standard error. Means followed by the same letter do not have a significant difference, p>0.05 (one-way ANOVA with Tukey's *post hoc* test).

4. DISCUSSION

Commiphora leptophloeos is an important medicinal plant widely distributed in Brazil and Bolivia [13,49] . Recently, this plant species has aroused great interest in the scientific community, especially for its pharmacological properties, including potent antioxidant, anti-inflammatory, and antimicrobial activities [12,13,15,17,50]. Previous phytochemical studies have shown that the leaves of this species are rich in C-glycosylated flavonoids derived from orientin, isoorientin, vitexin, isovitexin, isoquercetin, and quercetin [12,13]; while the stem barks are rich in condensed tannins derived from flavan-3-ol with type B binding [15,17,51]. Our results showed that the content of phenolic compounds in both extracts showed high levels of phenolic compounds, with no statistical difference between them. From the results, it can be seen that the six phenolic compounds found in the extract show no significant differences between plant bark and leaves. However, the leaf extract has a high flavonoid content (30-fold), rich in condensed tannins such as oligomeric procyanidins, confirming data from the literature [12,13,15,17,50]The content of phenolic compounds and flavonoids was used to determine the concentrations used in the *in vitro* tests.

The results of this study confirm the antioxidant and anti-inflammatory potential of *C. leptophloeos* hydroethanolic extract (HECL)[9,12]. First, we observed a significant antioxidant capacity, especially at concentrations of 100 and 200 µL of HECL-BA and at all concentrations of HECL-LE, where more than 80% of DPPH radicals were inhibited. This effect can be attributed to the presence of polyphenolic compounds and the flavonoid C-glycosides, in which the sugar is attached directly to the carbon of the flavonoid ring, increasing their chemical stability and bioavailability. They neutralize free radicals, prevent lipid peroxidation, chelate metals such as iron and copper, inhibit inflammatory enzymes and modulate cell signalling [45,46]. In our results, we observed that HECL extract has no denaturing activity, which means that tissue proteins are not damaged after exposure to these extracts which shows that they can be considered safe at the concentrations tested. Similar results were obtained with extracts of barbatimão[52], in which the authors observed that there was no protein denaturation in the extracellular matrix in wounds in rats [53]. In addition, both HECL extracts showed no hemolytic activity towards human erythrocytes, confirming the absence of toxicity to erythrocyte membranes, protecting the cells and not causing hemolytic activities. Studies support our findings and confirm that plant extracts rich in antioxidants and phenolic compounds can protect erythrocytes from hemolysis and oxidative damage and that the use of these extracts in topical formulations for wound treatment does not pose a risk of harm to blood cells [1,17,54–56]. In erythrocyte injury, free iron catalyzes the Fenton reaction, producing reactive oxygen species (ROS) as part of the

physiological host defense mechanism but in a vicious cycle, causing oxidative stress and leading to molecular, subcellular, cellular, and tissue damage [57,58]. The extract of *C. leptophloeos*, although not inhibiting the action of the enzyme trypsin, showed anti-inflammatory potential by modulating the expression of the pro-inflammatory gene and protein COX-2. The stem bark and leaves, rich in antioxidants and phytochemicals as in other studies[12,59,60], influence inflammatory processes by modulating the activity of proteases and inhibiting metalloproteinases (MMPs), reducing leukocyte migration and promoting resolution of inflammation[61].

HECL showed no cytotoxicity and maintained cell viability above 70% even after exposure to hydrogen peroxide, confirming its low toxicity and antioxidant potential, capable of controlling free radicals and ROS in cells. The same ability has been observed previously[13], confirming its low toxicity and antioxidant potential. This extract can control free radicals and ROS generated in the mitochondria inside cells. *C. leptophloeos* extracts have been shown to act as an electron receptor, stabilizing the OH* radical and reducing its action time in cells.

The study showed that the extract reduced the production of free radicals and inhibited the production of nitric oxide (NO) after LPS-induced stress, when compared to the positive control and the standard reference drug dexamethasone, a glucocorticoid known to inhibit inflammatory cytokines and cell adhesion and recruitment, which explains its anti-inflammatory effect [56]. NO, which is involved in several physiological processes, can form peroxynitrite (ONOO-), a reactive nitrogen species that causes cell damage [57,58]. Previous studies [32,33,59] confirm that the extract protects against oxidative stress by modulating antioxidant enzymes and reducing ROS levels.

