**UNIVERSIDADE CEUMA**
**PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE E BIOTECNOLOGIA DA REDE BIONORTE**

# EFEITO DO CINAMALDEÍDO EM MODELOS DE PATOLOGIA CUTÂNEAS CAUSADAS POR *Staphylococcus aureus.*

**CRISTIANE SANTOS SILVA E SILVA FIGUEIREDO**

**São Luís – MA**
**2019**

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Biodiversidade e Biotecnologia da Rede BIONORTE, na Universidade Ceuma, como requisito parcial para a obtenção do Título de Doutora em Biotecnologia.

**Orientador (a)**: Prof. Dr. Luís Cláudio Nascimento da Silva.
**Co-orientador (a)**:Prof. Dr. Marcos Augusto Grigolin Gristotto.

**São Luís – MA**
**2019**

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# RESUMO

FIGUEIREDO, Cristiane santos Silva e Silva. **Efeito do cinamaldeído em modelos de patologia cutâneas causadas por *Staphylococcus aureus*.** 2019. 155 f. Tese (Doutorado em Biotecnologia-Rede Bionorte) - Universidade Ceuma, 2019.

Feridas graves resultam em grandes lesões e/ou perda da função nas áreas afetadas e o tratamento tem desafiado os profissionais de saúde devido à sua complexidade, principalmente em pacientes com doenças crônicas (ex.: diabetes), e a presença de patógenos como *Staphylococcus aureus* e *Pseudomonas aeruginosa*. Levando isso em consideração, o desenvolvimento de novas terapias para a cicatrização de feridas requer atenção imediata. Verificou-se que várias preparações obtidas da família Asteraceae mostram boa eficácia quando avaliadas em ensaios clínicos de feridas complicadas, incluindo úlceras de perna venosas e úlceras de pé de pacientes diabéticos. Cinamaldeído (CNM) possui ação *in vitro* antimicrobiana de amplo espectro relacionada com instabilidade da membrana/parede celular e alterações no metabolismo energético. CNM ao ser administrado em feridas cutâneas infectadas por *Staphylococcus aureus*, que por ser da microbiota da pele, é apontado como microrganismo patogênico de maior ocorrência em feridas cutâneas, melhora o processo de cicatrização de lesões cutâneas infectadas pelo patógeno, possivelmente devido sua ação antimicrobiana e anti-inflamatória, possivelemente devido a sua ação antimicribiana e anti-inflamatória. Já que reduziu tanto a carga antimicrobiana como a produção de citocinas inflamatórias como TNF-α e IL-6 e acelerou o processo de cicatrização.

**Palavras-Chave:** *Staphylococcus aureus*. Cicatrização. Cinamaldeído.

## ABSTRACT

FIGUEIREDO, Cristiane santos Silva e Silva. **Effect of cinnamaldehyde on skin pathology models caused by *Staphylococcus aureus.*** 2019. 155 f. Tese (Doutorado em Biotecnologia-Rede Bionorte) - Universidade Ceuma, 2019.

Severe injuries result in serious injury and or loss of function in the affected areas and treatment has challenged health professionals because of its complexity, especially in patients with chronic diseases (eg.: diabetes), and the presence of pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Given this, the development of new wound healing therapies requires immediate attention. Several preparations obtained from the Asteraceae family have been found to show good efficacy when evaluated in complicated wound clinical trials, including venous leg ulcers and foot ulcers of diabetic patients. Cinnamaldehyde (CNM) has broad spectrum in vitro antimicrobial action related to membrane cell wall instability and changes in energy metabolism. CNM, when administered to *Staphylococcus aureus* infected skin wounds, which is considered to be the most common pathogenic microorganism in skin wounds, improves the healing process of pathogen-infected skin lesions, possibly due to its antimicrobial action. anti-inflammatory, possibly due to its anti-microbial and anti-inflammatory action. Since it reduced both antimicrobial load and production of inflammatory cytokines such as TNF-α and IL-6 and accelerated the healing process


**Keywords:** *Staphylococcus aureus.* Cicatrization. Cinnamaldehyde.

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## 1. INTRODUÇÃO

As doenças infecciosas causadas por microrganismos ainda representam importante causa de morbidade e mortalidade entre os seres humanos, especialmente nos países em desenvolvimento como o Brasil (DE SOUZA; MACHADO, 2019; MACHADO et al., 2017). Sem dúvida, a descoberta dos antibióticos na década de 1930 revolucionou a medicina e mudou o tratamento de doenças infecciosas, resultando em aumento da expectativa e da qualidade de vida humana (NICOLAOU; RIGOL, 2018). No entanto, desde que essas drogas foram introduzidas, a resistência microbiana evoluiu e se espalhou muito rapidamente, não estando mais restrita aos ambientes hospitalares (DE OLIVEIRA SANTOS et al., 2019; SHRESTHA; BARAL; KHANAL, 2019). Atualmente, um ponto crítico foi atingido, pois as novas drogas não estão sendo desenvolvidas no ritmo necessário para conter esta capacidade natural dos patógenos em adquirir resistência aos antibióticos (NICOLAOU; RIGOL, 2018)

Neste contexto, o tratamento de infecções cutâneas tem sido cada vez mais desafiador para os serviços de saúde em todo o mundo (KADAM et al., 2019). As lesões cutâneas constituem porta de entrada para microrganismos que expressam diferentes fatores de virulência relacionados com a destruição de células do hospedeiro e a evasão do sistema imune (GEISINGER; ISBERG, 2017; JACQUET et al., 2019). Por isso, as infecções microbianas são destacadas como as causas mais importantes de feridas crônicas; estando geralmente associadas com a formação de biofilmes, que são notoriamente recalcitrantes aos antibióticos convencionais (KADAM et al., 2019).

*Staphylococcus aureus* é uma bactéria, com elevado número de linhagens, as quais exibem complexas combinações de genes de virulência e resistência (BUKOWSKI et al., 2019; JACQUET et al., 2019). Este patógeno Gram-positivo é encontrado como parte da microbiota normal em vários locais do corpo humano tais como vias aéreas e pele (DARISIPUDI et al., 2018), o que facilita a contaminação de feridas por esse microrganismo. De fato, *S. aureus* é um dos patógenos mais comumente encontrados em lesões cutâneas (HOBBS et al., 2018; PETRY et al., 2014). Tomados em conjunto, todos estes fatores mostram a urgente necessidade de novas abordagens visando o tratamento de infecções causadas por *S. aureus*.

Os produtos naturais são apontados como boa alternativa para desenvolvimento de novos agentes antimicrobianos e cicatrizantes. Tem-se por exemplo, o cinamaldeído (CNM), um composto presente no óleo de plantas do gênero *Cinnamomum* (Lauraceae) especialmente a

canela, que tem sido destacado por suas propriedades antimicrobianas (ALBANO et al., 2019; FRIEDMAN, 2017). O CNM possui comprovada ação cicatrizante (YUAN et al., 2018), e outras ações farmacológicas que facilitam este processo como o efeito anti-inflamatório (MENDES et al., 2016) e antioxidante (MATEEN et al., 2019). Há evidências *in vitro* e *in vivo* da ação imunomoduladora do cinamaldeído na regulação de mediadores inflamatórios (citocinas, óxido nítrico (NO) e prostaglandina E2) e na modulação da migração celular, em diferentes modelos de inflamação (MENDES et al., 2016) (KIM, M. E.; NA; LEE, 2018).

O cinamaldeído é capaz de inibir diferentes fatores de virulência de *S. aureus* relacionados com a instauração de infecções cutâneas como a hemolisina e a formação de biofilmes (FERRO, T. A. et al., 2016; KOT et al., 2018). O tratamento com este composto aumentou a sobrevivência de larvas de *Galleria mellonella* infectadas por *S. aureus*. Recentemente, foi demonstrado que o uso tópico de CNM acelerou a cicatrização de feridas infectadas por *Pseudomonas aeruginosa*, reduzindo a carga bacteriana no tecido e as concentrações de mediadores inflamatórios (interleucina-17, fator de crescimento endotelial vascular e óxido nítrico) (FERRO, T. A. F. et al., 2019). As ações anti-inflamatória e cicatrizante do CNM estão relacionadas com a ativação do Receptor de Potencial Transiente Ankiryn 1 (TRPA1) (FERRO, T. A. F. et al., 2019; MENDES et al., 2016).

Todos estes atributos sugerem que o cinamaldeído é um excelente alvo para o desenvolvimento de agentes para o tratamento de infecções causadas por *S. aureus*. No entanto, não há relatos da ação terapêutica deste composto em modelos de infecção em murinos provocados por este patógeno. O presente estudo teve como objetivo principal avaliar o efeito da administração tópica do cinamaldeído no tratamento de lesões cutâneas infectadas por *S. aureus*.

# 2. REVISÃO BIBLIOGRÁFICA

## 2.1 Fundamentos de fisiologia e imunologia da pele

A pele é o maior e o mais visível órgão do corpo estando assim susceptível aos mais diversos tipos de injúrias. Formado por células justapostas, a pele é uma barreira física que recobre todo o corpo e tem a função de proteger o organismo de variações de temperatura, traumas, patógenos, toxinas, substâncias alergênicas e raios UVA e UVB. Faz também o controle dos níveis de água, eletrólitos e macromoléculas; além de atuar na percepção de sensações como frio calor, dor e tato (FORE, 2006; PIOTROWSKA; VISSCHER et al., 2015; WIERZBICKA; ZMIJEWSKI, 2016). A visão atual é que a pele é um órgão ativamente envolvido no metabolismo de proteínas, lipídios e outras moléculas sinalizadoras, e faz parte dos sistemas imunológico, nervoso e endócrino (HSU, Y. C.; LI; FUCHS, 2014; PAUS, 2016; JIA, Y. et al., 2018).

Duas camadas distintas e firmemente unidas compõe a pele: epiderme (mais superficial) e derme (mais profunda). Uma terceira camada, chamada de hipoderme, é localizada mais profundamente é constituída principalmente por tecido adiposo. A composição e a espessura da epiderme e derme variam dependendo da localização no corpo. A epiderme é constituída principalmente por queratinócitos que estão intimamente ligados uns aos outros, formando uma barreira limitando o acesso ao ambiente interno (YOUSEF; ALHAJJ; SHARMA, 2019). Os queratinócitos também participam da inflamação, pois podem secretar citocinas e apresentar antígenos via complexo principal de histocompatibilidade (MHC-II). (TAKAGI et al., 2006; ASAHINA; MAEDA, 2017). Outras células presentes na epiderme são os melanócitos (produzem melanina e expressam MHC-II, células de Langerhans (tipo de células dendríticas que apresentam antígenos móveis) e células de Merkel (com características neuroendócrinas e epiteliais) (MALISSEN; TAMOUTOUNOUR; HENRI, 2014; OGAWA; KAWAMURA; SHIMADA, 2016; KAPLAN, 2017). Od linfócitos B de memória também podem ser encontradas na epiderme, enquanto neutrófilos são recrutados em resposta ao dano tecidual (RICHMOND; HARRIS, 2014; LI, Y. H. et al., 2015). Existem três apêndices epidérmicos: as glândulas sudoríparas (que são termorreguladoras e secretam peptídeos antimicrobianos); os folículos pilossebáceos que produzem os pelos e as excreções sebáceas; e as unhas que cobrem as falanges distais (FORE, 2006; LOSQUADRO, 2017).

A derme é composta principalmente de proteínas da matriz extracelular que dão estrutura e elasticidade à pele, permitindo a livre migração de várias populações celulares. Esta estrutura tem a função de fornecer nutrientes e suporte circulatório a epiderme (RICHMOND; HARRIS, 2014). A derme e a epiderme são separadas pela membrana basal, uma fina camada de proteínas da matriz extracelular que regula o movimento de células e proteínas entre essas duas camadas. O tecido conjuntivo da derme é formado por proteínas fibrosas como colágeno, elastina e reticulina, que se encontram dispostas em uma substância amorfa de mucopolissacarídeos (FORE, 2006; LOSQUADRO, 2017).

Os fibroblastos são os principais tipos celulares da derme que realizam a síntese de proteínas estruturais como colágeno, elastina e substância fundamental amorfa para formação da matriz extracelular (RICHMOND; HARRIS, 2014). Estas células atuam no reparo da pele lesionada, pois fornecem apoio estrutural e guiam a migração das células imunes, assegurando o importante contato célula-célula (VAN LINTHOUT; MITEVA; TSCHOPE, 2014; WOODLEY, 2017), tendo a capacidade de produzir citocinas (NASTRI et al., 2018). Outras células presentes na derme são os mastócitos, plasmócitos, células dendríticas, células assassinas naturais (NK), macrófagos e linfócitos T (FEUERSTEIN; KOLTER; HENNEKE, 2017). Nesta região encontram-se também vasos sanguíneos, linfáticos, terminações nervosas, folículos pilosos, músculos eretores do pelo, glândulas sebáceas e sudoríparas (FORE, 2006; LOSQUADRO, 2017).

A função de barreira da pele é de importância crítica, pois quando é rompida uma cascata de reações bioquímicas, visando o processo de cicatrização é ativada para reparar o dano (TAKEO; LEE; ITO, 2015). O funcionamento adequado da pele nesses papéis requer estreita comunicação e colaboração entre vários tipos de células, incluindo células estromais (queratinócitos, fibroblastos, células endoteliais e adipócitos), bem como aquelas derivadas da medula óssea (células dendríticas, macrófagos, células NK, mastócitos, células T e outros) (ALI; ROSENBLUM, 2017; MALISSEN; TAMOUTOUNOUR; HENRI, 2014).

Este complexo órgão possui uma variedade de células residentes que atuam na detecção de microorganismos patogênicos (ou na prevenção de infecções). Quando ocorre dano tecidual, são liberados sinais moleculares denominados DAMPs (padrões moleculares associados ao dano; do inglês *Danger-associated molecular patterns*) que podem ser detectados por receptores para padrões moleculares presentes em células residentes na pele. Estes receptores

também podem identificar padrões moleculares associados aos patógenos (PAMPs; do inglês *Pathogen-associated molecular patterns*) (RICHMOND; HARRIS, 2014). As principais estruturas envolvidas no reconhecimento de DAMPs e PAMPs são os chamados receptores semelhantes ao *Toll* (TLR; do inglês *Toll-like receptors*) (CHEN, L.; DIPIETRO, 2017; EGERT; SIMMERING; RIEDEL, 2017).

A ativação destes receptores resulta na ativação da resposta imune inata (caracterizada por ações rápidas, porém inespecíficas) levando ao recrutamento de células inflamatórias do sangue para o tecido (neutrófilos, monócitos). (RICHMOND; HARRIS, 2014; CHEN, L.; DIPIETRO, 2017) Em seguida, a resposta imune adaptativa (mais lenta e específica) é ativada garantindo a depuração definitiva dos patógenos além de prover células de memória para proteger contra futuras reinvasões. Neste caso estas células de memória são mantidas no tecido após a resolução da lesão/infecção (ALI; ROSENBLUM, 2017; SUWANPRADID; HOLCOMB; MACLEOD, 2017; DEBES; MCGETTIGAN, 2019).

### 2.2 As feridas como um problema de saúde pública

As feridas constituem um sério problema de saúde pública devido ao crescente número de pessoas acometidas e as consequências sociais, psicológicas e econômicas relacionadas. Este quadro contribui para o aumento dos gastos públicos, além de interferir na qualidade de vida da população (GUEST et al., 2017; MITCHELL; CURTIS; BRAITHWAITE, 2017). É evidente que as lesões acometem pessoas independente do sexo, idade ou etnia. No entanto, a prevalência de feridas vem crescendo em decorrência da mudança do perfil da população mundial que envolvem o aumento da longevidade, acompanhado de hábitos de vida inadequados que estão relacionados a altos índices de doenças crônicas como diabetes mellitus e patologias vasculares (CHENG et al., 2013; DALYS; COLLABORATORS, 2018).

O tratamento das feridas tem desafiado os sistemas de saúde por todo o mundo por exigir acompanhamento e tratamento adequado para que as lesões não progridam culminando na perda de função da região acometida (DE LEON et al., 2016; JARBRINK et al., 2017; KAPP; MILLER; SANTAMARIA, 2018). O tratamento de feridas crônicas frequentemente envolve longos períodos de internação e/ou diversas sessões de consultas ambulatoriais com intervenções terapêuticas avançadas dispendiosas (SNYDER; FIFE; MOORE, 2016; NEWTON, 2017).

As feridas aumentam substancialmente o risco de contaminação microbiana, já que as

lesões constituem uma porta de entrada para os microrganismos (BUCH; CHAI; GOLUCH, 2019; GEISINGER; ISBERG, 2017; JACQUET et al., 2019). As infecções microbianas são destacadas como as causas mais importantes de feridas crônicas, geralmente associadas com a formação de biofilmes, pouco sensíveis, aos antibióticos convencionais (KADAM et al., 2019; MORGAN et al., 2019). Com isso, a avaliação e classificação das feridas é um fator importante para o direcionamento da equipe de profissionais de saúde (CHILDS; MURTHY, 2017; NEWTON, 2017; WELSH, 2018), pois, o manejo correto das feridas (principalmente daquelas classificadas como crônicas) é um processo complexo. Também é indispensável reconhecer os agentes etiológicos, as diferentes fases do processo de cicatrização, os produtos e intervenções disponíveis para o tratamento (HARDING, 2015; NEWTON, 2017; WELSH, 2018).

### 2.3 Classificação das feridas

A classificação das feridas é realizada a partir de um vasto número de instrumentos de verificação, escalas de classificação e pontuação. Todos destinados a classificar as feridas por tipo, descrever suas características, medir sua gravidade e prever a evolução do processo de cicatrização. Cada sistema possui finalidades distintas e sua seleção deve ser avaliada de acordo com a necessidade do profissional (ARNDT; KELECHI, 2014; FRYKBERG; BANKS, 2015). No processo de avaliação e classificação das lesões são analisados diversos fatores como a localização, tipo de tecido, secreção, odor, bordas, edema, medida e dor, assim como profundidade e grau de contaminação (KIM, J.; SIMON, 2018; KOTTNER; CLARK, 2019).

Em geral, as feridas podem ser classificadas de acordo com a complexidade, quanto a profundidade, evolução, grau de contaminação, características da cicatrização e de acordo com o agente causal da ferida (ARNDT; KELECHI, 2014; KIM, J.; SIMON, 2018). As lesões consideradas como simples evoluem espontaneamente para resolução da ferida. Diferentemente, as feridas que não apresentam o processo normal de cicatrização (inflamação, proliferação e remodelagem tecidual) são denominadas como complexas (SEEBAUER et al., 2019). As feridas complexas representam ameaça à viabilidade do membro afetado por acometerem áreas extensas e/ou profundas e necessitam de recursos especiais para sua resolução (KIM, J.; SIMON, 2018; MIYAGI; SHARMA; PRICE, 2011).

A classificação quanto à profundidade da ferida, geralmente é utilizada para descrever queimaduras e, às vezes, lesão de pressão. Nestes casos a profundidade é uma medida

de gravidade (MARTIN, N. A.; FALDER, 2017). A avaliação precisa da profundidade das queimaduras é uma tarefa crítica para fornecer o tratamento e cuidados corretos (RANGARAJU et al., 2019; SAIKO, 2017). Clinicamente, é necessário identificar a epiderme, a derme e o tecido subcutâneo e diferenciar o tecido granulado e o músculo, tendões e ossos. Dessa forma, a ferida é classificada em superficial ou parcial quando atinge apenas a epiderme, ou derme sem atravessá-la; e profunda ou total quando envolve também o subcutâneo, músculos e ossos (MARTIN, N. A.; FALDER, 2017; SAIKO, 2017).

As feridas podem ainda ser classificadas em relação a evolução em agudas ou crônicas (DAS; BAKER, 2016; HSU, J. T. et al., 2019). As feridas agudas são consideradas desde um simples arranhão até uma lesão profunda que conseguem uma cura completa, com o mínimo ou nenhuma formação de cicatrizes, em um prazo de três semanas. As feridas agudas podem ocorrer intencionalmente ou de forma traumática. As agudas intencionais são as feridas cirúrgicas, que na maioria das vezes são submetidas à cicatrização por primeira intenção, com a junção da borda por fios cirúrgicos ou grampos (NORMAN; DUMVILLE; CROSBIE, 2016; O'MATHUNA, 2016). As agudas traumáticas são aquelas que podem ocorrer por acidentes cortantes, automobilísticos, entre outros, e o seu processo cicatricial depende do agente causador da lesão, do tipo de contaminação que foi exposto, tempo do início do atendimento e materiais cicatrizantes utilizados (MANSOOR et al., 2015; UBBINK et al., 2015).

As feridas crônicas são aquelas onde existe múltipla associação de fatores (metabólicos, psicológicos, ambientais e comportamentais) influenciam negativamente o processo natural de cicatrização (superior a três semanas) (WERNICK; STAWICKI, 2019). Estas lesões estão relacionadas como grandes desafios para os sistemas de saúde em todo mundo (MUKHERJEE et al., 2014; KATHAWALA et al., 2019; KOSARIC; KIWANUKA; GURTNER, 2019). As feridas crônicas podem ser referidas como úlcera do pé diabético, úlceras venosas da perna e úlcera por pressão, infecção do sítio cirúrgico, abscesso ou úlceras por trauma. Na maioria das vezes, as feridas crônicas estão associadas a históricos de doenças pregressas, como diabetes mellitus, obesidade, hipertensão arterial, neoplasias, hanseníase, estresse, tabagismos. Podendo também ser resultado de agregação de duas ou mais doenças (comorbidades) (SATHIARAJ; NORMAN; RICHARD, 2010; ROSOWSKI; HUTTENLOCHER, 2015; POWERS et al., 2016; YAO et al., 2016; BUCH; CHAI; GOLUCH, 2019).

A idade e o estado nutricional (deficiência em proteínas, vitaminas e sais minerais retardam a cicatrização) do paciente também desempenham papéis importantes no desenvolvimento de feridas crônicas (MOLNAR; VLAD; GUMUS, 2016; KATHAWALA et al., 2019). Como características estas feridas apresentam elevado nível de exsudato (em razão do prolongamento ou estacionamento no período de inflamação), destruição dos tecidos adjacentes o que geralmente leva a uma predisposição às infecções (TURI et al., 2016; ZHAO, R. et al., 2016; SIBBALD et al., 2017).

### 2.4 Visão geral do processo de cicatrização de feridas

A cicatrização das feridas é um processo bioquímico, celular e imunológico complexo que visa restaurar e restabelece as funções do tecido lesionado (SEEBAUER et al., 2019). O processo cicatricial é comum a todas as feridas, independentemente do agente que a causou, é sistêmico e dinâmico e está diretamente relacionado às condições gerais do organismo (WERNICK; STAWICKI, 2019). Este processo caracteriza-se por uma cascata de eventos celulares, moleculares e bioquímicos que interagem para que ocorra a reconstituição tecidual (KRISHNASWAMY; MINTZ; SAGI, 2017; WANG, P. H. et al., 2018; WERNICK; STAWICKI, 2019). Em indivíduos saudáveis, o restabelecimento de uma barreira epidérmica funcional é altamente eficiente, ao passo que o reparo da camada dérmica mais profunda é menos perfeito e resulta na formação de cicatriz com uma perda substancial da estrutura e função tecidual original. Quando a resposta normal do reparo dá errado, há dois resultados principais: defeito cutâneo ulcerativo (ferida crônica) ou formação excessiva de tecido cicatricial (cicatriz hipertrófica ou queloide) ( PAZYAR et al., 2014; MARTIN, P.; NUNAN, 2015).

O processo de cicatrização consiste em fases distintas e superpostas que são denominadas de (i) hemostasia e inflamação, (ii) proliferação e (iii) remodelação tecidual (Figura 1) (CARVALHO et al., 2018; WERNICK; STAWICKI, 2019). A primeira etapa da cicatrização ocorre com o rompimento da integridade tecidual, ocorre a liberação imediata de tromboxano A2 e prostaglandina que medeiam a vasoconstrição. Essa fase se prolonga em média de 24 a 72 horas. Paralelamente a este processo, inicia-se a cascata de coagulação. As plaquetas chegam induzindo a produção de um coágulo sanguíneo para conter o sangramento, e viabilizar a formação de uma matriz rica em fatores de crescimento e quimiocinas (HOFFMAN, 2018; RODRIGUES et al., 2019). Esses fatores quimiotáticos incluem o fator de crescimento endotelial

vascular (VEGF), fator de crescimento derivado de plaquetas (PDGF), fator de crescimento epidérmico (EGF), e fator de crescimento de fibroblastos (FGF) e citocinas, sendo, o fator de necrose tumoral alfa (TNF-α) e interleucinas (IL-1, IL-6), que em conjunto, promovem a migração de células inflamatórias e células estromais para o local da ferida (REINKE; SORG, 2012; RODRIGUES et al., 2019; WANG, P. H. et al., 2018).

A fase inflamatória é marcada pela ação de neutrófilos e macrófagos. A presença de microrganismos patogênicos e/ou tecidos necrosados irá estender o período de inflamação, provocando atrasos significativos no processo de cicatrização (RAHIM et al., 2017). Os neutrófilos migram para as margens da ferida em aproximadamente 24 horas. Estas células são responsáveis pelos processos de esterilização (e prevenção de possíveis contaminantes) e degradação dos restos celulares através da produção de espécies reativas e citocinas pró-inflamatórias que amplificam a resposta inflamatória (RODRIGUES et al., 2019; WANG, P. H. et al., 2018).

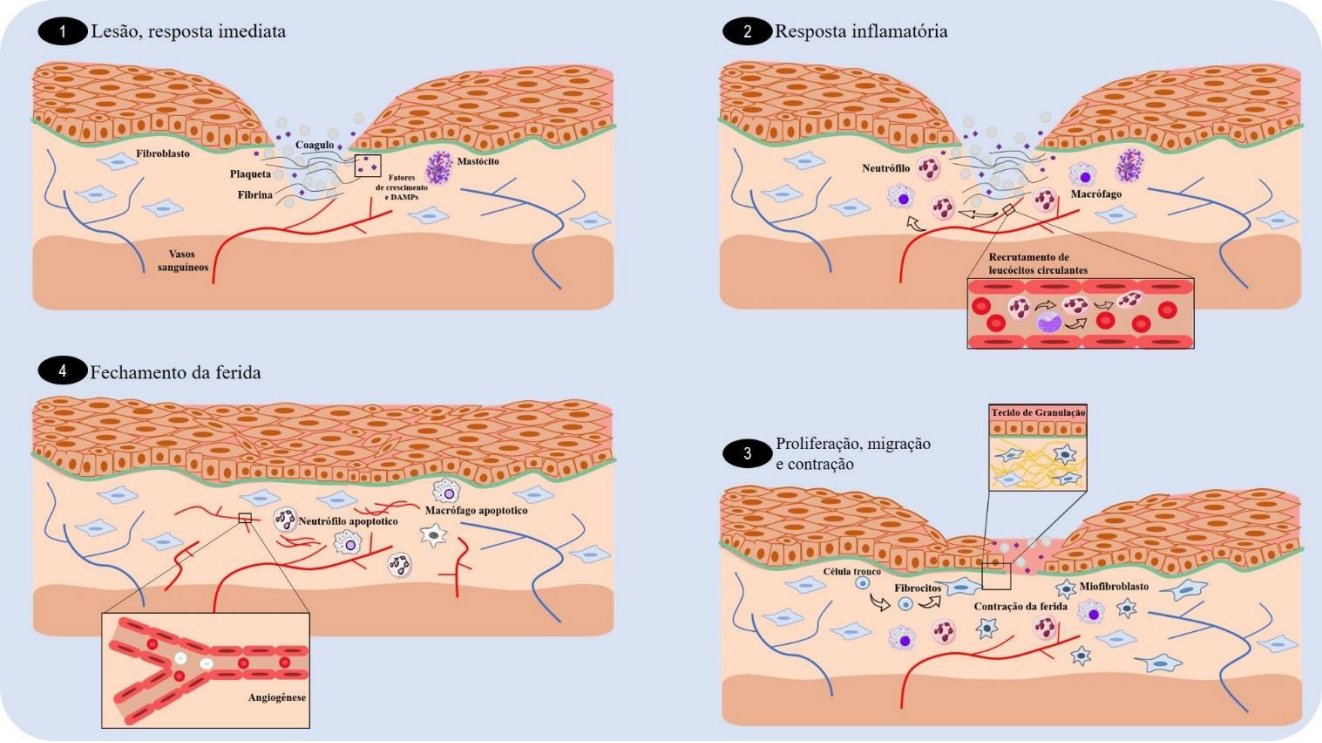


**FIGURA 1.** Visão geral dos estágios essenciais da cicatrização de feridas. Existem quatro estágios fundamentais envolvidos na cicatrização de feridas: a resposta imediata (1), a inflamação (2), a proliferação (3) e o fechamento da ferida (4). Fonte: Carvalho, 2018.

Neutrófilos também produzem Serinas Proteases e Metaloproteinases da Matriz (MMPs), enzimas cruciais para o correto andamento do processo cicatricial

(KRISHNASWAMY; MINTZ; SAGI, 2017; ROHANI; PARKS, 2015). MMPs são dependentes de zinco e regulam eventos relacionadas a morte celular e inflamação influenciando indiretamente o comportamento celular (ROHANI; PARKS, 2015). No entanto, a atividade elevada destas proteínas pode induzir a formação de feridas crônicas (LAZARO et al., 2016).

Com a diminuição dos níveis de neutrófilos, os macrófagos tornam-se o tipo de célula predominante nas feridas. Os macrófagos têm dois papéis principais no processo de cicatrização: (i) englobamento de neutrófilos necróticos ou apoptóticos, fornecendo um importante mecanismo de depuração, (ii) e produção de citocinas, quimiocinas e fatores de crescimento que estimulam a reação inflamatória, ajudam a recrutar mais células inflamatórias e promovem a fase proliferativa de reparação, incluindo angiogênese e regeneração de tecidos (MIRZA et al., 2015; SORG et al., 2017).

Desta maneira, os macrófagos desempenham papéis chaves na progressão da fase inflamatória para a fase proliferativa (KOH; DIPIETRO, 2011; MANTOVANI et al., 2013). Os macrófagos (residentes na pele ou diferenciados a partir dos monócitos infiltrantes) assumem primeiramente um fenótipo pró-inflamatório conhecida com subgrupo M1 (ativação clássica) associados à atividade fagocitária, sequestro e produção de mediadores pró-inflamatórios (como TNF-α e IL-6) (HESKETH et al., 2017; VANNELLA; WYNN, 2017). Mais tarde, os macrófagos se diferenciam em um fenótipo reparativo denominado de subconjunto M2 (ativação alternativa) que estão envolvidos na síntese de mediadores anti-inflamatórios e na produção de matriz extracelular, no início da proliferação de fibroblastos, bem como nos processos angiogênicos (KOH; DIPIETRO, 2011; SNYDER et al., 2016).

Os macrófagos M2 também fagocitam os neutrófilos (ou seja, efferocitose), bactérias e detritos celulares, a fim de evitar mais danos ao local da ferida nas fases posteriores da cicatrização. Se a transição de M1 para M2 não ocorrer de maneira correta, não haverá progressão para a fase proliferativa, levando a formação de feridas crônicas ou não curativas (KOTWAL; CHIEN, 2017; MANTOVANI et al., 2013). O predomínio de macrófagos M2 sinaliza então para o início do segundo estágio da cicatrização, a fase proliferativa subdividida em reepitelização (movimentação das células epiteliais oriundas tanto da margem como de apêndices epidérmicos localizados no centro da lesão), fibroplasia e angiogênese (marcada pela formação do tecido de granulação responsável pela ocupação do tecido lesionado) (PAZYAR et al., 2014; SORG et al., 2017). O sucesso da epitelização depende dos aspectos específicos da

ferida, tais como a localização, a profundidade, o tamanho, a contaminação microbiana, bem como as condições de saúde relacionadas com o paciente, a genética e a epigenética (RITTIE, 2016; RITTIE et al., 2016; SORG et al., 2017).

A reepitelização começa pela conversão de queratinócitos estacionários em queratinócitos migratórios planos, que ativados reorganizam seu citoesqueleto e migram a fim de restaurar o tecido de forma rápida e suficiente para evitar perda de fluido ou infecção adicional (QING, 2017; RITTIE, 2016). No decorrer do processo, os contatos firmes de célula-célula são restabelecidos e os queratinócitos readquirem seu fenótipo em forma de paralelepípedo quiescente seguido de estratificação epidérmica (CHOMISKI et al., 2016; LAW et al., 2017). O pré-requisito para uma epitelização eficaz é uma matriz extracelular apropriada que facilite a migração dos queratinócitos (RITTIE, 2016; WANG, P. H. et al., 2018). Em paralelo, diversos fatores de crescimento (como o FGF e EGF) são direcionados aos fibroblastos e fazem com que estas células migrem dos tecidos vizinhos para o local da lesão (fibroplasia). Os fibroblastos secretam substâncias importantes para a cicatrização como colágeno, fibronectina, elastina, glicosaminoglicanas e ácido hialurônico (LAW et al., 2017; SORG et al., 2017).

Outro fator crucial é a angiogênese já que a intensa atividade celular, durante todo o processo de reparo da ferida, demanda o transporte de oxigênio e nutrientes. Além disso, há necessidade de novos vasos para migração de células imunológicas (MARTIN, P.; NUNAN, 2015). O TNF-α e VEGF agem nas células endoteliais ativando da angiogênese. Diversos estudos demonstram que angiogênese necessita ser finamente regulada para influenciar positivamente o processo cicatricial (DIPIETRO, 2016). É importante ressaltar que o nível de angiogênese nas feridas geralmente se correlaciona com a resposta inflamatória, principalmente porque as células inflamatórias produzem uma abundância de mediadores pro-angiogênicos (CHEN, L.; DIPIETRO, 2017).

A cicatrização continua com a produção de um tecido de granulação constituído de macrófagos, fibroblastos, vasos sanguíneos, glicoproteína, fibronectina e ácido hialurônico (KIM, D. J.; MUSTOE; CLARK, 2015). O tecido de granulação é a principal evidência macroscópica da deposição de novo tecido conjuntivo e possui aspecto granuloso e coloração avermelhada, devido à inúmeros capilares enovelados circundando células (macrófagos, fibroblastos) e a matriz extracelular. A formação de tecido de granulação saudável no leito da ferida é um dos fatores que facilita a cicatrização de feridas, sendo que a ausência deste favorece a formação de feridas

crônicas (SINGH et al., 2017; WANG, P. H. et al., 2018).

A cascata de eventos cicatriciais tem fim com a fase de remodelação, que leva a formação de uma cicatriz (rica em colágeno) que substitui o tecido original. Esta fase é caracterizada por etapas de produção, deposição, digestão e reorganização das fibras de colágenos (produzidas pelos fibroblastos presentes na ferida) (PAZYAR et al., 2014). Inicialmente, é produzido um colágeno provisório (mais fino que o encontrado no tecido original; denominado tipo III) que deve ser substituído por um colágeno mais espesso e organizado (apresentando fibras maiores, com maior número de fibrilas e com quantidade significativa de ligações cruzadas entre elas; denominado tipo I). As fibras tipo I são depositadas obedecendo a orientação e organização das fibronectinas e a direção da natureza das forças de tensão aplicadas sobre o tecido (SORG et al., 2017).

O sucesso desta fase de remodelagem é garantido pelas MMPs (produzidas pelos macrófagos, neutrófilos, fibroblastos e células epiteliais) que são responsáveis pela digestão das fibras de colágeno tipo III (SORG et al., 2017). Esta ação deve ser equilibrada com a síntese de novas fibras tipo I, graças o efeito de inibidores de MMPs (AYUK; ABRAHAMSE; HOURELD, 2016). As novas fibras são organizadas conforme o sentido das fibras do tecido conjuntivo adjacente. Como resultado há formação de fibras de colágeno maiores e uma cicatriz com aparência mais uniforme ( LAZARO et al., 2016; KRISHNASWAMY; MINTZ; SAGI, 2017).

Os miofibroblastos desempenham papel essencial na contração final da ferida. Estas células são derivadas de fibroblastos que sofreram alterações fenotípicas durante a síntese de tecido de granulação (SORG et al., 2017). A contração ocorre ao longo da direção das linhas de tensão cutânea e depende de vários mediadores (serotonina, bradicinina, epinefrina, angiotensina) e da comunicação entre as células e a matriz extracelular (fibronectina). Os miofibroblastos produzem fibronectina, colágeno, ácidos aminoglicanos e trombospondina. A fibronectina é responsável pela ligação dos fibroblastos à matriz extracelular. Terminada esta etapa, há uma regeneração limitada dos anexos da pele, como folículos pilosos e glândulas. Ocorre diminuição da vascularização e os vasos neoformados sofrem apoptose fenômeno denominado regressão endotelial. O resultado é uma cicatriz de coloração similar ao tecido original e que pode apresentar 80% da força de tensão original (MARTIN, P.; NUNAN, 2015; DIPIETRO, 2016).

### 2.5 *Staphylococcus aureus* e a infecção de feridas

Como mencionado anteriormente, a contaminação de uma ferida por microrganismos patogênicos retarda significativamente o processo de cicatrização. Estes patógenos são identificados pelos receptores das células imune através dos PAMPs. Eles também liberam fatores de virulência capazes de induzir danos nas células do hospedeiro, levando a liberação de substâncias sinalizadoras denominadas de DAMPs. A persistência dos microrganismos no local da ferida leva a um quadro de resposta imunológica exacerbada (PORTOU et al., 2015; CHEN, L.; DIPIETRO, 2017). Inflamação persistente pode resultar em cicatrização tardia da ferida, formação de cicatriz ou feridas crônicas (WANG, P. H. et al., 2018; WERNICK; STAWICKI, 2019).

Os principais patógenos envolvidos em contaminação de feridas são *Staphylococcus aureus*, *Acinetobacter baumannii, Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp., *Clostridium* spp., *Peptostreptococcus* spp. entre outras (PARK et al., 2016; FLEMING et al., 2017; RAHIM et al., 2017). Por ser uma bactéria que frequentemente coloniza a pele, *S. aureus* é um dos principais agentes responsáveis por infectar feridas cutâneas (GEOGHEGAN; IRVINE; FOSTER, 2018; MCNEIL; FRITZ, 2019). *S. aureus* é um microrganismo Gram-positivo, produtor de coagulase, pertencente ao grupo das eubactérias, que geralmente forma colônias amarelas, acinzentadas ou laranja, em função da grande quantidade de carotenóides localizados na membrana celular, como a estafiloxantina (GOLDMANN; MEDINA, 2018; TIWARI; GATTO; WILKINSON, 2018).

Caracterizado como patógeno oportunista associado à colonização assintomática de pele e mucosa, provoca infecção quando essas barreiras fisiológicas são rompidas, permitindo a invasão bacteriana (KRISMER et al., 2017; NGUYEN, A. T.; TALLENT, 2018; YANG, J. J. et al., 2018). Esta bactéria tem-se destacado devido à capacidade de expressar variedade de fatores de virulência que facilitam a adesão celular, a evasão do sistema imune, danos à célula hospedeira provocando os sinais e sintomas da doença (SHAHINI SHAMS ABADI et al., 2017; COPIN; SHOPSIN; TORRES, 2018; GOLDMANN; MEDINA, 2018). Esta versatilidade está relacionada com o amplo espectro de doenças que englobam desde infecções localizadas dos tecidos moles (por exemplo, impetigo e dermatite) e também complicações sistêmicas (por exemplo, bacteremia, sepse e síndrome do choque tóxico) ( PERES et al., 2015; HUNTER et al., 2016; VAN WAMEL, 2017).