We suggest that antioxidant compounds in the extracts are responsible for the increase in catalase activity, particularly at the 350 µg/mL concentration of HECL-LE, with as significant finding. Catalase is essential for breaking down hydrogen peroxide (H_2O_2), a toxic by-product of cellular metabolism, thus protecting cells from oxidative stress [60,61]. The increased activity of this enzyme suggests that HECL-LE has the ability to reduce H_2O_2 levels, thereby helping to maintain cellular homeostasis. On the other hand, the absence of catalase activity at lower concentrations of the bark extract (HECL-BA) and the reduction at higher concentrations of HECL-LE suggest that the dosage and composition of the extract are critical factors in the antioxidant efficacy.

The OxInflammation process is the new bridge to understanding the relationship between inflammation and oxidative stress inside the cells[26]. To understand this mechanistic theory deeply, the macrophage cells are essential. They coexist through phenotypic plasticity in inflammation's regulation, duration, and intensity and orchestrate the transition between the initial and resolution

phases[62]. Classical macrophages (Macrophages 1- M1) promote acute inflammation by releasing pro-inflammatory cytokines that induce vasodilation and recruit immune cells to the injury site, initiating the inflammatory cascade[63]. In contrast, Macrophages 2 (M2) are involved in the resolution of inflammation by producing anti-inflammatory cytokines, facilitating the removal of cellular debris [64,65]. Therefore, controlling the transdifferentiation of these cells during the inflammation process can be the key to controlling OxInflammation and avoiding the development of chronic inflammation diseases. It is already known that in the early phase of inflammation, molecular patterns associated with damage (DAMPS) and molecular patterns associated with pathogens (PAMPS) initiate a pro-inflammatory cascade in the cytosol of M1 macrophages, particularly through the activation of non-specific receptors such as toll-like receptors. Therefore, the synergy between toll-like membrane receptors and the inhibition of *NF-κβ* through phosphorylation of inhibitory molecules mediated by *Iκβ* (Iκκ kinase), including *Iκβα* [66,67], is responsible for the translocation of *NF- κβ* to the nucleus and initiates the transcription of pro-inflammatory cytokines such as interleukin 6 (*IL-6*), *TNFα*, *COX-2* and *BAX*. Our results showed that the B-type procyanidins identified in the stem bark extract and the C-glycoside flavonoids identified in the leaf extract can bind to cell receptors such as Toll-like 4 (*TLR4*), promote negative regulation and inhibit a central signalling pathway (*NF-κβ*) in macrophages, specifically the 50 and 100 µg/mL concentrations of stem bark. This control will promote the negative regulation of other pathways and influence the negative expression of pro-inflammatory markers during inflammatory phases. We observed a reduction in the apoptotic cytokine *BAX* and the pro-inflammatory cytokines *IL-6*, *TNF-α* and *COX-2*. This multifaceted property of HECL extracts allows them to modulate different biochemical pathways and interact with other inflammatory mediators, resulting in a synergistic effect that enhances their anti-inflammatory properties.

In our study, we observed that one of the consequences of controlling pro-inflammatory gene expression was control of the pro-apoptotic genes family, such as the *BAX* gene. *BAX* protein allows membrane depolarization and release of mitochondrial contents, including cytochrome c, via a Ca^{+} dependent opening of Mitochondrial pores [68–70]. Morphological changes in the apoptotic cell include pyknosis, cell shrinkage, and membrane blebbing, releasing vesicles containing cellular contents that will be phagocytosed by surviving cells, and promoting more inflammation [71–74]. Our results showed that all extracts concentrationscarried out a down-regulation in the dose-dependent profile, for *BAX*. Thus, reducing excessive apoptosis will help restore tissue homeostasis and limit the inflammatory response, as observed in extracts from other species of the *Commiphora* genus. These results are consistent with previous studies evaluating the anti-inflammatory[12,13], antioxidant[12–14], antifungal, and antibiofilm [15–17] potential of *C. leptophloeos* and highlight the efficacy of this plant extract in modulating cellular processes essential for recovering tissue after chronic inflammation wound.