Além disso, um número cada vez maior de linhagens de *S. aureus* tem demonstrado

resistência aos agentes antimicrobianos; por esta razão, *S. aureus* resistente à meticilina (MRSA) e *S. aureus* multirresistente (MDRSA) têm sido reconhecidos como as principais causas de infecções hospitalares ( HUNTER et al., 2016; FURTADO et al., 2019; NARAYANAN, N. et al., 2019). Embora tradicionalmente ligadas ao ambiente hospitalar, linhagens resistentes de *S. aureus* têm emergido na comunidade e aumentando tanto a frequência quanto a gravidade da infecção por esta bactéria (RICHARDSON et al., 2019). De fato, níveis alarmantes de resistência já são detectados em algumas localizações, chegando ao ponto de alguns isolados de *S. aureus* possuírem resistência para fármacos considerados como de última escolha, a exemplo de vancomicina (OLUFUNMISO; TOLULOPE; ROGER, 2017; VANEGAS MUNERA et al., 2017).

### 2.6 Óleos essenciais de plantas no tratamento das lesões cutâneas infectadas por *S. aureus*

O tratamento de lesões cutâneas ao longo do tempo passou por profundas transformações e cada vez mais novas abordagens terapêuticas para tratamento de feridas são estudadas (KUMAR et al., 2019). Cabe salientar que o cuidado de feridas é um processo dinâmico, complexo e que requer atenção especial principalmente quando se refere a uma lesão crônica (HAN; CEILLEY, 2017; HSU, J. T. et al., 2019). As feridas crônicas geralmente se caracterizam pela presença de bactérias, porém a sua presença no leito da ferida, não indica necessariamente que há infeção ( SORIA; CARRASCOSA, 2007; JOHNSON et al., 2018). Com o surgimento de linhagens bacterianas resistentes aos antibióticos, o tratamento de feridas infectadas tornou-se bem mais complicado devido a ineficácia dos antimicrobianos comumente utilizados (SALISBURY et al., 2018).

O tratamento tópico pelo uso de curativos, gazes e apósitos são historicamente utilizados pelo fácil manuseio, acessibilidade a população e por não necessitarem de receita médica (SOULIOTIS et al., 2016; CHAGANTI et al., 2019). Porém, é necessário a busca por alternativas que possam combater a infecção e controlar o desenvolvimento de resistência bacteriana ( REZVANIAN et al., 2017; KHAN; MUJAHID, 2019; ZENG et al., 2018). A partir disso, o uso de plantas com propriedades medicinais, antimicrobianas e cicatrizantes surgem como excelentes fontes para o tratamento de feridas (CARVALHO et al., 2018). O uso de fitoterápicos na cicatrização de feridas tem sido incrementado nos últimos anos com a busca de

princípios ativos, isolados de plantas, que apresentem efetivo papel no processo de cicatrização da ferida (ABD JALIL; KASMURI; HADI, 2017; SCHEFF; ANSARI, 2017).

Óleos essenciais (OE) são compostos aromáticos extraídos de várias partes de diversas plantas. São voláteis de baixo peso molecular, incluindo-se monoterpenos e sesquiterpenos. Os monoterpenos são responsáveis por cerca de 90% dos OE e são dotados de uma ampla gama de propriedades biológicas e farmacológicas com atividades antibacterianas e antifúngicas (LANGEVELD; VELDHUIZEN; BURT, 2014) além de efeitos antiinflamatórios (COSTA et al., 2019) e analgésicos (GUIMARAES; QUINTANS; QUINTANS, 2013). Essas propriedades fazem desses compostos fortes candidatos para o desenvolvimento de produtos para reparo de tecidos.

Uma revisão sistemática recente abordou a capacidade cicactrizante de óleos essências, e o potencial destes no desenvolvimento de preparações poliméricas. Os autores destacam que os OE de Lavandula, Croton, Blumea, Eucalyptus, Pinus, Cymbopogon, Cedrus, Abies, Rosmarinus, Origanum, Salvia e Plectranthus apresentaram resultados promissores em feridas de roedores; sendo capaz de aumentar a taxa de fechamento das lesões, melhorar a deposição de colágeno e/ou aumentar a proliferação de fibroblastos. Todos esses OE foram compostos principalmente por terpenos (timol, 1,8-cineol, linalol, limoneno ou pinenos). (PEREZ-RECALDE; RUIZ ARIAS; HERMIDA, 2018).

Aplicações de OE em humanos acometidos por feridas também são descritas na literatura. Por exemplo, uma criança hospitalizada na Unidade de Terapia Intensiva que foi tratada com OE para queimadura não desenvolveu nenhuma infecção na corrente sanguínea e teve um período de internação hospitalar de quatro dias a menos em relação a uma outra criança hospitalizada, na mesma unidade, que recebeu o tratamento padrão para queimaduras sendo ainda diagnosticada com duas infecções na corrente sanguínea e quatro condições adquiridas no hospital (JOPKE; SANDERS; WHITE-TRAUT, 2017).

Os OE também foram efetivamente utilizados para tratar feridas experimentais em camundongos infectadas por MRSA. Neste caso, a administração tópica de pomada contendo óleo essencial de endro (*Anethum graveolens*) (DEO) durante o processo de cicatrização de feridas induzidas experimentalmente por MRSA em camundongos BALB/c preveniu o crescimento bacteriano e também reduziu a área da ferida em comparação com o grupo controle, além de reduzir significativamente a fase inflamatória e acelerou a reepitelização, angiogênese,

fibroblastos e deposição de colágeno (MANZUOERH et al., 2019).

Em pomadas administradas, contendo óleo essencial de *Mentha piperita*, em feridas infectadas por *Pseudomonas aeruginosa* e *Staphylococcus aureus* em camundongos acelerou a cicatrização. Observou-se ainda que a contagem bacteriana tecidual total, o edema e o nível de inflamação foram reduzidos. Já a migração de fibroblastos, síntese de colágeno e reepitelização foram aumentadas nas feridas dos animais tratados com o óleo essencial de *M. piperita*. Além disso, os níveis de expressão TNF-α, VEGF e FGF-2 foi regulada negativamente em comparação com o grupo controle. Estes resultados indicam que o óleo essencial de *M. piperita* pode ser usado para este tipo de tratamento (MODARRESI; FARAHPOUR; BARADARAN, 2019).

### 2.7 Cinamaldeído e o tratamento de infecções cutâneas

Os produtos naturais de plantas representam alvos interessantes para a prospecção de novas drogas. Um exemplo é o cinamaldeído, o composto predominante do óleo essencial das cascas de *Cinnamomum cassia*. Cinamaldeído (CNM) é um composto conhecido por exercer atividade antimicrobiana de amplo espectro (CHEN, W. et al., 2015; UTCHARIYAKIAT et al., 2016). Tem sido demonstrado que o cinamaldeído também tem ação anti-inflamatória, além de mediador de monócitos e macrófagos (KIM, M. E.; NA; LEE, 2018), cicatrizante, (GILL; HOLLEY, 2004), anticancro e é de baixa toxicidade para os seres humanos (KIM, M. E.; NA; LEE, 2018).

A aplicação do CNM para o tratamento de infecções cutâneas se dá principalmente por suas propriedades antimicrobianas, imunomoduladoras e cicatrizantes (FERRO, T. A. F. et al., 2019; FERRO, T. A. et al., 2016; MENDES et al., 2016). Nos tópicos a seguir discutiremos cada uma destas atividades farmacológicas do CNM. Além disso, CNM tem sido descrito como agente antioxidante, anti-inflamatório e antiapoptótico em diversos modelos *in vitro* e *in vivo* (MENDES et al., 2016; ABOU EL-EZZ et al., 2018). As ações antiapoptóticas do cinamaldeído têm sido relacionadas com o aumento da defesa contra radicais livres através da ativação da via do Nrf2 (NF-E2 - related fator 2) (WANG, F. et al., 2015; ABOU EL-EZZ et al., 2018; MITAMURA et al., 2018).

#### 2.7.1 Cinamaldeído como agente antimicrobiano contra *S. aureus*

CNM é considerado um agente antimicrobiano de amplo espectro com ação contra

patógenos Gram-positivos (*S. aureus*, *Clostridium* ssp., *Enterococcus* ssp., *Streptococcus* ssp.) (FERRO, T. A. et al., 2016; ROSHAN; RILEY; HAMMER, 2017; RIBEIRO et al., 2018), Gram-negativos (*Acinetobacter baumannii*, *Salmonella enterica*, *Escherichia coli*, *P. aeruginosa*) (NARAYANAN, A. et al., 2017; KARUMATHIL et al., 2018; SILVA et al., 2018; FERRO, T. A. F. et al., 2019) leveduras (*Candida* ssp., *Cryptococcus* ssp. e *Malassezia pachydermatis*) ( KUMARI et al., 2017; BAKHTIARI et al., 2019; SIM et al., 2019) fungos filamentosos (*Aspergillus* ssp., *Fusarium* ssp., *Microsporum* ssp., *Penicillium* ssp., *Trichophyton* ssp.) ( OOI et al., 2006; MORCIA et al., 2017; DENG et al., 2018; SCHLOSSER; PRANGE, 2018). Os mecanismos de ação relatados para CNM contra bactérias e fungos foram recentemente revisados ( SHREAZ et al., 2016; FRIEDMAN, 2017). Em resumo, a ação antimicrobiana do cinamaldeído está relatada com a inibição da divisão celular via FtsZ (do inglês, *filamentation temperature sensitive protein Z*), (LI, X. et al., 2015), redução da produção de energia e alterações na membrana bacteriana (GILL; HOLLEY, 2004).

Em particular, o efeito inibitório ações do CNM contra *S. aureus* está relacionado a mudanças na polaridade e permeabilidade da membrana celular (HAMMER; HEEL, 2012). CNM mostrou inibir a expressão de *sarA* (regulador positivo da formação de biofilme) em *S. aureus* em concentrações sub-inibitórias (JIA, P. et al., 2011). Este efeito é relacionado à indução de vias de estresse em *S. aureus* que, por sua vez, levam à expressão de genes associados a biofilme (SCHILCHER et al., 2016). Cinamaldeído também tem a capacidade de diminuir a hemólise induzida por *S. aureus* e protegeu larvas de *Galleria mellonella* da infecção por esta bactéria (FERRO, T. A. et al., 2016).

### 2.7.2 Cinamaldeído como agente imunomodulador

Diversos trabalhos têm destacado a ação imunomoduladora de CNM em modelos de inflamação induzida por microrganismos ou toxinas ( HAGENLOCHER et al., 2015; YANG, J. J. et al., 2018; FERRO, T. A. F. et al., 2019; YANG, G. et al., 2019). Dados recentes obtidos de modelo de inflamação induzida por LPS (administrado por via intraperitoneal) em camundongos sugerem que o CNM exerce efeitos anti-inflamatórios que são mediados em parte pela ativação do Receptor de Potencial Transiente Ankiryn 1 (TRPA1), resultando na atenuação na severidade da síndrome de resposta inflamatória sistêmica (MENDES et al., 2016).

Outro estudo demonstrou que CNM reduz o dano inflamatório provocado por LPS

em células cardíacas através da regulação da autofagia e da produção de espécies reativas de oxigênio (ROS). Os autores dessa pesquisa mostraram que a via de sinalização envolvida nesta proteção era relacionada com inibição da expressão de TLR4, NOX4 e MAPK resultando em menores níveis das citocinas TNF-α, IL-1β e IL-6 (ZHAO, H. et al., 2016). Posteriormente, foi demonstrado que o CNM possuía um efeito protetor em camundongos submetidos a uma alta dose de LPS (15 mg/kg; via intraperitoneal), evidenciada pela diminuição dos níveis de IL-1β e redução da ativação do inflamassoma NLRP3 (através da inibição da catepsina B e proteína P2X7R) (XU et al., 2017).

### 2.7.3 CNM como agente cicatrizante

No processo de cicatrização CNM também estimulou a angiogênese *in vivo* e *in vitro*, regulando positivamente o fator de crescimento endotelial vascular (VEGF) e a expressão de Flk-1/KDR (CHOI et al., 2009). Em outro estudo, verificou-se que o CNM apresentou propriedades pró-angiogênicas em células endoteliais da veia umbilical humana (HUVECs). Demonstrou-se que nas HUVECs, o composto estimulou a proliferação, migração, formação de tubos, ativou as vias da fosfatidilinositol 3-quinase (PI3K) e da proteína quinase mitogênica (MAPK). Além disso, a secreção do fator de crescimento endotelial vascular (VEGF) das HUVECs foi aumentada (YUAN et al., 2018).

*In vivo*, o óleo restaurou parcialmente os vasos intersegmentares no peixe-zebra pré-tratado com PTK787, um inibidor seletivo do receptor do fator de crescimento endotelial vascular (VEGFR), mostrando eficácia pró-angiogênica. CNM reduziu o tamanho de ferida em modelo de ferida cutânea, e proteína VEGF elevada e densidade vascular (células $CD31^{+}$) na margem dessas feridas. Esses resultados sugerem que o CNM acelera a cicatrização de feridas induzindo a angiogênese na área afetada. O provável mecanismo envolve a ativação das vias de sinalização PI3K/AKT e MAPK (YUAN et al., 2018).

Outro exemplo do potencial cicatrizante de CNM foi obtido em um modelo de feridas experimentais contaminadas por *Pseudomonas aeruginosa*. Inicialmente, os autores demonstraram que concentrações sub-inibitórias de CNM reduziram a capacidade desta bactéria de formar biofilme e causar hemólise. Em aplicações tópicas, CNM provocou redução da carga bacteriana no tecido e aceleração da cicatrização. Além disso, baixa concentração de Interleucina-17 (IL-17), VEGF e óxido nítrico foram detectadas ao nível tecidual. Os autores

também demonstraram que ações do CNM são dependentes da ativação do receptor TRPA 1 (FERRO, T. A. F. et al., 2019).

Com base nessas observações o objetivo deste trabalho é avaliar o efeito de cinamaldeído em modelo *in vivo* de disfunção de células cutâneas contaminadas por *S. aureus*, possibilitando o estudo dos mecanismos envolvidos nas ações de cinamaldeído em nível sistêmico e celular.

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**ARTIGO 1**

# Use of Some Asteraceae Plants for the Treatment of Wounds: From Ethnopharmacological Studies to Scientific Evidences

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Severe wounds result in large lesions and/or loss of function of the affected areas. The treatment of wounds has challenged health professionals due to its complexity, especially in patients with chronic diseases (such as diabetes), and the presence of pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Taking this into consideration, the development of new therapies for wound healing requires immediate attention. Ethnopharmacological studies performed in different countries have shown the use of several plants from the Asteraceae family as wound-healing agents. Evidences gained from the traditional medicine have opened new ways for the development of novel and more efficient therapies based on the pharmacological properties of these plants. In this article, we discuss the literature data on the use of Asteraceae plants for the treatment of wounds, based on the ethnopharmacological relevance of each plant. Special attention was given to studies showing the mechanisms of action of Asteraceae-derived compounds and clinical trials. *Ageratina pichinchensis* (Kunth) R.M. King and H. Rob. and *Calendula officinalis* L. preparations/compounds were found to show good efficacy when assessed in clinical trials of complicated wounds, including venous leg ulcers and foot ulcers of diabetic patients. The compounds silibinin (from *Silybum marianum* (L.) Gaertn.) and jaceosidin (from *Artemisia princeps* Pamp.) were identified as promising compounds for the treatment of wounds. Overall, we suggest that Asteraceae plants represent important sources of compounds that may act as new and efficient healing products.

Keywords: *Ageratina pichinchensis*, *Calendula officinalis*, silibinin, jaceosidin, drug development, ethnomedicine

## INTRODUCTION

Wounds, especially of chronic nature, cause a serious public health concern as they negatively affect the quality of life of a large number of people, showing psychological, social, and economic impacts (Vowden and Vowden, 2016; Guest et al., 2017). When not properly treated, their associated lesions can become larger and result in the loss of function of the affected areas. Wounds are classified on the basis of the various factors such as location, borders, size, tissue type, secretion, odor, edema, and pain (Frykberg and Banks, 2015; Wernick and Stawicki, 2018). Based on these characteristics,

they can be simple or complex, deep or superficial, acute or chronic, sterile or contaminated, or even defined by the type of healing (Morton and Phillips, 2016; Karthik et al., 2018).

Based on its complexity, a wound is considered as simple when it is able to spontaneously evolve to resolution or as complex when lesions are extensive and/or deep and require special resources or more specialized treatment for its complete healing (Wernick and Stawicki, 2018). Based on its depth, a wound is superficial when it is restricted to the epidermis or dermis or deep when it affects the subcutaneous tissue, muscles, and/or bones (Shin et al., 2017; Podd, 2018). Wounds can be also classified as acute or chronic, with the former achieving resolution within 3 weeks and minimal or no scar tissue formation, whereas the latter may take several weeks to heal and can often lead to the loss of function of the affected tissues (Frykberg and Banks, 2015; Jørgensen et al., 2016). The healing time is influenced by different factors such as the presence of comorbidities (diabetes, hypertension, neurological lesions, among others), infection, aging, nutritional status, personal care, and appropriate and timely treatment (Manrique et al., 2015; Kulprachakarn et al., 2017; Hou and Kim, 2018; Long et al., 2018; Yuan et al., 2018).

The treatment of chronic wounds is complex and can include the administration of vascular endothelial growth factor or erythropoietin, which may not always be efficient and present high costs and has short half-life and side effects (Hong and Park, 2014; Arslantas et al., 2015; Yu et al., 2018). Moreover, the treatment of chronic wounds often requires the use of antimicrobials due to their multifactorial nature (Karthik et al., 2018). Considering this aspect, the development of new therapies for wound healing requires immediate attention. Plant-derived products have presented protective actions in wound care, and the healing activity of several active compounds has been shown (Agar et al., 2015; Neamsuvan and Bunmee, 2016; Jaric et al., 2018). Ethnopharmacological studies performed in different countries have shown that many plants from the Asteraceae family may be useful as sources of healing agents and therefore aid in the treatment of different types of wounds. As such, their pharmacological potential has been explored in an attempt to develop novel and more efficient therapies to accelerate healing and diminish the loss of function of tissues in the wounded area (Agar et al., 2015; Neamsuvan and Bunmee, 2016; Jaric et al., 2018). This article discusses the scientific evidences supporting the use of Asteraceae plants and their derived compounds as healing therapies. The main focus was given to plants with ethnopharmacological relevance, especially to the mechanism of action of their isolated compounds and clinical trials assessing their efficacy.

## OVERVIEW OF WOUND HEALING

Wound healing consists of a coordinated cascade of cellular and biochemical events that interact with the tissue reconstitution (Eming et al., 2014; Lee and Jang, 2018; Nagle and Wilbraham, 2018). This process consists of three distinct and superposed phases: inflammation, proliferation, and tissue remodeling (**Figure 1**) (Eming et al., 2014). Inflammation occurs soon after lesion. At this stage, a blood clot is formed to cease bleeding and also to make a viable matrix rich in growth factors and chemokines, which in turn contribute to the migration of leukocytes and stromal cells (Rousselle et al., 2018). After 24 h, neutrophils appear at the margins of the wounds, and the process of sterilization and waste degradation begin. This first stage of the healing process is normally completed within 3 days following surgical or acute wounds (Gurtner et al., 2008; Shaw and Martin, 2009).

Proliferation is the phase responsible for the wound closure, occurring 4 days following a wound. It involves re-epithelization (movement of epithelial cells), formation of granulation tissue (responsible for filling the injured tissue), and angiogenesis (Kanji and Das, 2017; Zomer and Trentin, 2018). Fibroblasts produce the new extracellular matrix necessary for the cell growth, whereas the new blood vessels carry oxygen and the nutrients necessary for the local cellular metabolism (Shaw and Martin, 2009; Rousselle et al., 2018). Two weeks after the lesion, there is a vasculature regression, and the granulation tissue is converted into an avascular scar without inflammation, which is covered by intact epithelium, as a result of collagen deposition (Broughton et al., 2006; Eming et al., 2014). Scar contraction occurs in large lesions due to the activity of myofibroblasts—fibroblast-like cells that have the contractile ability of smooth muscle cells (Wernick and Stawicki, 2018; Zomer and Trentin, 2018).

Finally, the remodeling phase begins approximately 3 weeks after the lesion. This phase is characterized by a random deposition of collagen, and then, metalloproteinases (produced by macrophages, neutrophils, fibroblasts, and epithelial cells) regulate the degradation and deposition of extracellular matrix, which are essential for wound re-epithelization (Gurtner et al., 2008; Caley et al., 2015; Kanji and Das, 2017; Rousselle et al., 2018; Zomer and Trentin, 2018). Consequently, larger collagen fibers are formed and organized according to the direction of the adjacent connective tissue. At the end of this stage, there is a limited regeneration of the skin attachments, such as hair follicles and glands, and a pale-colored scar with up to 80% of the original tensile strength present (Broughton et al., 2006; Gurtner et al., 2008; Eming et al., 2014).

## PLANTS FROM ASTERACEAE FAMILY AS WOUND-HEALING AGENTS

Plants have been used for medicinal purposes for many years as shown in previous studies (Abd Rani et al., 2018; Ricardo et al., 2018; Tiwari et al., 2018). In this context, plants from the Asteraceae family are well known for their ethnopharmacological importance for many communities (Rodriguez-Chavez et al., 2017; Tewari et al., 2017; Saleh and Van Staden, 2018), and this family is widely distributed and is considered to be the largest family of flowering plants in the world (Gao et al., 2010). Due to their distribution and ethnopharmacological importance, several plant-derived products from this family have been studied, with some of their pharmacological activities already identified. These include anti-inflammatory (George Kallivalappil and Kuttan, 2017), antimicrobial (Ghaderi and Sonboli, 2018), antioxidant

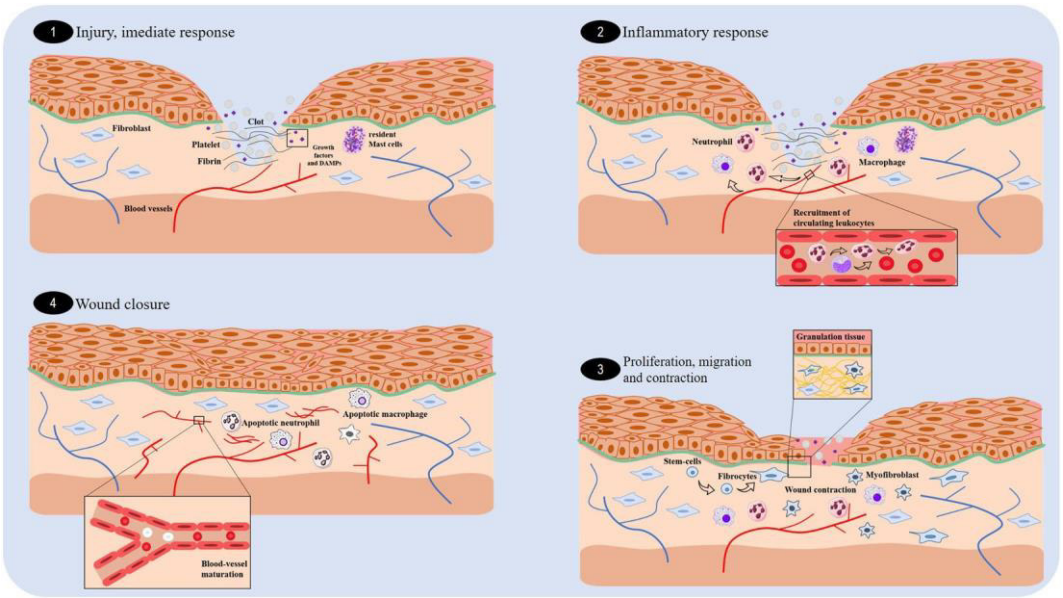


FIGURE 1 | Overview of the essential stages of wound healing. There are four fundamental stages involved in wound healing: the immediate response (1), inflammation (2), proliferation (3), and wound closure (4).

(Babota et al., 2018), anti-protozoa (Garcia et al., 2017), and healing activities (Ozbilgin et al., 2018). Some species [such as *Calendula officinalis* L., *Achillea millefolium* L., *Neurolaena lobata* (L.) R.Br. ex Cass.] have been specially described in the literature due to their therapeutic potential for the treatment of wounds (Parente et al., 2012; Nayak et al., 2014). Their efficacy has been suggested to be related to their ability to promote the proliferation of keratinocytes and thus the remodeling of the extracellular matrix (Speroni et al., 2002; Rosa Ados et al., 2014). Hence, the therapeutic use of these plants will now be discussed. The most relevant studies (i.e., those that provide insights into the mechanism of action) are summarized in **Table 1**.

## *Blumea balsamifera* (L.) DC.

*Blumea balsamifera* (L.) DC. is a plant used in the traditional medicine of several Asiatic countries, where it is popularly known as Ainaxiang (De Boer and Cotingting, 2014; Ong and Kim, 2014; Sujarwo et al., 2015). Its leaves are rich in volatile compounds such as L-borneol (major compound), terpenoids, fatty acids, phenols, alcohols, aldehydes, ethers, ketones, pyridines, furans, and alkanes (Pang et al., 2014a), which may contribute to the healing properties of *B. balsamifera*. Indeed, the topical application of the volatile oil obtained from the leaves of *B. balsamifera* in wounded Kun-Ming mice enhanced angiogenesis and collagen deposition, and additionally induced epithelial deposition and formation of granular tissue. This effect on the proliferation phase of healing was suggested to be associated with the increased production of the neuropeptide substance P (Pang et al., 2014b). The volatile oil also accelerated the healing of Sprague-Dawley rats with burn injuries by triggering the release of growth factors in the tissue and decreasing the plasma concentrations of pro-inflammatory cytokines (TNFα and IL-1) (Fan et al., 2015).

Pang et al. (2017) in their study evaluated the healing actions of a flavonoid-rich leaf extract from *B. balsamifera* on skin wounds of Sprague-Dawley rats. This extract caused wound contraction, capillary regeneration, collagen deposition, and re-epithelization 7 days following treatment. These alterations were associated with the enhanced expression of vascular endothelial growth factor, transforming growth factor-β1, and CD68 antigen in rat wound tissues. Different compounds were detected in the extract including 16 flavonoid aglucons, 5 flavonoid glycosides, 5 chlorogenic acid analogs, and 1 coumarin (Pang et al., 2017).

## Silibinin From *Silybum marianum* (L.) Gaertn.

*Silybum marianum* (L.) Gaertn. is another plant of ethnopharmacological importance in wound healing (Hudaib et al., 2008; Aziz et al., 2016). Evidences have shown that silymarin, an extract from its seeds, increases epithelization and decreases inflammation in albino rats subjected to the excision wound (Sharifi et al., 2012). It was also shown that this extract protects human fibroblasts from lipopolysaccharide (LPS)-induced oxidative stress (Sharifi et al., 2013). Similarly, the silymarin-derived compound silibinin (flavonoid) accelerated the closure of skin wounds in rats by upregulating the expression of stromelysin 1 hydroxyproline, glycosaminoglycans, and collagen (important constituents of extracellular matrix) (Tabandeh et al., 2013). This compound was also found to reduce the toxic effects caused by nitrogen mustard in the mouse skin (Balszuweit et al., 2013). This action was associated with an inhibition of oxidative stress and inflammation (Jain et al., 2015). As shown in another

TABLE 1 | Use of some Asteraceae plants for the treatment of wounds *in vivo* and *in vitro*.

| Species | Popular name | Product | Type of study | Conclusions | Reference |
|---|---|---|---|---|---|
| *Blumea balsamifera* (L.) | Sambong | Leaf extract | *In vivo* study with Sprague-Dawley rats | The extract induced wound contraction, capillary regeneration, collagen deposition, and re-epithelization | Pang et al., 2017 |
| | | Volatile oil | *In vivo* study with Kun-Ming mice | The topical application of the volatile oil promoted capillary regeneration, blood circulation, collagen deposition, granular tissue formation, epithelial deposition, and wound contraction | Pang et al., 2014b |
| | | Silibinin-based gel | *In vivo* study with Swiss mice | The formulation induced the production of collagen fibers, fibroblasts, and proliferating blood capillaries (angiogenesis) | Samanta et al., 2016 |
| *Acmella oleracea* | Jambu | Rhamnogalacturonan | *In vivo* study in Wistar Rats with gastric ulcers | The treatment reduced the gastric lesions due to its anti-inflammatory and antioxidant mechanisms It also induced cellular proliferation | Maria-Ferreira et al., 2014 |
| *Achillea asiatica* | | Ethanolic extract | *In vivo* study with Sprague-Dawley rats and *in vitro* study with Hs68 fibroblasts | The extract enhanced healing by promotion of keratinocyte differentiation and motility and anti-inflammatory effects. It induced the expression of β-catenin, collagen, and keratinocyte differentiation markers | Dorjsembe et al., 2017 |
| *Artemisia princeps* Pampanini | Korean wormwood, Korean mugwort, and Japanese mugwort | Jaceosidin | *In vitro* study with HUVEC[1] | Jaceosidin promoted proliferation, migration, differentiation of human endothelia cells, and angiogenesis | Lee et al., 2014 |
| *Calendula officinalis* | Pot marigold | Hydroalcoholic extract | *In vivo* study with BALB/c mice and *in vitro* study with HDF[2] | The extract was able to induce tissue granulation, proliferation, and cell migration | Dinda et al., 2016 |
| | | Tincture | *In vitro* study with HI-38[3], NIH-3T3[4], HeLa[5], HDF[2] | The treatment potentiated wound healing by stimulating fibroblast proliferation and migration in a PI3K-dependent pathway | Dinda et al., 2015 |
| | | Oil | *In vivo* study in foot ulcers of diabetic patients | The use of low-intensity laser therapy associated with *C. officinalis* oil caused analgesic and reduced inflammation | Carvalho et al., 2016 |
| *Achyrocline alata* | Jateí-ka-há | Extract | *In vivo* study with mice | The extract accelerated the healing by decreasing the initial inflammatory response and promoted re-epithelization and collagen remodeling | Pereira et al., 2017 |

*[1]HUVECs, human umbilical vascular endothelial cells; [2]HDF, human primary dermal fibroblast cells; [3]HI-38, human lung fibroblast cells; [4]NIH-3T3, Swiss albino mouse fibroblast cells; [5]HeLa, human cervical carcinoma cells.*

study, the repeated topical application (14 days treatment) of a silibinin-based gel resulted in an efficient wound healing strategy, by acting on tissue re-epithelization, collagen production, and deposition of granulation tissue (as shown in **Figure 2**) (Samanta et al., 2016).

## *Calendula officinalis* L.

*Calendula officinalis* L. (or calendula) is a species used in the treatment of wounds in Europe since 13th century, and a large number of cosmetic and personal care products have been developed using its compounds (Parente et al., 2012). Its use as a healing agent is supported by different *in vivo* and *in vitro* studies. In one of these studies, the ethanolic extract obtained from *C. officinalis* flowers, and its dichloromethane and hexanic fractions were found to increase angiogenesis in both chorioallantoic membranes (CAMs) of embryonated eggs and rat with skin wounds. This effect on vessels was related to discrete infiltration of inflammatory cells and increased the collagen deposition (Parente et al., 2011, 2012). Recently, a cream containing the glycolic extract from *C. officinalis* flowers was found to enhance collagen organization in the initial phase of the healing process, and this was correlated with an increase in the concentrations of hydroxyproline, an indicator of the collagen content in the tissue (Aro et al., 2015).

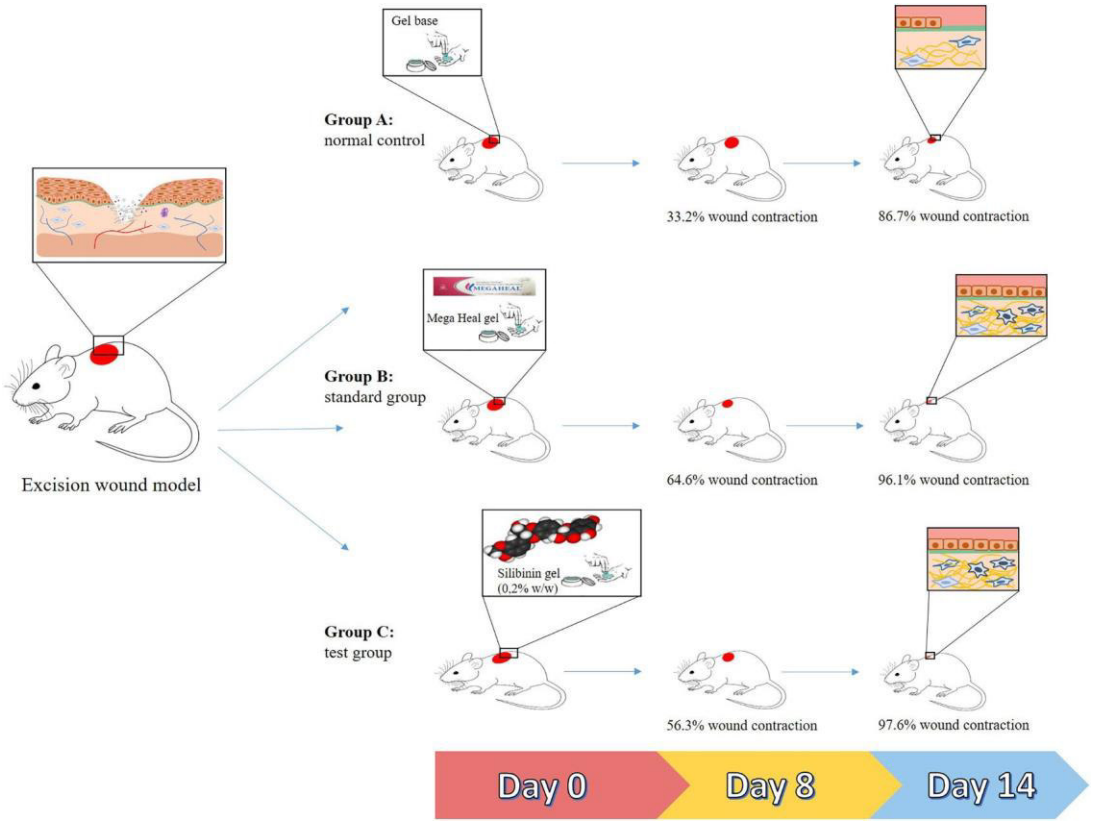


FIGURE 2 | Summarized representation of the *in vivo* actions of silibinin (from *Silybum marianum*) in wound healing. Group A: mice treated with a gel base (controls). Group B: mice treated with a commercially available gel (Mega Heal gel; Aristo Pharmaceuticals Ltd.; positive control). Group C: mice treated with a silibinin-based gel (0.2% w/w). After 14 days of treatment, the mean sizes of the wounds from mice of groups B and C were smaller than those of group A.

*In vitro* studies were performed to provide more insights into the mechanisms of action involved in the healing action of a product based on the hydroalcoholic extract of *C. officinalis* (approved by the European Medicines Agency (Nicolaus et al., 2017). *C. officinalis* tincture was able to increase the proliferation and the migration of fibroblasts in a PI3K-dependent pathway, with activation of FAK and Akt. Flavonol glycosides were the major compounds detected in this extract (Dinda et al., 2015). Human keratinocytes treated with *C. officinalis* flower extracts (*n*-hexanic and ethanolic extracts) exhibited the increased expression of IL-8 and activation of the transcription factor NF-κB, in addition to the enhanced migration ability. The ethanolic extract of this plant was also able to inhibit collagenase activity in human dermal fibroblasts. These effects were attributed to the presence of flavonoids and saponins in the extract (Nicolaus et al., 2017).

The hydroalcoholic extract and its aqueous fraction of *C. officinalis* (rich in rutin and quercetin-3-*O*-glucoside) exhibited significant *in vitro* effects on the proliferation and migration of human dermal fibroblasts, in addition to the increased expression of connective tissue growth factor and α-smooth muscle actin, proteins that favor healing by activating cell proliferation, migration, adhesion, and tissue repair. The topical application of hydroalcoholic extract or its aqueous fraction of *C. officinalis* on excisional wounds of BALB/c mice accelerated wound contraction by increasing the tissue levels of connective tissue growth factor and α-smooth muscle actin (Dinda et al., 2016).

Particularly noteworthy was the commercially available product containing the hydroglycolic extract of *C. officinalis* (Plenusdermax) as it promoted wound epithelization, thus decreasing the healing time in patients with venous leg ulcers (Buzzi et al., 2016). Another study showed that the use of low-intensity laser therapy associated with *C. officinalis* oil causes analgesia, in addition to the reduction of lesions in foot ulcers of diabetic patients (Carvalho et al., 2016). Interestingly, *C. officinalis* has been considered as an alternative resource by national health surveillance agencies such as the one in Brazil.

## *Achillea* Genus

*Achillea* genus has been widely used in the traditional medicine as a source of healing products (Mohammadhosseini et al., 2017; Jaric et al., 2018). *Achillea millefolium* L. is the most studied species among others. *A. millefolium* is a herb, commonly known as yarrow, which is indigenous to the Northern Hemisphere of Europe and Asia, and it has been popularly used for over 3,000 years (Ali et al., 2017). Its pharmacological properties

include anti-inflammatory, antioxidant, antifungal, and healing actions (Karamenderes and Apaydin, 2003; Fierascu et al., 2015), which have been attributed to several chemical constituents such as sesquiterpenes and phenolic compounds (Fierascu et al., 2015).

An *in vitro* study, carried out in human skin fibroblasts, showed that the hydroalcoholic extract from the aerial parts of *A. millefolium* induces cell proliferation (Ghobadian et al., 2015). More recently, oil extracts from aerial parts of *A. millefolium* were shown to reduce skin irritation in healthy individuals. Two approaches were applied to obtain the extracts: (i) the aerial parts of *A. millefolium* were macerated with ethanol, followed by olive oil (E1) or sunflower oil (E2) and (ii) the maceration of plant material occurred only in the presence of olive oil (E3) or sunflower oil (E4). This double-blind study enrolled 23 volunteers who had 8% sodium lauryl sulfate applied to their skin to cause irritation. After 24 h, these subjects received a topical application of the oil extracts for 7 days. All oil formulations were able to stabilize the skin pH and to increase hydration while reducing erythema. However, E1 and E2 exhibited the highest anti-inflammatory action, whereas E3 and E4 promoted highest levels of skin hydration. The presence of compounds with reported anti-inflammatory actions in both E1 and E2 (luteolin, apigenin and their glycosides, caffeic, and chlorogenic acids as well as chlorophyll derivatives) may explain these results (Tadic et al., 2017).

Evidences have also suggested a healing potential of *Achillea asiatica* Serg. (synonym of *A. millefolium* var. *manshurica* Kitam), popularly known as Mongolian yarrow. *In vitro* incubation of the ethanolic extract from the aerial parts of this plant with Hs68 fibroblasts triggered the production of collagen by these cells; this involved the activation of transforming growth factor-β-mediated pathways. The same extract also enhanced the differentiation and motility of keratinocytes through the upregulation of β-catenin, Akt, and keratinocyte differentiation markers. Compounds such as chlorogenic acid, apigenin-7-*O*-glucoside, and schaftoside were identified and associated with the healing effects of *A. asiatica* ethanolic extract (Dorjsembe et al., 2017). A comparative analysis of the *in vitro* healing potential of extracts obtained from *A. coarctata*, *A. kotschyi*, and *A. lycaonica* was performed in cultured NIH-3T3 fibroblasts. *A. kotschyi* extract was the most effective, presenting chlorogenic acid, hyperoside, apigenin, hesperidin, rutin, kaempferol, and luteolin in its composition (Jain et al., 2015).