Corroborating *BAX* genes downregulation, the expression of *COX-2*, an inflammatory enzyme, was significantly downregulated for both parts of the plant, an important factor in reducing inflammation. Generally, *NF-κβ* activates *COX-2* expression and promotes positive feedback, producing prostaglandins and exacerbating inflammation. Prostaglandins can, in turn, influence the activity of *NF-κβ*, highlighting the importance of this interaction in modulating the inflammatory response. Also, *COX-2* can be induced by cytokines (IL-1, IL-2, and TNF-α) and other mediators at sites of inflammation such as growth factors and endotoxins [75–77]. On the other hand, *COX-2* expression can be inhibited by glucocorticoids, IL-4, IL-10, and IL-14 [76,78]. As shown by gene expression, the different concentrations of bark and leaves extracts reduced the expression of pro-inflammatory cytokines and *COX-2* activators and decreased inflammation, showing the great potential of *C. leptophloeos* to control the inflammation. Similar results were observed using *Grindelia squarrosa* extracts associated with grindelic acid [79] in inflammation after LPS exposure, in which was observed a reduction of NF-κβ and inhibition of cytokine synthesis (IL-8, TNF-α, IL-1β and IL-6), indicating complex interactions involved in inflammatory, immune and tissue regeneration responses [79].

In the context of the anti-inflammatory process, we observed the up-regulation of NRF-2, particularly at the 50µg/mL concentration of the stem bark, reinforcing the anti-inflammatory potential of the extract. The mutual interaction between NRF-2 and NF-κβ is essential for balancing inflammatory and antioxidant responses [80] in response to oxidative stress and also in the control of inflammation [31,81]. NRF-2 is a key regulator of the antioxidant response, activating the expression of genes that encoding antioxidant enzymes and detoxification proteins. When activated, NRF-2 stimulates antioxidant enzymes, such as GSH, and reduces oxidative stress [82], which in turn can suppress the activation of NF-κβ, since oxidative stress activates this pathway. Phenolic compounds act in a similar way to Nonsteroidal Anti-Inflammatory Drugs (NSAIDs), but some of them inhibit pro-inflammatory mediators other than COX, inhibiting their activity or gene expression [83,84]. We follow the hypothesis that crude plant extracts are complex chemical mixtures that act on multiple targets, unlike an isolated substance present in NSAIDs. Furthermore, extract components such as condensed tannins and oligomeric procyanidins have never been described before in *C. leptophloeos*.

Our results showed a negative regulation of HO-1, HIF-1 and IL-10 at all concentrations compared to the positive control. Several authors have demonstrated the activation of NRF-2 and consequently HO-1, but the literature describes that it is unclear whether NRF2-dependent HO-1 induction is part of a general protective response [85] or whether it specifically reduces oxidative stress and inflammation [86]. However, many authors believe that this pathway is fundamental to understanding the relationship between oxidative stress and inflammation, known as OxInflammation [86–90].

At this point, our question is: what antioxidant pathway and mechanism, which we haven't been able to measure, is controlling the oxidative stress in the tissue, given that the cytokines HO-1 and HIF-1 have been downregulated? We know that HO-1 acts by breaking down heme [91] into less toxic products such as carbon monoxide, biliverdin and free iron [92], reducing the formation of free radicals and modulating the inflammatory response [93]. In situations of acute or chronic inflammation, the persistent activation of NF-κβ can inhibit the expression of HO-1, triggering negative feedback mechanisms, which leads to a reduction in the anti-inflammatory effects mediated by HO-1 [94]. This situation can lead to an imbalance between pro- and anti-inflammatory mechanisms, favoring the perpetuation of the inflammatory response. Cytokines such as TNF-α, IL-1β, IFN-γ, and activation of the MAPK pathways, namely JNK and p38, can antagonize HO-1 expression. The activity of these kinases in response to oxidative or inflammatory stress can reduce the expression of anti-inflammatory genes such as HO-1 [95,96]. These cytokines activate inflammatory pathways that antagonize the expression of anti-inflammatory genes, including HO-1. A priori, HIF-1 regulates the transcription of genes that help cells survive in low oxygen conditions and is involved in many cellular processes, such as angiogenesis and glycolysis [59,97], modulates the expression of IL-6 and influences inflammation [98]. The IFN-γ[99] and PI3K/Akt[100] pathways activate HIFs whose activity is regulated by their a-subunits (HIF-1α and HIF-2α), particularly under in hypoxic conditions. It has recently been reported that the activity of HIF-1α and HIF-2α in macrophages can be differentially regulated differently by cytokine stimuli [99,101]. HIF-1α inhibits proliferation, controls glycolysis and is stabilized in hypoxic macrophages [101]. In contrast, HIF-2α promotes cell division, suppresses p53, stimulates LPS and IFN-γ and is not depend on ON [102]. Cross-regulation between pathways may occur in situations of acute inflammation; the environment may not be sufficiently hypoxic to stabilize HIF-1, resulting in its low expression [101,103]. Concluding the resolution phase of inflammation, we observed downregulation of IL-10 in macrophages exposed to *C. leptophloeos* at all concentrations of stem bark and at 350 µg/mL of leaf. IL-10 exerts a significant influence on the NF-κβ pathway, inhibiting its activation in immune cells such as macrophages and dendritic cells through the induction of inhibitory proteins such as Iκβα. However, IL-10 downregulation may be due to a variety of signaling pathways and multifactorial pro-inflammatory cytokines: TNF-α[104], IL-1β[103,105], IFN-γ[103], MAPKs [106,107]pathway and miRNAs (miR106a) [108]. This inhibition prevents the translocation of NF-κβ to the nucleus, thus reducing the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [109,110] and, at the same time, promotes the expression of anti-inflammatory genes, contributing to the resolution of inflammation and tissue healing [111].