## *Pluchea* Genus

Plants from the *Pluchea* genus have been used as healing agents by different communities (Gridling et al., 2009; Schmidt et al., 2009; Ab Rahman et al., 2014). *Pluchea indica* (L.) Less. healing actions have been attributed to its antioxidant and anti-inflammatory properties (Buapool et al., 2013). These evidences have been further supported by recent studies showing that nanoparticles containing *P. indica* leaf ethanolic extract increase the migration of oral mucosal cells *in vitro*. This preparation presented characteristics (size, charge, polydispersity index, increased colloidal stability) that support its use as an oral spray (Buranasukhon et al., 2017). Furthermore, the size of *Leishmania amazonensis*–induced cutaneous lesions in BALB/c mice was found to be reduced by the intralesional treatment with an essential oil obtained from the leaves of *Pluchea carolinensis* (Jacq.) D.Don. The main component of this essential oil was selin-11-en-4α-ol (Garcia et al., 2017).

## *Artemisia princeps* Pamp. and Isolated Compounds

Another plant with the ethnopharmacological relevance is *Artemisia princeps* Pamp., which is traditionally used to treat inflammatory-related diseases and had its properties scientifically proven in various *in vitro* and *in vivo* models (Min et al., 2009; Chen et al., 2016). Jaceosidin is extracted from this plant, which has also been identified as the main constituent of other plants from the *Artemisia* genus such as *A. argyi* with ethnomedicinal use as a healing agent (Li et al., 2018). It has the ability to inhibit the production of pro-inflammatory mediators such as TNF-α, IL-1β, and $PGE_2$ (Min et al., 2009). *In vitro*, this flavonoid induces the proliferation, migration, and differentiation of human umbilical vascular endothelial cells (**Figure 3**) (Lee et al., 2014). It also stimulates the formation of microvessels in rat aortic tissue, and this effect has been associated with the activation of VEGFR2/FAK/PI3K/AKT/NF-κB signaling pathways (Lee et al., 2014). Overall, all these studies suggest Jaceosidin as an interesting pro-angiogenic compound.

Isosecotanapartholide, isolated from *A. princeps*, has also exhibited *in vitro* proliferative properties. Isosecotanapartholide (and the extract from *A. princeps*) inhibited the production of IL-33 by human keratinocytes (HaCaT), and this was associated with reduced levels of signaling molecules such as signal transducer and activator of transcription-1 (STAT-1), thymus and activation-regulated chemokine (TARC/CCL17), and adhesion molecule-1 (Ali et al., 2017).

## *Ageratina pichinchensis* (Kunth) R.M. King and H. Rob.

*Ageratina pichinchensis* (Kunth) R.M. King and H. Rob. is a plant with ethnopharmacological relevance in Mexico, and its several pharmacological activities have been confirmed in murine models and clinical trials *in vivo*, such as onychomycosis (Romero-Cerecero et al., 2009), interdigital tinea pedis (Romero-Cerecero et al., 2012b), stomatitis (Romero-Cerecero et al., 2015b), and vulvovaginal candidiasis (Romero-Cerecero et al., 2017). Despite its use for wound healing, the first study showed that the daily topical application of an aqueous extract from the aerial parts of *A. pichinchensis* heals wounds in rats without inducing skin irritation (Romero-Cerecero et al., 2011). Based on these results, a bio-guided purification revealed that 7-*O*-(β-D-glucopyranosyl)-galactin is the major compound associated with the effects of *A. pichinchensis* in cell proliferation (Romero-Cerecero et al., 2013). Later, two extracts (aqueous and hexane) with standardized concentrations of 7-*O*-(β-D-glucopyranosyl)-galactin were shown to promote the healing of skin lesions in rats with streptozotocin-induced diabetes (Romero-Cerecero et al., 2014).

The healing properties of this plant were also assessed in human clinical trials. For instance, the effectiveness of a

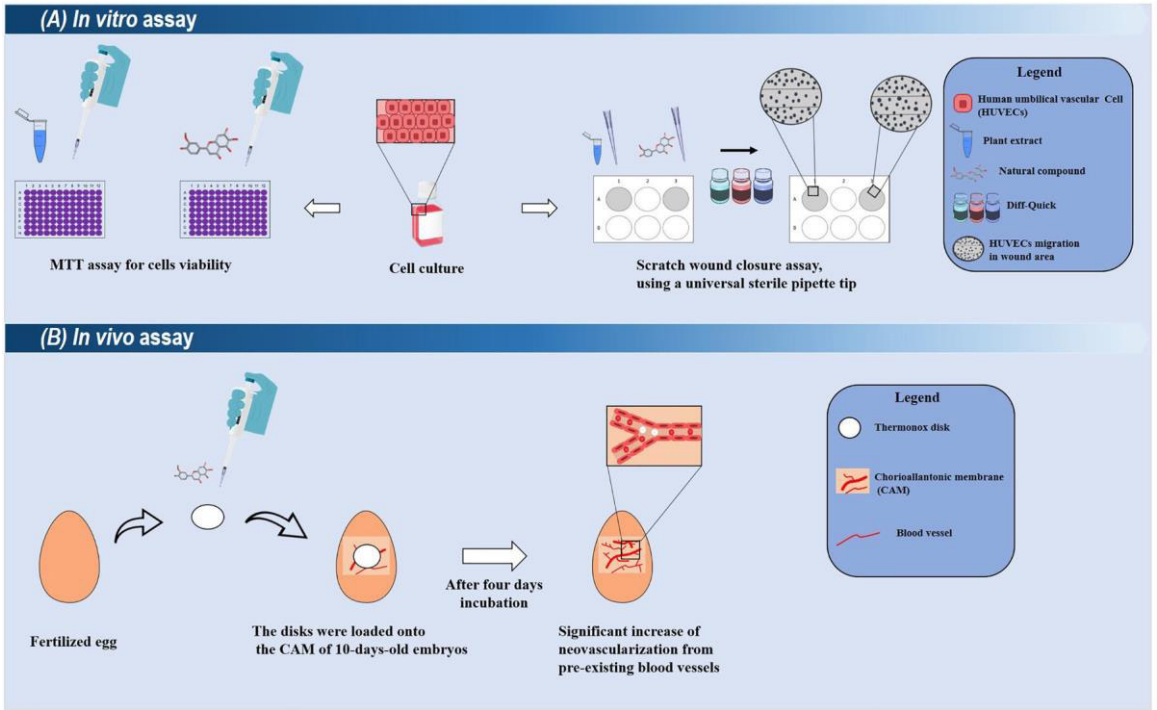


FIGURE 3 | Summarized representations of *in vitro* and *in vivo* techniques used for evaluating the healing potentials of plant-derived compounds. (A) In *in vitro* assays, the cytotoxic and proliferative effects of a compound/extract can be evaluated toward different cell cultures (such as human umbilical vascular cells). Their proliferative effects can be analyzed through the scratch wound closure assay. Compounds with promising results can be selected for *ex vivo* and *in vivo* models. (B) A simple *in vivo* model using chick Chorioallantoic membrane (CAM). In this assay, the damaged CAM is treated with the selected compound (applied into a disk), and after few days, the neovascularization is measured.

standardized extract of *A. pichinchensis* was proved to heal chronic venous leg ulcers (Romero-Cerecero et al., 2012a). In another study, a cream containing an extract of *A. pichinchensis* was topically used by diabetic patients with foot ulcer; the results showed this treatment decreases healing time and lesion size although no significant differences were observed. The authors attributed this fact to the sample size, but they concluded that a large clinical trial could prove the action of *A. pichinchensis* in this type of wound (Romero-Cerecero et al., 2015a).

## *Achyrocline alata* (Kunth) DC. and *Achyrocline satureioides* (Lam.) DC.

Plants from the *Achyrocline* genus play an important role in traditional medicine and are commonly found in Latin American countries (Retta et al., 2012; Alerico et al., 2015; Bolson et al., 2015). Ethnobotanical surveys performed in the Brazilian state of Rio Grande do Sul indicated that *Achyrocline satureioides* (Lam.) DC. is widely used for healing. It was shown that the ethanolic extracts from the aerial parts of this plant induce the proliferation of HaCaT keratinocytes (Alerico et al., 2015). The healing activity of an essential oil of *A. satureioides* inflorescences incorporated into hydroxyethyl cellulose films was also demonstrated in Wistar rats (Yamane et al., 2016).

A recent study evaluated the use of the extracts from inflorescences of *Achyrocline alata* (Kunth) DC. and *A. satureioides* for the repair of cutaneous wounds in mice. Both extracts showed positive results, but only *A. alata* accelerated wound closure, presenting a higher probability of healing in a shorter time of treatment. The authors attributed this effect to higher concentrations of phenolic compounds in *A. alata*. Moreover, it was possible to observe that animals treated with *A. alata* extract present less mast cells at the site of inflammation, better re-epithelization and granulation of the injured tissue, and reduction of the initial inflammatory reaction (Pereira et al., 2017).

## *Acmella oleracea* (L.) Spreng.

*Acmella oleracea* (L.) Spreng. (jambu) is a native plant from Brazil that is used to treat skin and gastrointestinal disorders and also as a female aphrodisiac (Neamsuvan and Bunmee, 2016; Neamsuvan and Ruangrit, 2017; Da Rocha et al., 2018). A polysaccharide extracted from *A. oleracea*, named rhamnogalacturonan, was found to inhibit ethanol-induced gastric ulcers in rats (Nascimento et al., 2013). This effect was better elucidated later, as this compound was shown to protect against both acute (intraperitoneal treatment) and chronic lesions (oral administration) induced by ethanol (Maria-Ferreira et al., 2014). Rhamnogalacturonan also enhanced the gastric cell proliferation and mucus content while decreasing inflammation and oxidative stress in the stomach (Maria-Ferreira et al., 2014).

Another study reported the development of hydroxyethyl cellulose (HCE) films containing an ethanolic extract from the aerial parts of *A. oleracea* and an essential oil obtained from the inflorescences of *Achyrocline satureioides*. The HCE films containing these two plant materials demonstrated wound healing activity in Wistar rats, an effect that was associated with increased levels of collagen deposition in wounds. α-Humulene and spilanthol were detected in the essential oil of *A. satureioides* and the extract of *A. oleracea*, respectively (Yamane et al., 2016).

### *Artemisia* Plants

The genus *Artemisia* plays an important role in the traditional medicine (Shenkman and Krivenkov, 1986; Kadioglu et al., 2017; Ota and Ulrih, 2017) and in the development of anti-inflammatory and anticancer drugs (Kadioglu et al., 2017; Coricello et al., 2018; Konstat-Korzenny et al., 2018). The pharmacological potentials of these plants have also been evaluated in healing models. For example, the extract from *Artemisia asiatica* (Pamp.) Nakai ex Kitam was efficient against gastric injuries induced by ethanol (Park et al., 2008), while *Artemisia argyi* H.Lév. and Vaniot healed oral ulcers in rats (Yin et al., 2017). Another study showed that the essential oil from *Artemisia montana* (Nakai) Pamp improves the proliferation of human keratinocytes and enhances their capacity to produce type IV collagen. These effects were associated with the phosphorylation of Akt and ERK 1/2. *In vivo* assays showed that the essential oil from *A. montana* promotes the healing of rats with dorsal wounds (Yoon et al., 2014). The aqueous extract from *Artemisia campestris* L. also reduced the number of inflammatory cells in the wounded area and presented a positive effect in the progress of wound healing (Ghlissi et al., 2016).

## CONCLUSION

This review described the aspects involved in the healing properties of some Asteraceae plants. In fact, several plants from this family have ethnopharmacological relevance for the treatment of wounds due to their direct effects on healing and in some cases due to their anti-inflammatory actions. The discussed studies provided the scientific basis for the ethnopharmacological usage of these plants, since different products derived from them (isolated compounds, oils, and extracts) are effective in the models of healing *in vitro* and *in vivo*. Silibinin (from *S. marianum*) and jaceosidin (from *A. princeps*) were identified as promising compounds for the development of healing agents. Furthermore, the results obtained in clinical trials with *A. pichinchensis* and *C. officinalis* are exciting and highlight their importance for the treatment of wounds. These evidences suggest that Asteraceae plants are important sources for the development of new efficient drugs for healing.

## AUTHOR CONTRIBUTIONS

AC, RD, MS, CF, AZ, MG, EF, and LdS contributed to conception and design and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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**ARTIGO 2**

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# Cinamaldeído para o tratamento de infecções microbianas: evidências obtidas de modelos experimentais

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**Resumo**

A canela é utilizada como tempero, aromatizante e na medicina tradicional, sendo obtida a partir da casca das plantas do gênero *Cinnamomum* (Lauraceae). Cinamaldeído (CNM) é o principal componente ativo do óleo essencial de canela e é aprovado pela *Food and Drug Administration* (FDA) para uso. Possui várias atividades farmacológicas que incluem ação antimicrobiana, antioxidante, anti-inflamatória e antitumoral. Neste artigo discute-se a aplicação do cinamaldeído em modelos experimentais de infecção *in vivo* induzidos por bactérias. Foram selecionados artigos com a aplicação do cinamaldeído purificado (CNM) em modelos experimentais de infecção induzidas por bactérias. O CNM possui ação *in vitro* antimicrobiana de amplo espectro relacionada principalmente com o comprometimento da integridade da membrana/parede celular e alterações no metabolismo energético. Além disso, CNM é capaz de inibir diversos fatores de virulência bacterianos. CNM foi aplicado com sucesso no tratamento de infecções causadas por *Cronobacter sakazakii, Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus* e *Vibrio* ssp. Aliado a

isso, os efeitos imunomoduladores deste composto aprimora a resposta do hospedeiro, ajudando no controle da infecção. Estas evidências experimentais incentivam o uso do cinamaldeído como molécula líder para o desenvolvimento de novos fármacos anti-infectivos.

**Palavras-chave:** *Cinnamomum* sp., óleos essenciais, relação patógeno-hospedeiro, agentes anti-infectivos, imunomodulação, atividade antimicrobiana.

## 1. Introdução

A canela é amplamente utilizada como especiaria tendo uma longa história de uso como tempero e aromatizante, também é empregada na medicina tradicional em todo o mundo [1-3]. Esta especiaria é obtida da casca de diversas plantas do gênero *Cinnamomum* (Lauraceae) [4]. Aproximadamente 250 espécies de *Cinnamomum* foram identificadas, sendo amplamente cultivadas em países asiáticos como Sri Lanka, Madagascar, Indonésia e no sul da China. As espécies mais comumente utilizadas são *C. cassia*, *C. zeylanicum*, *C. burmannii*, *C. camphora*, *C. osmophloeum*, *C. verum* [3, 5, 6].

Os constituintes químicos mais importantes da canela são os óleos voláteis (cinamaldeído, eugenol e ácido cinâmico), além de conter diterpenos e proantocianidinas [5]. O cinamaldeído (CNM; $C_9H_80$) é um álcool terpeno cíclico apontado como o principal componente ativo do óleo essencial de canela (60-75%). CNM (também conhecido como aldeído cinâmico) é um composto que possui isomeria ótica, sendo o isômero trans-cinamaldeído de ocorrência natural e mais comercializado [7]. CMN é aprovado pela *Food and Drug Administration* (FDA) e tem sido amplamente utilizado para produção de gomas, sorvetes, doces, bebidas, pães, cereais. No entanto, a aplicação do CNM na conservação de alimentos é limitada devido seu sabor particular, volatilidade e natureza lipofílica [8, 9].

O CNM possui várias atividades farmacológicas que incluem ação antimicrobiana de amplo espectro [7, 10] e efeitos antioxidantes [11, 12] anti-inflamatório [13] e antitumoral [14]. Este composto é apontado com um emergente recurso para o tratamento da diabetes [14], doença de Alzheimer e outras patologias relacionadas à neuroinflamação [2, 15]. As ações anti-inflamatórias e cicatrizantes do cinamaldeído estão relacionadas com a ativação do Receptor de Potencial Transiente Ankiryn 1 (TRPA1) [16, 17].

Outra propriedade importante do CNM é a capacidade de inibir importantes fatores de virulência de patógenos como *Candida albicans*, *Campylobacter jejuni*, *Clostridium difficile*,

*Escherichia coli*, *Pseudomonas aeruginosa* e *Staphylococcus aureus* [18-22]. Estas características associadas aos potenciais antimicrobianos, anti-inflamatórios e antioxidantes têm sugerido que este composto constitui um agente potencial para o tratamento de infecções causadas por bactérias e fungos [23, 24].

Em um cenário em que a emergência de linhagens multirresistentes tem tornado o tratamento um desafio global, o desenvolvimento de novas terapias antimicrobianas eficientes é crucial [25, 26]. Nesta revisão não exaustiva, destacamos a aplicação do cinamaldeído em modelos experimentais de infecção induzidas por bactérias e fungos. Estudos utilizando ensaios de infecção baseado em células e invertebrados também foram incluídos. Os artigos foram pesquisados na plataforma PubMed do *National Center for Biotechnology Information*. Para um melhor entendimento das ações farmacológicas do CNM, foram selecionados apenas os artigos que avaliaram o composto purificado em modelos experimentais de infecção *in vivo* (usando vertebrados ou invertebrados); enquanto os estudos empregando óleos essenciais e extratos/frações (mesmo que contendo CNM) foram excluídos.

## 2. Cinamaldeído como um potente agente antimicrobiano

Como descrito acima, a canela tem sido utilizada para o tratamento de infecções na medicina popular há séculos [27, 28]. Este uso empírico levou a avaliação de seus constituintes como agentes antimicrobianos. CNM, como composto majoritário, tem sido associado aos efeitos da canela e diversos estudos demonstram a ação antimicrobiana de CNM contra um vasto espectro de patógenos bacterianos e fúngicos (incluindo leveduras e fungos filamentosos) [5, 7, 10]. Uma visão geral dos patógenos inibidos pelo CNM é apresentada na tabela 1.

Os mecanismos de ação relatados para CNM contra bactérias e fungos foram recentemente revisados [7, 10]. Devido ao caráter hidrofílico, CNM tem a capacidade de interagir com os lipídios presentes na membrana microbiana, alterando sua permeabilidade e levando a disfunção celular [29-31]. De fato, a composição dos ácidos graxos de membrana foi demonstrada como crucial para a inibição de *Listeria monocytogenes* pelo CNM [32]. O tratamento com CNM reduziu a expressão de porinas de *Cronobacter sakazakii* (proteínas relacionadas como a permeabilidade da membrana), deixando a bactéria mais suscetível a estresses ambientais [33].

Estudos com *E. coli* sugerem que além de alterar a proporção de ácidos graxos saturados e

insaturados na membrana, CNM se liga ao sulco menor do DNA genômico [31]. Análises de metabolômicas demonstraram que CNM age como um composto pró-oxidante, alterando o metabolismo de macromoléculas (proteínas, ácidos nucléicos, lipídios e carboidratos) [34]. A relação do CNM com a indução de estresse oxidativo foi confirmada pelo aumento da expressão de mediadores da defesa antioxidante (malondialdeído e superóxido dismutase) em *E. coli* tratada com concentrações sub-inibitórias deste composto [29].

Evidências que CNM perturba a produção de energia e o metabolismo celular também foram obtidas utilizando *Bacillus subtilis*, *E. coli*, *P. aeruginosa, Salmonella enterica*, e *S. aureus* [35, 36]. No entanto, CNM não induz a expressão do gene *recA*, um regulador geral de vias associadas ao reparo do DNA [35]. CNM também inibe a divisão celular bacteriana através da ligação à proteína FtsZ de *Bacillus cereus*, *E. coli* e *S. aureus* [5, 37]. Esta propriedade foi explorada para o desenho de moléculas análogas ao CNM com alta capacidade de desregular o processo de divisão celular de *S. aureus* [37].

CNM também apresentou ação inibitória contra *Mycobacterium tuberculosis*, *M. avium*, *M. bovis* [38-41]. O mecanismo de ação contra *M. avium* subsp. *paratuberculosis* envolve a liberação de íons potássio para o meio extracelular e diminuição dos níveis intracelulares de ATP [41]. Em adição, CNM aumentou a expressão do gene *clgR* de *M. tuberculosis*, indicando comprometimento da membrana celular [30]. Estes resultados impulsionaram o uso do CNM como molécula líder para o desenvolvimento de composto com ação contra *M. tuberculosis* [40, 42].

A membrana celular também é o alvo da ação do CNM contra *C. albicans*, além de inibir a produção de ergosterol [43, 44]. CNM foi também reportado como um potente inibidor não competitivo da enzima beta-(1,3)-glucano sintase e um inibidor misto da quitina sintetase 1 da levedura *Saccharomyces cerevisiae*, ambas importantes na síntese da parece celular fúngica [45]. A parede celular foi associada como alvo de ação do CNM contra *Aspergillus fumigatus* [23], *Aspergillus ochraceus* [46] e *Geotrichum citri-aurantii* (importante fitopatógeno) [47].

ATPases de bactérias e fungos são bloqueadas pelo CNM como demonstrado para *C. sakazakii*, *E. coli*, *L. monoytogenes*, *Salmonella enterica* serovar Typhimurium e *C. albicans* [5, 10, 48]. CNM e seus derivados inibem fortemente as ATPases dependentes de $H^+$ presentes na membrana de *Candida* ssp. induzindo acidificação do compartimento intracelular e consequente morte da levedura. Estes efeitos foram acompanhados por ruptura celular e severas alterações

morfológicas [49].

## 3. Cinamaldeído e o tratamento de infecções bacterianas

### 3.1. *Cronobacter sakazakii*

*Cronobacter sakazakii* e um patógeno associado ao consumo de alimentos associado com a patogênese da enterocolite necrozante, e casos graves de meningites e sepse em indivíduos imunocomprometidos [50, 51]. Um estudo demonstrou que o tratamento com CNM inibiu a formação de biofilme em cinco linhagens de *C. sakazakii*, seguida pela expressão diminuída de genes associados a síntese de celulose, produção de biofilme e componentes flagelares [52]. A habilidade de *C. sakazakii* de resistir a altas temperaturas e outras situações de estresse foi significantivamente comprometida após o tratamento com CNM, dado pela baixa regulação de genes envolvidos na tolerância e sobrevivência da bactéria [33].

CNM apresentou eficácia na redução da expressão de genes associadas com a capacidade de adesão e invasão, e a produção de endotoxinas (como *fliD*, *flhD*, *motA*, *motB*, *flgJ*, *ompA*, *ompX*, *uvrY*, *wzx*, *lpx* e *sod*). Os autores também destacaram que as bactérias tratadas tiveram menos sucesso em infectar e sobreviver em diferentes linhagens celulares (células do epitélio intestinal de ratos, células epiteliais de cérebro humano e macrófagos humanos) [53].

Modelos *in vivo* de inflamação intestinal induzida em camundongos recém-nascidos avaliaram o efeito terapêutico de CNM contra *C. sakazakii*. Os resultados mostraram que o tratamento reduziu o número de unidades formadoras de colônias do patógeno no tecido íleo e minimizou os danos morfológicos nos tecidos intestinais. Este efeito protetor foi confirmado pela baixa expressão gênica de IL-6, TNF-α, importantes citocinas pro-inflamatórias, além de inibir a ativação da via NF-κB induzida pela bactéria [54].

### 3.2. *Escherichia coli*

A espécie *Escherichia coli* é uma bactéria Gram-negativa, normalmente encontrada na microbiota de animais e humanos, desempenhando um papel importante durante a digestão e síntese de vitaminas [55]. Entretanto, enquanto algumas linhagens mantém um relacionamento comensal com o organismo, outras linhagens têm sido relatadas em diversos casos de infecção, como infecções do trato urinário [56, 57], gastrointestinal [58, 59], meningite [60, 61] e sepse [62, 63]. Essa patogenicidade é devido a algumas propriedades como fatores de virulência que

possibilitam a capacidade de adesão celular, assim como a produção de exotoxinas que contribuem para o estabelecimento da infecção [64-66]. Compostos naturais com amplo potencial antimicrobiano, como por exemplo o CNM, tem sido estudado na tentativa de combater o avanço da infecção.

A eficácia do uso de compostos derivados de plantas como tratamento alternativo em infecções bacterianas tem impulsionado estudos que elucidem a ação desses compostos na inibição de fatores de virulência bacteriana [67-69]. A exemplo disso, um trabalho com *E. coli* O157:H7 enterohemorrágica (EHEC) em linhagens de células humanas Caco-2 observou que CMN não diminuiu a capacidade de adesão e invasão celular da bactéria, porém reduziu a motilidade e formação de biofilme, apresentando diminuição da expressão de genes codificadores de fatores de virulência [18]. Entretanto, foi demonstrado em outro trabalho que CNM diminuiu a capacidade de invasão e adesão de duas linhagens de *E. coli* uropatogênicas (UPEC). Este efeito foi associado a baixa expressão de genes de virulência (*fimA*, *fimH*, *focA*, *sfaA*, *sfaS* e *papG*) importantes para patogênese da infecção no trato urinário [70].

Essa ação inibitória do CNM sob a expressão de genes de virulência de *E. coli* foi também observada na interação de EHEC com células epiteliais de intestino bovino [71]. Posteriormente, foi relatado que o CNM é capaz de reduzir diversos fatores de virulência da EHEC pela modulação da expressão de genes como $stx_1$ e $stx_2$ (responsáveis pela produção de verotoxinas VT1 e VT2), *eae* (codificação de adesinas), *fliC*, *fimA*, *hcpA*, e *lpfA* (envolvidos na codificação de flagelina A, fimbria tipo1, *pilus* hemorrágico e fimbrias longas, respectivamente) [72]. Assim como, outros trabalhos relatam que o tratamento com CNM preveniu eficientemente a formação de biofilme em duas linhagens de *E. coli* uropatogênicas [73] e isolados clínicos de amostra de urina obtidas de mulheres hospitalizadas [74].

Os resultados benéficos do CNM na inibição de fatores de virulência de *E. coli* foram confirmados em um modelo de infecção por UPEC. Neste estudo foi realizado utilizando um modelo onde camundongos da linhagem C57BL/6 (fêmeas) foram infectados com UPEC (via transuretral) e receberam comida contendo diferentes doses de CNM (0,1%, 0,2% e 0,4%). A suplementação com CNM reduziu a infecção do trato urinário causada por UPEC. Dentre as três concentrações testadas, CNM a 0.4 % foi a que obteve a maior eficácia na redução das populações de UPEC na bexiga [24]. Similarmente, porcos submetidos a uma dieta contendo CNM e outros elementos apresentaram menor incidência de diarreia pós-desmame causada por *E.*

*coli* enterotoxigênica (ETEC) que os animais tratados com zinco [75].

### 3.3. *Listeria monocytogenes*

*L. monocytogenes* é uma bactéria que, apesar de apresentar uma incidência consideravelmente baixa, possui um alto índice de mortalidade, sendo caracterizada como um dos principais patógenos em alimentos contaminados [76]. Devido à sua característica intracelular facultativa, a patogenicidade deste microrganismo está relacionada à sua capacidade sobreviver à ação fagocítica das células do hospedeiro e superar as barreiras fisiopatológicas [77].

A ação anti-*L. monocytogenes* de CNM tem sido relacionada com danos na membrana plasmática e alterações na geração de energia [32, 78]. CNM também é capaz de aumentar a ação de estreptomicina contra biofilmes e células planctônicas de *L. monocytogenes* [79]. Em relação aos efeitos antivirulências, tem sido relatado que CNM induz redução na motilidade, adesão e invasão bacteriana, além de provocar diminuição na produção de hemolisina e fosfolipase. Foi observado também a inibição na expressão de diversos genes responsáveis pela virulência desta bactéria [80]. CNM apresenta capacidade de potencializar a atividade antibiofilme de Nisina A (conservante alimentar natural) e seu derivado M21A [81], e também da estreptomicina [79].

O único relato de aplicação do CNM em modelos experimentais de listeriose foi realizado utilizando larvas de *G. mellonella*. Nesse estudo foi demonstrado que CNM aumentou a sobrevivências das larvas infectadas, sendo observado também um aumento na síntese de peptídeos antimicrobianos como galerimicina e lisozima [82]. Estes resultados têm incentivado o desenvolvimento de produtos contendo CNM para aplicação nas indústrias de alimentos [83].

### 3.4. *Pseudomonas aeruginosa*

Os efeitos inibitórios de CNM em diversos fatores de virulência de *P. aeruginosa* tem sido demonstrado em alguns trabalhos. Este composto reduz características de virulência por linhagens com perfis fenotípicos variados, incluindo a produção de hemolisinas, proteases, elastase, piocianina e de formar biofilme [17, 19]. Esta ação está relacionada com a inibição de vias de *quorum sensing* [19].

Aplicações tópicas diárias de CNM em feridas infectadas por *P. aeruginosa*, resultou em significativa redução da carga bacteriana no tecido e aceleração da cicatrização. Além disso encontrou-se, ao analisar o tecido, baixa concentração de Interleucina-17 (IL-17), fator de

crescimento endotelial vascular (VEGF) e óxido nítrico. As ações do CNM são referentes a ativação do Receptor de Potencial Transiente Ankiryn 1 (TRPA 1), alvo farmacológico do cinamaldeído [17].

### 3.5. *Salmonella enterica*

A eficácia do CNM em reduzir a formação de biofilme tem sido demonstrada em diferentes serovars de *Salmonella enterica* [36, 84, 85]. O mecanismo de ação de CNM em biofilmes de *S. enterica* serovar Typhimurium está relacionado com a inibição de proteínas envolvidas no metabolismo energético como Peroxirredoxinas e ATPases [36]. Em adição, CNM aumentou a ação da estreptomicina contra biofilmes de *S. enterica* serovar Typhimurium [79]. Além disso, CNM foi capaz de inibir o biofilme formado por *S. aureus* e *S. enterica* [86].

Posteriormente, a eficácia do CNM em reduzir a expressão de fatores de virulência foi demonstrada em *S. enterica* serovar Enteritidis. Os genes regulados negativamente são envolvidos na invasão celular (*sipABCD*) e OMPs (*ompW*, *ompC*, *ompS1*, e *nmpC*) [87]. De fato, a habilidade de invasão de *S. enterica* serovar Typhimurium na presença do CMN foi reduzida, como demonstrada em um modelo de infecção *in vitro* utilizando linhagens de células do epitélio intestinal (IPEC-J2) [88].

Estudos em aves fornecem confirmações adicionais do potencial de CMN na redução da expressão de genes de virulência envolvidos na motilidade bacteriana (*flhC* e *mota*) e invasão bacteriana (*hilA*, *hilD* e *invF*) com consequente diminuição da colonização em aves infectadas com *S. enterica* serovar Enteritidis [89]. Da mesma forma, foi demonstrado que aves alimentadas com ração suplementada com CNM apresentaram maior sobrevivência a infecção por *S. enterica* serovar *Heidelberg,* além de reduzir a incidência de transmissão horizontal do patógeno entre outras aves [90].

### 3.6. *Staphylococcus aureus*

Apesar de *S. aureus* compor a microbiota normal dos seres humanos, esta bactéria pode provocar uma diversidade infecções no hospedeiro, principalmente quando ocorre falha na resposta imunológica [91]. Produção de toxinas (hemolinas, leucidinas), enzimas (lipase, protease e hialuronidase), assim como a síntese de adesinas e proteínas de superfície estão entre as principais propriedades deste microrganismo que caracterizam a sua patogenicidade [92-94].

CNM tem ação antimicrobiana contra diferentes linhagens de *S. aureus*, incluindo aquelas com fenótipos de resistência [95, 96]. Dentre todos os possíveis alvos de ação antivirulência do CNM, a atividade antibiofilme tem sido relatada em diferentes estudos [22, 95, 97-100]. Além disso, tem sido demonstrado que CNM inibe significativamente a capacidade de *S. aureus* de formar biofilme tanto em superfícies de poliestireno e aço inoxidável [98]; como também diminuindo a capacidade desta bactéria de aderir-se ao látex [95]. Reduções na expressão de *sarA*, um regulador positivo de biofilme mediado por Bap, são apontadas como uma das causas que atribuem estas características inibitórias ao composto [97].

Apesar de todos estes indícios da eficácia do CNM contra *S. aureus* (tendo como alvo a viabilidade e virulência desta bactéria), a aplicação deste composto em modelos murinos de infecção provocada por esta bactéria ainda não foram publicados. No entanto, quando testado em modelos alternativos de infecção baseado em larvas de *Galleria mellonella*, CNM diminuiu a mortalidade das larvas infectadas por *S. aureus* e reduziu a carga bacteriana na hemolinfa [95].

### 3.7. *Vibrio* ssp.

CNM (e compostos derivados) também são capazes de reduzir a expressão de diversos fatores de virulência (formação de biofilme, produção de pigmentos e proteases) de espécies de *Vibrio*, através da inibição do *quorum sensing* mediado por A2 [101-103]. Esta ação se dá pela redução de LuxR ao DNA bacteriano. Estes dados foram confirmados em um modelo alternativo de infecção utilizando *Artemia*, onde CNM foi capaz de proteger este organismo da infecção por *Vibrio harveyi* [102].

CNM encapsulado em lipossomos foram demonstrados como eficazes agentes contra a infecção causada por *Vibrio vulnificus* em *Danio rerio* (*zebrafish*). Foi demonstrado que os peixes tratados com esta formulação apresentaram maior sobrevivência, o que estaria relacionada com uma resposta imunológica mais eficaz pela inibição de citocinas pró-inflamatória (como IL-1β, IL-6, IL-15 e TNF-α) e indução de citocinas anti-inflamatórias (IL-10) e da porção C3b do complemento [104].

## 4. Conclusão

Cinamaldeído tem diversos mecanismos que possibilitam o uso deste óleo essencial para o tratamento de doenças infecciosas caudas por bactérias. Destaca-se seu potencial antimicrobiano

de amplo espectro e a capacidade de reduzir a expressão de diversos fatores de virulência de diversas bactérias. Aliado a isso, os efeitos imunomoduladores deste composto aprimora a resposta do hospedeiro, ajudando no controle da infecção. Estas evidências experimentais incentivam o uso do cinamaldeído como molécula líder para o desenvolvimento de novos fármacos anti-infectivos.

**Tabela 1.** Microrganismos de relevância clínica com crescimento inibido pelo cinamaldeído.

| | Microrganismos | Referências |
|---|---|---|
| **Gram-positivos** | *Bacillus cereus* | [105] |
| | *Bacillus subtilis* | [35] |
| | *Clostridium botulinum* | [106] |
| | *Clostridium difficile* | [107] |
| | *Enterococcus faecalis* | [95] |
| | *Listeria monocytogenes* | [78] |
| | *Mycobacterium avium* | [41] |
| | *Mycobacterium tuberculosis* | [30] |
| | *Staphylococcus aureus* | [95] |
| | *Staphylococcus epidermidis* | [108] |
| | *Streptococcus agalactiae.* | [104] |
| | *Streptococcus dysgalactiae,* | [109] |
| | *Streptococcus mutans* | [110] |
| | *Streptococcus pyogenes* | [111] |
| | *Streptococcus uberis* | [109] |
| **Gram-negativos** | *Acinetobacter baumannii* | [112] |
| | *Aeromonas hydrophila* | [113] |
| | *Acinetobacter baumannii* | [112] |
| | *Aeromonas hydrophila* | [113] |
| | *Escherichia coli* | [24] |
| | *Enterobacter aerogenes* | [114] |
| | *Salmonella enterica* | [36] |
| | *Shewanella putrefaciens* | [115] |
| | *Pseudomonas aeruginosa* | [116] |
| | *Porphyromonas gingivalis* | [117] |
| | *Proteus vulgaris* | [114] |
| | *Vibrio cholerae* | [114] |
| | *Vibrio parahaemolyticus* | [114] |

| | | |
|---|---|---|
| | *Vibrio vulnifucus* | [104] |
| **Leveduras** | *Candida albicans* | [118] |
| | *Candida glabrata* | [119] |
| | *Candida tropicalis* | [114] |
| | *Candida krusei* | |
| | *Cryptococcus neoformans* | [120] |
| | *Cryptococcus laurentii* | [120] |
| | *Malassezia pachydermatis* | [121] |
| **Fungos filamentosos** | *Aspergillus ochraceus* | [46] |
| | *Aspergillus fumigatus* | [23] |
| | *Aspergillus westerdijkiae.* | [122] |
| | *Fusarium sporotrichioides* | [123] |
| | *Fusarium graminearum* | [123] |
| | *Fusarium langsethiae* | [123] |
| | *Microsporum gypseum* | [114] |
| | *Penicillium expansum* | [124] |
| | *Penicillium italicum* | [125] |
| | *Penicillium verrucosum* | [122] |
| | *Trichophyton rubrum* | [114] |
| | *Trichophyton mentagraphytes* | [114] |

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**ARTIGO 3**

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# Cinamaldeído melhora a inflamação associada a lesões cutâneas infectadas por *Staphylococcus aureus*.

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## Resumo

*Staphylococcus aureus* é apontado como microrganismo patogênico de maior ocorrência em feridas cutâneas. Os variados perfis de resistência e virulência de linhagens desta espécie têm estimulado o desenvolvimento de novas alternativas terapêuticas para o tratamento de infecções causadas por este patógeno. Nesse contexto destaca-se o cinamaldeído (CNM) por apresentar potentes efeitos antimicrobianos, cicatrizantes e anti-inflamatórios. No presente trabalho avaliou-se a ação do CNM em feridas cutâneas experimentais infectadas por *S. aureus*. Camundongos *Swiss* (n=12/grupo) foram alocados aleatoriamente em três grupos (animais com lesões não

infectadas; animais com lesões infectadas e sem tratamento; animais com feridas infectadas e tratadas com CNM). A infecção ocorreu logo após a indução da lesão, enquanto o tratamento iniciou um dia após e durou por dez dias. O tratamento de feridas com CNM melhora o processo de cicatrização de lesões cutâneas infectadas por *S. aureus*. A severidade da infecção também foi atenuada pela aplicação tópica do CNM, um efeito associado a diminuição da carga bacteriana na ferida. Foi também observado que o número de neutrófilos e a concentração de TNF-α e IL-6 estavam diminuídos nas lesões tratadas com CNM. Tomados em conjuntos, estes dados fornecem mais evidências da eficácia do CNM para o tratamento de lesões cutâneas infectadas.

**Palavras-chave:** Óleos essenciais.Cinamaldeído. *Staphylococcus aureus*. Cicatrização.

## INTRODUÇÃO

A cicatrização de lesões cutâneas ocorre por um complexo processo que envolve a participação de diferentes moléculas e células, que se organizam: hemostasia, inflamação, proliferação e remodelação (Boniakowski et al., 2017; Han and Ceilley, 2017; Komi et al., 2019; Phillipson and Kubes, 2019). Diversos fatores locais ou sistêmicos podem retardar o processo de cicatrização, podendo ser destacados: doenças metabólicas, quadros de imunossupressão, insuficiência venosa e presença de microrganismos patogênicos (Han and Ceilley, 2017; Malone et al., 2017).

A invasão de patógenos no tecido cutâneo lesado induz intensa resposta inflamatória que predispõe ao desenvolvimento de feridas crônicas e retarda no processo de reparo tecidual (Rosique et al., 2015; Rahim et al., 2017). Nestes quadros ocorre uma intensa produção de citocinas pró-inflamatórias (como TNF-α e IL-6) que atuam, dentre outras ações, no recrutamento de leucócitos para o local da ferida assim aumentam o dano tecidual (Boniakowski et al., 2017; Brockmann et al., 2017).