During the different phases of inflammation, the NF-κβ pathway was inhibited mainly when treated with the stem bark. We found that the stem bark extracts had therapeutic potential for reducing

ox-inflammation through the action of condensed tannins, demonstrating their preclinical efficacy in vitro. In addition, the leaf extract showed great potential probably due to its high concentration of flavonoid C-glycosides, a potent antioxidant that could be used in the cosmetic industry, protecting the skin against premature ageing and damage caused by free radicals, UV rays and pollution. Therefore, the stem bark and leaf extracts of *C. leptophloeos* contribute to the development of therapies for inflammatory conditions and highlight the need for further studies to fully understand the mechanisms of action and the clinical and/or cosmetic efficacy of the extract.

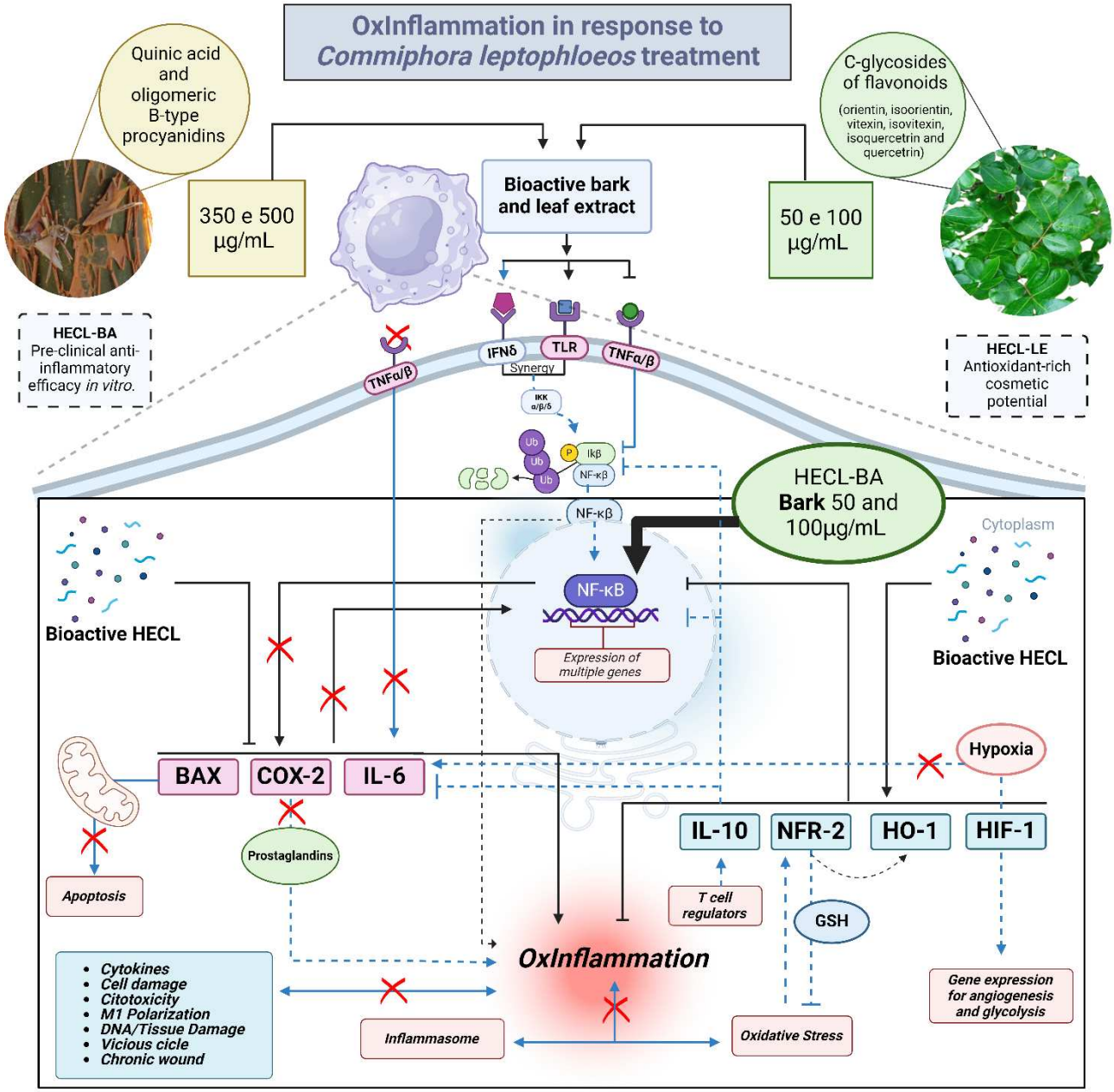


Figure 9. *TLR4/NF-κB/*inflammasome axis is involved in *IL-6*, *COX-2*, and *BAX* blocking after *Commiphora leptophloeos* extract exposure. Blue arrows (mechanisms described in the

literature); red arrows (mechanisms from our results). Figure created in BioRender.com: OxInflammation.

5. CONCLUSION

Commiphora leptophloeos extracts exhibited no protein-denaturing activity and no hemolytic activity against human erythrocytes. Furthermore, they inhibited over than 80% of the DPPH radical and demonstrated no cytotoxicity, maintaining cell viability above 70% even under oxidative stress induced by hydrogen peroxide. These findings highlight their low toxicity and significant antioxidant potential, capable of mitigating free radicals and reactive oxygen species (ROS) within cells. Moreover, the stem bark extract at concentrations exceeding 100 µg/mL and the leaf extract at concentrations above 500 µg/mL effectively reduced nitric oxide levels in LPS-stimulated cells.