*Staphylococcus aureus*, por ser frequente integrante da microbiota da pele, é apontado

como microrganismo patogênico de maior ocorrência em feridas cutâneas (Ryu et al., 2014; Rahim et al., 2017). Este patógeno possui adesinas que facilitam sua aderência ao tecido e favorecem a formação de biofilme e o desenvolvimento de infecções crônicas (Serra et al., 2015; Bassetti et al., 2017). Além disso, diversas linhagens de *S. aureus* apresentam fatores de virulência relacionados aos danos nas células hospedeiras e a evasão do sistema imunológico, contribuindo assim para a permanência do patógeno e para a gravidade da lesão tecidual (Shallcross et al., 2013; Oliveira et al., 2018). A presença de *S. aureus* em lesões cutâneas é um fator preocupante, visto que o número de casos de cepas resistentes à antibióticos tem aumentado nos últimos anos (Foster, 2017).

Assim a necessidade de desenvolver novas alternativas terapêuticas para o tratamento das lesões infectadas por microrganismos é crescente (Serra et al., 2015). Os óleos essenciais têm sido apontados como promissores candidatos para combater infecções devido aos seus efeitos antimicrobianos e anti-inflamatórios (Pandey et al., 2016; Swamy et al., 2016). No contexto do tratamento de feridas contaminadas, também é importante que este candidato a fármaco possua, além de ação antimicrobiana, efeitos cicatrizantes e imunomoduladores (Carvalho et al., 2018). Diversas evidências sugerem que o cinamaldeído (CNM), um dos principais compostos presente no óleo essencial da Canela (*Cinnamomun* sp.), consegue cumprir todos estes requisitos (Chen et al., 2017; Vasconcelos et al., 2018; Ferro et al., 2019).

CNM é destacado por possuir efeito antimicrobiano de amplo espectro além de inibir a virulência de diversas espécies de bactérias (Shreaz et al., 2016; Friedman, 2017; Vasconcelos et al., 2018). Este composto é capaz de reduzir a viabilidade de biofilmes formados por *S. aureus*, além de inibir a capacidade deste patógeno de aderir a superfícies e liberar substâncias citotóxicas (Jia et al., 2011; Ferro et al., 2016). Recentemente, foi demonstrado que o tratamento tópico com CNM acelerou o processo de reparo tecidual de feridas contaminadas por *Pseudomonas*

*aeruginosa*, um efeito associado com a diminuição da severidade da infecção e mediado pela interação com o receptor TRPA-1 (*transient receptor potential ankyrin 1*) (Ferro et al., 2019). Assim, o presente estudo teve como objetivo avaliar os efeitos do tratamento tópico com CMN em feridas cutâneas experimentais contaminadas com *S. aureus*.

## MATERIAL E METÓDOS

### Obtenção do Cinamaldeído

Cinamaldeído oriundo da espécie vegetal *Cinnamomum cassia* foi obtido comercialmente da Sigma-Aldrich® (Darmstadt, Alemanha), pureza de > 95%.

### Linhagem bacteriana

Para os ensaios antimicrobianos foi utilizada a linhagem de *S. aureus* ATCC 6538 gentilmente cedidas pela Bacterioteca (Laboratório de Microbiologia) da Universidade CEUMA.

### Animais

O presente estudo foi realizado no Biotério da Universidade CEUMA em São Luís (MA), após a aprovação pela Comissão de Ética de Uso de Animais (CEUA) da instituição, sob o Parecer nº 129/17. Foram utilizados 36 camundongos *Swiss*, de ambos os sexos com 6 a 8 semanas de vida e apresentando peso corpóreo entre 19 a 30 g, procedentes do biotério da Universidade CEUMA. Os animais foram alojados em gaiolas individuais de polipropileno, forradas com maravalha e colocadas em uma estante ventilada, com microisoladores de sistemas independentes de insuflamento e exaustão de ar, diminuindo o risco de outros tipos de infecções. O laboratório para alojamento dos animais apresentava-se arejado e com temperatura média de

21ºC, com sistema de ventilação e ciclo claro-escuro de 12h. Os animais foram alimentados com ração específica para roedores e água *ad libitum* sendo obedecidos os princípios éticos em experimentação animal preconizados pelo CEUA sob protocolo n° 129/2017.

**Protocolo para Indução da Ferida**

Para realização do procedimento cirúrgico experimental, os camundongos foram previamente anestesiados com cloridrato de xilazina (1 mg/kg) e cloreto de cetamina (50 mg/kg) por via intramuscular. Após a anestesia, foi realizado a tricotomia e a assepsia da região dorsal torácica com álcool etílico a 70% e solução salina estéril (NaCl a 150 mM). A pele foi demarcada usando-se um molde de papel adesivo (8 mm), previamente esterilizado e a ferida cutânea foi produzida por excisão da pele com tesoura cirúrgica e pinça de dissecção (Ferro et al., 2019). Em seguida, as feridas foram contaminadas utilizando uma suspenção contendo *S. aureus* ATCC 6538 (80 μL, 1,5 x $10^8$ UFC/mL). O grupo de animais não infectados recebeu o mesmo volume de solução salina sobre o leito da ferida.

**Grupos Experimentais e Tratamento Tópico**

Os animais foram agrupados (n=12 por grupo) de acordo com o tipo de tratamento tópico realizado e a análise da cicatrização, sendo:

- Grupo controle não infectado (CON): Animais com feridas assépticas e tratados diariamente com 50 μL de solução salina (150 mM);
- Grupo controle infectado (Sa): Animais com feridas experimentais infectadas tratados diariamente com 50 μL de solução salina (150 mM);

- Grupo infectado + cinamaldeído (Sa + CNM): Animais com feridas experimentais infectadas tratados diariamente com 50 µL de uma solução contendo cinamaldeído (200 µg por animal).

Os curativos foram feitos diariamente dentro da câmara de fluxo laminar precedidos de atividades antissépticas para evitar qualquer tipo de contaminação, no mesmo horário, com a aplicação dos respectivos tratamentos até o 3° ou 10º dias. Antes da aplicação do produto na ferida, foi feito o registro fotográfico da lesão. Uma análise macroscópica das lesões quanto ao tamanho e parâmetros clínicos (presença de esfacelo, crosta, edema, exsudato, necrose, cor e tipo de tecido. Em seguida, foi adicionado uma cobertura da lesão (curativo tipo *band-aid*) para evitar contaminações por outros microrganismos. Para melhor controle do quadro clínico, os camundongos foram pesados e a temperatura verificada diariamente (resultados não mostrados por não ter diferença entre os grupos).

**Contagem de Bactérias Viáveis**

A parte dorsal da ferida (tecido) foi removida e usada para contagem de bactérias (24h após termino do tratamento de 3 e 10 dias). Para recuperação das bactérias presentes nas feridas, amostras da lesão infectada foram coletadas em tubos contendo 1mL de PBS com homogeneização por 90 segundos, seguido por centrifugação por 5 minutos a 2500 RPM. Os sobrenadantes da solução foram diluídos, plaqueados em Agar Manitol Salgado. Após incubação a 37°C por 24 h, os valores de UFC foram determinados por método padrão de contagem de colônias.

**Avaliação Clínica da Lesão**

As lesões foram avaliadas diariamente após 24 horas da infecção, observando-se na área circunscrita à ferida os seguintes parâmetros: hiperemia, edema, hematoma, sangramento,

secreção, odor, prurido, presença e característica das crostas, presença necrose, coloração e aspecto do tecido de granulação e cicatricial (tabela 1) suplementar 1). Os gráficos representam a somatório dos valores obtidos em cada parâmetro. Todas as feridas foram fotografadas e seus diâmetros maior (comprimento) e menor (largura), devido a tração da pele, foram medidos diariamente pós-infecção precedendo as biópsias. Para a mensuração da área da lesão foi utilizado a seguinte a equação:

$$A = \pi * R * r$$

"A" representa a área ($cm^2$); "R" (diâmetro/2), o raio maior e "r", o raio menor. O grau de contração será expresso em percentual, será mensurado pela equação:

$$100 * (Ai - Af) = \%\ de\ contração$$

Ai= área inicial da ferida; Af = área final.

**Dosagem de Mediadores Inflamatórios**

As concentrações de citocinas [Interleucina-2 (IL-2), Interleucina-4 (IL-4), Interleucina-6 (IL-6), Interferon-γ (IFN-γ), Fator de Necrose Tumoral (TNF), Interleucina-17A (IL-17A) e Interleucina-10 (IL-10)] foram quantificados em amostras de lesão (pele), coletadas no 3º dia e 10° dia (resultados não mostrados por não haver a presença de citocinas na amostra), pelo Kit CBA e foram mensuradas por citometria de fluxo (Accuri C6 BD®). Amostras de tecido foram pesadas e uma igual quantidade de cada amostra foi macerada em 1 mL de PBS com homogeneização por 90 segundos, seguido por centrifugação por 5 minutos a 2500 RPM. O sobrenadante foi coletado para dosagem de citocinas. Os valores foram expressos em pg/mL.

**Caracterização de fenótipo celular por citometria de fluxo**

As amostras de pele foram trituradas em meio RPMI e filtradas por Corning® cell strainer (Sigma) com poros de 40 µm. Após a filtração, as amostras foram lavadas duas vezes com tampão fosfato salino (PBS) e ressuspensas em meio RPMI suplementado (10% de soro fetal e penicilina-estreptomicina). Aproximadamente, um milhão de células foram adicionadas a cada poço de uma placa de microdiluição e marcadas durante com 7AAD (7-aminoactinomicina D; Thermo Fisher Scientific) para viabilidade, e com diferentes anticorpos monoclonais (ver quadro 1) conjugados a fluorocromos (FITC, PE, PerCP, APC), todos da eBiosciences (Brasil). As amostras foram analisadas em um citometro de fluxo BD Accuri C6 (BD Biosciences - Immunocytometry Systems). Os eventos foram analisados com o software FlowJo 7.6.1 (TreeStar-CA).

Quadro 1: anticorpos Monoclonais utilizados após coleta do tecido

| APC | PE | PercP | FITC |
|---|---|---|---|
| CD 3e | Ly-6G | CD4 | Ly-6C |
| CD 62 L | CD 14 | CD 8 | F4/80 |

**Avaliação Histológica de Reparo Tecidual**

Após eutanásia dos animais nos terceiro e décimo dias pós-infecção, procedeu-se a coleta de fragmentos de pele com fixação em formol tamponado com PBS pH 7,2, em seguida desidratação em concentrações crescentes de álcool etílico, diafanização em xilol, embebição e inclusão em parafina. O bloco de parafina obtido, foi submetido a cortes no micrótomo de 3 a 5 µm de espessura, com posterior coloração da lâmina contendo amostra tecidual em Hematoxilina e Eosina (HE). As lesões foram analisadas em microscópio de luz (Leica), aproximadamente 10 campos, nos aumentos de 40 a 400 X. Os critérios avaliados foram: debris celulares (presença ou ausência); infiltrado inflamatório; reepitelização (presença ou ausência); vascularização e padrão

de distribuição das fibras colágenas/fibroblastos. Estabeleceu-se os seguintes escores para o infiltrado inflamatório: ausência (não observado em nenhum campo), leve (1 a 3 campos observados), moderado (4 a 6 campos observados) e intenso (acima de 7 campos observados).

**Análise Estatística**

As porcentagens de inibição do crescimento bacteriano foram calculadas como a média das inibições obtidas para cada experimento individual. Os gráficos e a avaliação estatística dos resultados foram realizados por meio de análise de variância (ANOVA) no programa Graphpad Prism 5.0, seguida pelo teste de Boferroni ou Kruskal – Wallis, para dados paramétricos e não paramétricos respectivamente. Adotou-se o nível de significância $< 0,05$.

## RESULTADOS

### Tratamento tópico com Cinamaldeído reduz o tempo de cicatrização das feridas contaminadas por *S. aureus*

Neste trabalho foi avaliada a ação do CNM, administrado topicamente e de forma diária, em feridas contaminadas por *S. aureus*. A dose de CNM usada foi determinada através da avaliação da atividade antimicrobiana do CNM contra diferentes linhagens de *S. aureus*. Este composto é capaz de inibir o crescimento de todas as linhagens de *S. aureus* com uma mediana de concentração inibitória mínima (CIM) de 1000 µg/mL (dados não mostrados). A partir disto, o tratamento tópico foi baseado na administração de 50 µL de uma solução de CNM a 4000 µg/mL (quatro vezes a mediana da CIM), resultando na aplicação de 200 µg/mL por animal a cada dia.

Inicialmente, foi analisada a evolução dos tamanhos das lesões durante os dez dias de tratamento (Figuras 1 e 2). O grupo de animais infectados por *S. aureus* apresentou nos primeiros

seis dias menor contração das áreas das lesões, em relação aos camundongos não infectados (Figuras 2A e 2B). No entanto, no décimo dia a maioria dos animais dos dois grupos já apresentavam fechamento completo da ferida. Em contrapartida, no grupo infectado tratado com CNM o processo de cicatrização completo foi significativamente acelerado, sendo que todos animais já apresentavam as lesões totalmente reparadas no oitavo dia de tratamento.

No sétimo dia, os animais infectados e tratados com CNM apresentaram valores de contração média da ferida (95,50 ± 1,91%) significativamente superiores ($p < 0,05$) aos observados para os demais grupos (66,50 ± 14,99 e 72,50 ± 12,40, para os animais com feridas não contaminadas e animais com feridas contaminadas com *S. aureus*, respectivamente) (Figura 2C). Estes dados ficam ainda mais claros quando se observa as áreas sob a curva (AUC) obtidas a partir dos gráficos de evolução diária das áreas de lesão, evidenciando que os animais tratados com CNM apresentaram menores valores ($p < 0,05$) (Figura 2B).

**Tratamento tópico com Cinamaldeído restaura a integridade tecidual das feridas contaminadas por *S. aureus***

A análise histológica comprovou que o protocolo terapêutico à base de cinamaldeído intensificou o processo de cicatrização, com o processo de reepitelização bem evidenciado ao fim de 10 dias de tratamento. Neste estágio foi possível observar que a epiderme apresentou padrões semelhantes ao grupo controle, isto é caracterizada pelos estratos (basal, espinhoso, granuloso, lúcido-córneo), com queratinização bem evidenciada. A derme apresentou intensa celularidade (fibroblastos), distribuição uniforme de feixes de fibras colágenas, leve infiltrado inflamatório, ampla vascularização e ausência de anexos dérmicos (Figura 3).

Os fragmentos de pele observados aos 3 dias pós-tratamento, demonstraram a presença de debris celulares, ausência de reepitelização, moderada celularidade (fibroblastos), intenso

infiltrado inflamatório com predominância polimorfonuclear na derme e, também, associado ao tecido adiposo subcutâneo e ampla vascularização (Figura 3).

**O tratamento tópico com Cinamaldeído reduz a severidade da infecção nos animais com feridas contaminadas por *S. aureus***

Diariamente foram analisados diversos aspectos que em conjunto constituem um índice de severidade do processo inflamatório induzido pela lesão cutânea (Figura 4). Verificou-se que escore de severidade mais elevado nos camundongos infectados em relação ao grupo com feridas não contaminadas. Este efeito foi mais evidente entre o segundo e quinto dia (período que os dois grupos apresentaram diferenças estatísticas; $p < 0,05$), comprovando que a presença de *S. aureus* desencadeia um prolongamento na fase inflamatória.

Os animais com lesões infectadas e tratadas com CNM apresentaram um escore de severidade semelhante ao grupo controle. Em relação aos animais com feridas infectadas, o tratamento com CNM foi capaz de reduzir a severidade da infecção a partir do segundo dia ($p< 0,05$) (Figura 4A). Na análise da área sob a curva do gráfico dos parâmetros clínicos, os grupos Sa + CNM e COM também apresentaram valores significativamente menores que o grupo Sa ($p< 0,05$) (Figura 4B).

**O tratamento tópico com Cinamaldeído reduz a carga bacteriana nas feridas contaminadas por *S. aureus***

Em seguida, foi avaliado se o tratamento com CNM reduziria a carga bacteriana na região da lesão cutânea infectada por *S. aureus* após o décimo dia de tratamento. O grupo Sa apresentou uma média de 7,5 Log UFC/mL, enquanto que no grupo Sa + CNM houve uma redução significativa da carga bacteriana presente na região de lesão (4, 9 log UFC/mL; $p< 0,05$) (Figura

5).

**O tratamento tópico com Cinamaldeído reduz os níveis de citocinas pró-inflamatória nas feridas contaminadas por *S. aureus***

A presença de citocinas (IL-2, IL-4, IL-6, IFN-γ, TNF-α, IL-17A, IL-10) no tecido cutâneo após três e dez dias da indução da lesão foi avaliada através do Kit CBA (*Mouse Th1/Th2/Th17 Cytokine Kit*) a partir de fragmentos de tecido. Apenas as citocinas IL-6, TNF-α, e IL-17A foram detectadas no terceiro dia (Figura 6). O grupo Sa apresentou os maiores teores de IL-6 e TNF-α em relação aos demais grupo ($p < 0,05$). O tratamento com CNM promoveu redução nas concentrações teciduais de IL-6 e TNF-α na ordem de 76,04% e 79,32%, respectivamente (Figura 6A e 6B). Os níveis de IL-17 não se apresentaram alterados entre os grupos experimentais (Figura 6C). De igual modo, no décimo dia não foi detectado alterações nos níveis de citocinas.

**O tratamento tópico com Cinamaldeído reduz os níveis de neutrófilos nas feridas contaminadas por *S. aureus***

Desta forma, foram analisadas as populações de monócitos/macrófagos ($Ly6C^+$) e neutrófilos ($Ly6G^+$) em amostras de ferida e no sangue periférico dos camundongos dos diferentes grupos experimentais (Figura 7). Em relação aos macrófagos, não foi possível observar nenhuma alteração significativa nos níveis destas células no tecido cutâneo (Figura 7A). Por outro lado, os níveis de seus precursores no sangue (monócitos) foram aumentados (aproximadamente em 500%) no grupo de animais com lesões infectadas por *S. aureus* ($p < 0,05$). O grupo Sa + CNM apresentou níveis reduzidos de monócitos (em cerca de 35%) em relação aos animais com feridas infectadas e sem tratamento; no entanto, sem diferenças estatísticas (Figura 7B).

No tocante a quantificação dos neutrófilos na amostra de ferida, foi observado que a população de neutrófilos no tecido foi dramaticamente alterada mediante a infecção cutânea por *S. aureus* (aumento de 335%). Este quadro foi alterado no grupo submetido ao tratamento tópico com CNM, que apresentaram níveis de neutrófilos similares ao grupo sem infecção (Figura 7C). A infecção das feridas com *S. aureus* também alterou a quantidade de neutrófilos sanguíneos (redução de 159%). No grupo *Sa* + CNM foi detectados níveis reduzidos de neutrófilos no sangue em relação aos animais com feridas infectadas (aproximadamente 50%), porém sem diferenças estatísticas (Figura 7D).

## DISCUSSÃO

A ocorrência de processos infecciosos em feridas, em particular causados por bactérias multirresistentes, é um grave problema de saúde pública (Pereira-Franchi et al., 2017; Upreti et al., 2018; Dotel et al., 2019). Infecções graves de feridas podem causar maior morbidade e mortalidade em pacientes; acarretando internações mais longas, atraso na cicatrização bem como diminuição na qualidade de vida (Neves et al., 2019). Neste sentido, há uma grande necessidade de obter novas alternativas para o tratamento destas lesões (Pazyar et al., 2014; Neves et al., 2019), sendo o CNM (graças aos seus efeitos antimicrobianos, cicatrizantes e imunomoduladores) considerado um recurso atrativo (Yuan et al., 2018; Ferro et al., 2019).