The stem bark extract demonstrated greater anti-inflammatory potential at concentrations of 50 and 100 µg/mL compared to the leaf extract at concentrations of 350 and 500 µg/mL. Macrophages cells after exposure to *C. leptophloeos* extracts, presented a reduction in the expression of *TLR4* and consequently *NF-κβ*. This led to a decrease in pro-inflammatory cytokines such as *BAX*, *IL-6*, *TNF-α* and *COX-2*. Furthermore, an increase in *NRF-2* expression was observed when using the bark extract at 50 µg/mL. The NFR-2 controls oxidative stress and is involved in the resolution of acute inflammation. Our results indicate that the control of oxidative inflammation was mediated through the downregulation of *TLR4/NF-κβ* pathway and the upregulation of *NRF-2*, demonstrating a dose-dependent response. Additionally, we observed that the regulation of oxidative stress by *NRF-2* was associated with the expression of *HO-1* and hypoxia-induced factors. The decrease in *HO-1* and *HIF-1* expression may result from cross-regulation between *NF-κβ*, *HO-1* and *HIF-1*. The stem bark extracts demonstrated therapeutic potential in reducing oxidative inflammation and restoring cellular homeostasis, probably due to the presence of condensed tannins. Meanwhile, the leaf extract exhibited a notably high content of flavonoid C-glycosides, recognized as potent antioxidants with promising cosmetic potential. Overall, *C. leptophloeos* extracts possess bioactive compounds with antioxidant and anti-inflammatory properties, effectively modulating the OxInflammation process. However, further research is needed to fully elucidate the underlying mechanisms of action.

6. FUNDING SOURCES

FAPEMIG, (processes APQ-03519-22 and APQ-04164-22), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (310413/2023-0). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001. Reggiani Vilela Gonçalves is a research productivity fellow of the National Council for Scientific and Technological Development (CNPq) - Level 1D.

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