Os principais resultados obtidos neste trabalho sugerem que o tratamento tópico de feridas com CNM melhora o processo de cicatrização de lesões cutâneas infectadas por *S. aureus*; um efeito associado com a diminuição da carga bacteriana e modulação da resposta do hospedeiro. Destaca-se que CNM é considerado seguro em concentrações de até 8% no tecido cutâneo em humanos e de até 15% em outros mamíferos (Bickers et al., 2005), valores bem acima dos

utilizados neste estudo (0.4%).

O tratamento tópico com CNM foi capaz de acelerar o reparo tecidual, sendo observada ao fim do período experimental uma eficaz restauração das estruturas da pele (evidenciado pelas análises histológicas). O efeito benéfico de CNM no processo de cicatrização foi comprovado recentemente utilizando feridas experimentais assépticas e contaminadas com *P. aeruginosa* (Yuan et al., 2018;Ferro et al., 2019). Esta ação foi relacionada com a indução do processo de angiogênese no local da ferida através da ativação das vias de sinalização PI3K/AKT (fosfatidilinositol 3-cinase (PI3K)/AKT) e MAPK (proteína cinase ativada por mitógenos) (Yuan et al., 2018). Em feridas infectadas por *P. aeruginosa*, CNM reduziu a expressão de mediadores inflamatórios e a proliferação microbiana. Foi demonstrado que estes efeitos têm a participação do receptor TRPVA1 (Ferro et al., 2019).

Neste trabalho também foi possível observar que a administração de CNM reduziu a carga bacteriana no tecido cutâneo. Diversos relatos *in vitro* indicam que CNM interfere na viabilidade, propriedades adesivas (incluindo a formação de biofilme) e toxicidade (produção de hemolisinas) de *S. aureus* (Ferro et al., 2016; Ortines et al., 2018; Liu et al., 2019). Estes fatores de virulência, sobretudo os biofilmes bacterianos, estão relacionados com a cronicidade de lesões cutâneas (Anderson et al., 2018;Roy et al., 2019). No entanto, a eficácia deste composto em infecções causadas por *S. aureus* só foi avaliada utilizando o invertebrado *G. mellonella* (Ferro et al., 2016). Desta forma, a redução no crescimento e na expressão de fatores de virulência de *S. aureus* que levou a menores danos teciduais nos animais tratados com CNM.

Os níveis diminuídos de bactéria nas feridas devem estar associados com a menor severidade da infecção nos animais do grupo Sa + CNM (que apresentaram escores de severidade similares aos animais do grupo controle). Adicionalmente, evidências obtidas de diversos modelos de infecção microbiana demonstram que CNM possui propriedades anti-inflamatórias

(Muhammad et al., 2015; Chung et al., 2018; Ferro et al., 2019). Os dados obtidos nesta pesquisa demonstram que este composto reduz a inflamação exacerbada relacionada com a infecção por *S. aureus* através da diminuição da migração de neutrófilos para o local da lesão, assim como diminuição da liberação de citocinas pró-inflamatória (IL-6 e TNF-α).

O correto funcionamento da fase inflamatória da lesão é essencial para o processo de reparo tecidual. O prolongamento desta fase é relacionado com a progressão para as feridas crônicas, devido a destruição dos tecidos adjacentes o que geralmente leva a uma predisposição às infecções sistêmicas (Turi et al., 2016; Zhao et al., 2016; Sibbald et al., 2017). A presença de microrganismos patogênicos e/ou tecidos necrosados irá estender o período de inflamação, provocando atrasos significativos no processo de cicatrização (Rahim et al., 2017). Os elevados teores de neutrófilos, IL-6 e TNF-α nas lesões contaminadas por *S. aureus* demonstram este papel deletério da infecção sob a fase inflamatória.

IL-6 e TNF-α são citocinas que, em conjunto com outras moléculas quimiotáticas, promovem a migração de células inflamatórias e células estromais para o local da ferida (Reinke and Sorg, 2012; Wang et al., 2018; Rodrigues et al., 2019). Os neutrófilos são as primeiras a migrar para o local da infecção, sendo fortemente ativados pelas estruturas microbianas (PAMPs) e também por moléculas derivadas das células mortas (DAMPs) (Kovtun et al., 2018). Os neutrófilos desempenham funções cruciais para a defesa antimicrobiana e processo de reparo. No entanto, em situações de lesões severas, estas células podem contribuir para um estado inflamatório exacerbado e prejudicial ao hospedeiro (Kovtun et al., 2018).

Neste sentido, CNM devido ao seu caráter anti-inflamatório pode modular a expressão de citocinas pró-inflamatórias, levando assim a um menor recrutamento de células para a ferida infectada. Estes efeitos foram observados tanto clinicamente (escore de severidade de inflamação) como pela dosagem dos mediadores inflamatórios. Adicionalmente, o tratamento

com CNM também apresentou como tendência a diminuição da quantidade relativa de neutrófilos e monócitos no sangue.

## CONCLUSÃO

O presente estudo demonstrou que cinamaldeído promoveu a aceleração do processo de cicatrização de feridas contaminadas por *S. aureus*. O tratamento tópico com este composto foi eficiente na inibição da carga bacteriana no tecido lesionado, resultando em menores níveis de dano tecidual. Em adição, os animais tratados com cinamaldeído apresentaram menores teores de neutrófilos infiltrados nos tecidos cutâneos e, consequentemente, quantidades reduzidas de citocinas pró-inflamatória. Em conjunto, estes efeitos levaram a diminuição da severidade da infecção e maiores taxas de reparo tecidual. Estes dados corroboram com a aptidão terapêutica do cinamaldeído para o tratamento de feridas.

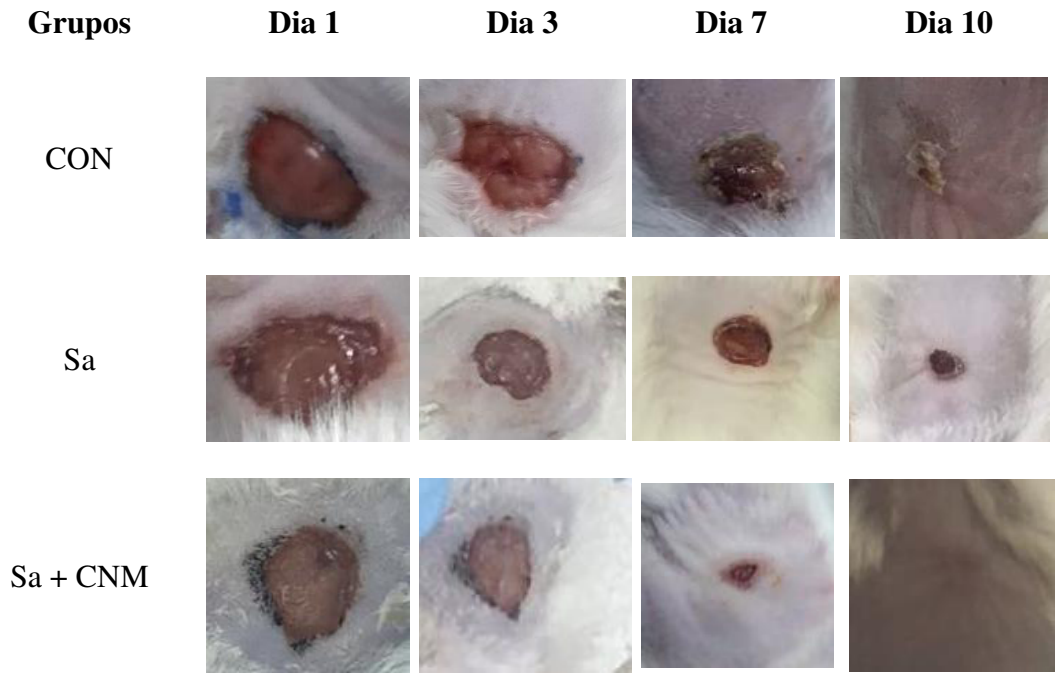


**Figura 1.** Avaliação macroscópica do processo de cicatrização dos grupos experimentais utilizados neste estudo. CON: animais com lesões cutâneas não infectadas; Sa: animais com lesões cutâneas infectadas com *S. aureus*; Sa + CNM: animais com lesões cutâneas infectadas com *S. aureus*. Os registros fotográficos foram realizados nos dias 1, 3, 7 após a indução e infecção da ferida.

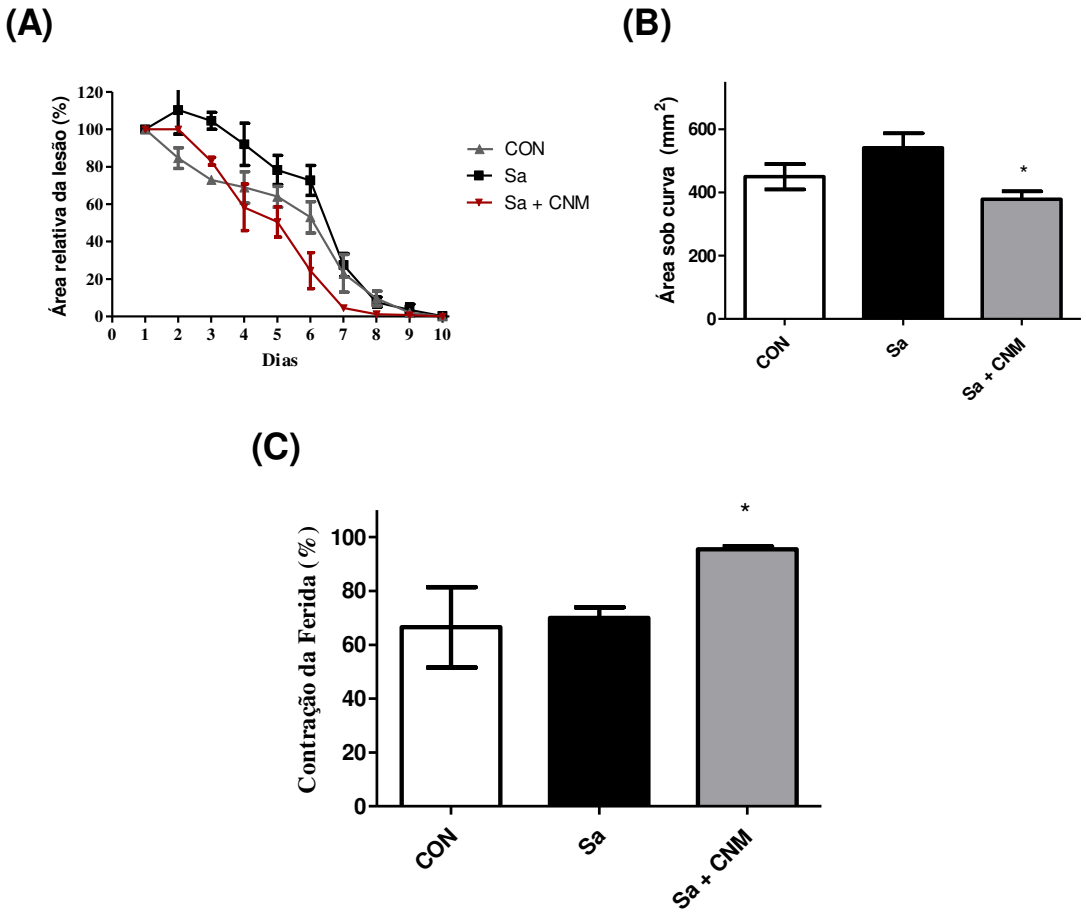


**Figura 2**. Efeito do tratamento tópico com Cinamaldeído na área da lesão cutânea contaminada por *Staphylococcus aureus.* (A) Avaliação das áreas relativas das lesões cutâneas; (B) Área sob a curva (AUC) obtida da análise de área relativa da lesão cutânea de acordo com cada grupo experimental; (C) Análise da contração da ferida após seis dias de tratamento (* P< 0,05). CON: animais com lesões cutâneas não infectadas; Sa: animais com lesões cutâneas infectadas com *S. aureus*; Sa + CNM: animais com lesões cutâneas infectadas com *S. aureus*.

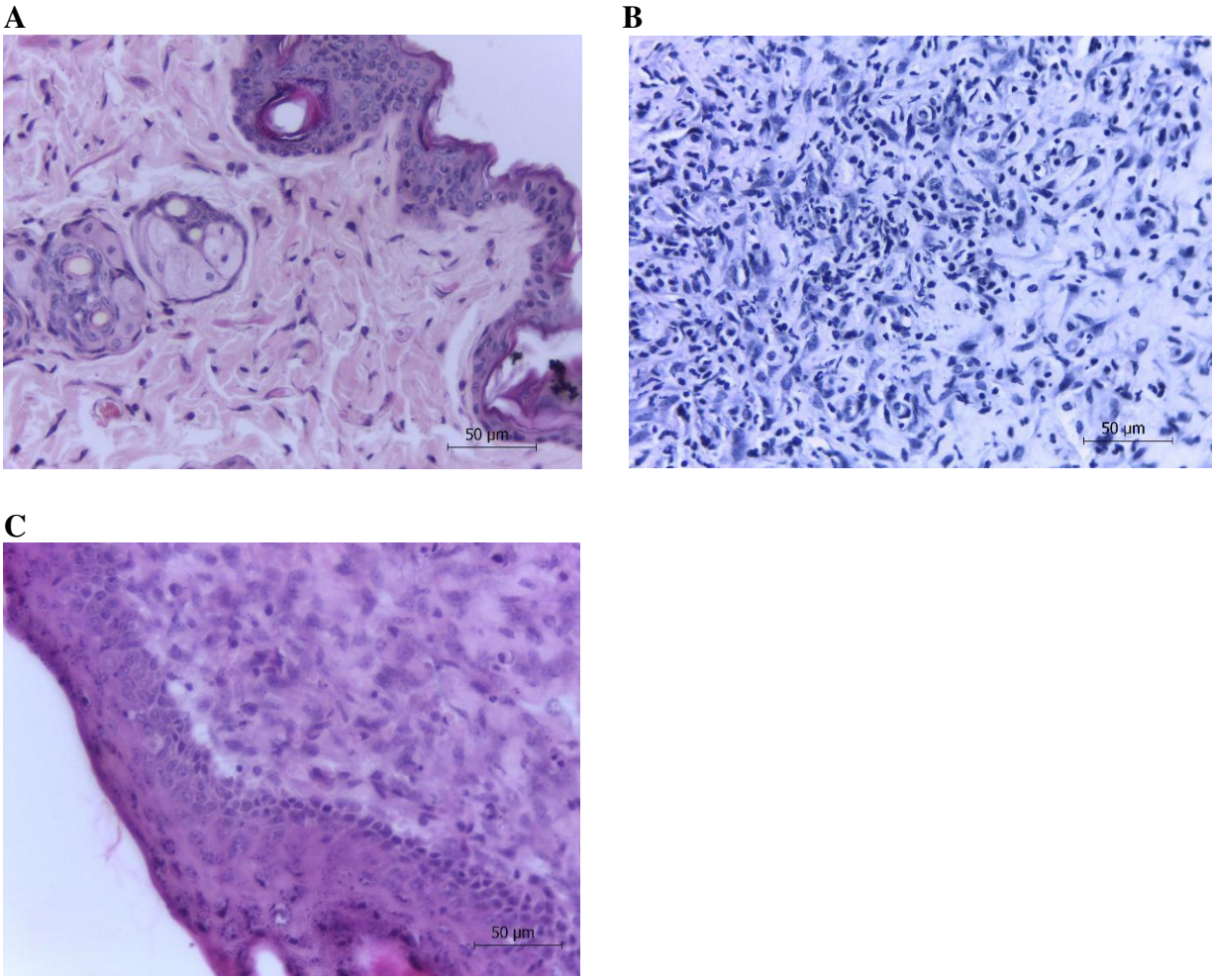


**Figura 3.** Imagens da Análise Histológica do Tecido após 10 dias de Infecção e/ou tratamento. A: Não Infectado; B:Infectado Não Tratado; C: Infectado Tratado com Cinamaldeído. Aumento (40X).Coloração: Hematoxilina/Eosina.

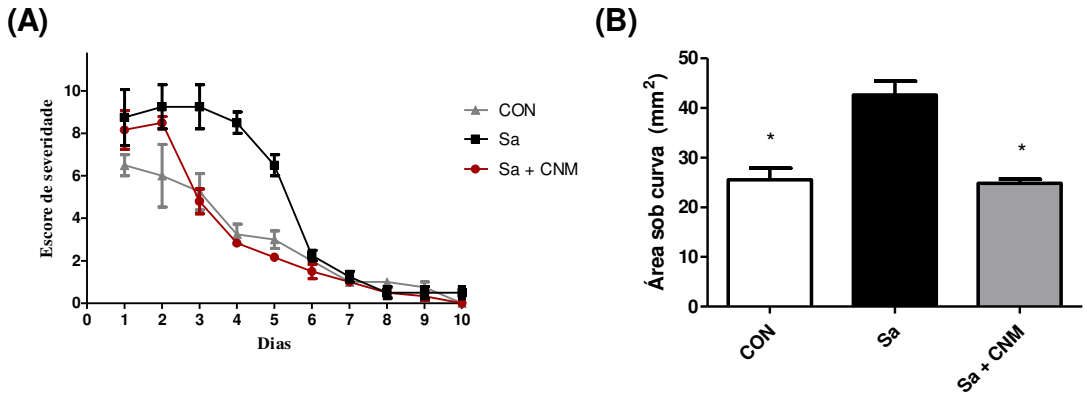


**Figura 4**. Efeito do tratamento tópico com Cinamaldeído na severidade da inflamação nos animais submetidos à lesão cutânea contaminada por *Staphylococcus aureus.* (A) Análise dos parâmetros clínicos dos grupos experimentais. (B) Análise da área sob a curva (AUC) dos parâmetros clínicos dos camundongos. CON: animais com lesões cutâneas não infectadas; Sa: animais com lesões cutâneas infectadas com *S. aureus*; Sa + CNM: animais com lesões cutâneas infectadas com *S. aureus*. (* P< 0,05)

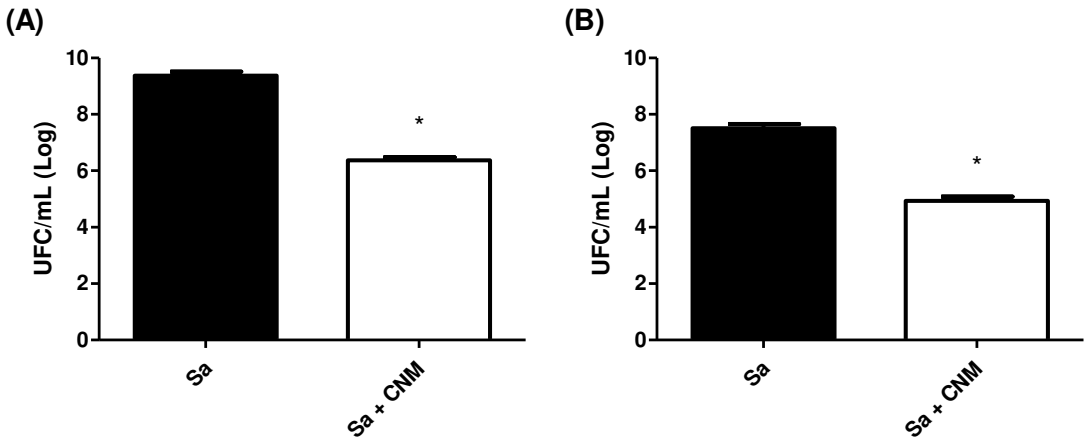


**Figura 5**. Efeito do tratamento tópico com Cinamaldeído na carga bacteriana no tecido das feridas contaminadas por *Staphylococcus aureus* após 3 (A) e 10 (B) dias após a indução e infecção da ferida. A carga bacteriana é expressa em Log de unidade formadora de colônia/mL (Log UFC/mL). CON: animais com lesões cutâneas não infectadas; Sa: animais com lesões cutâneas infectadas com *S. aureus*; Sa + CNM: animais com lesões cutâneas infectadas com *S. aureus*. (* P< 0,05).

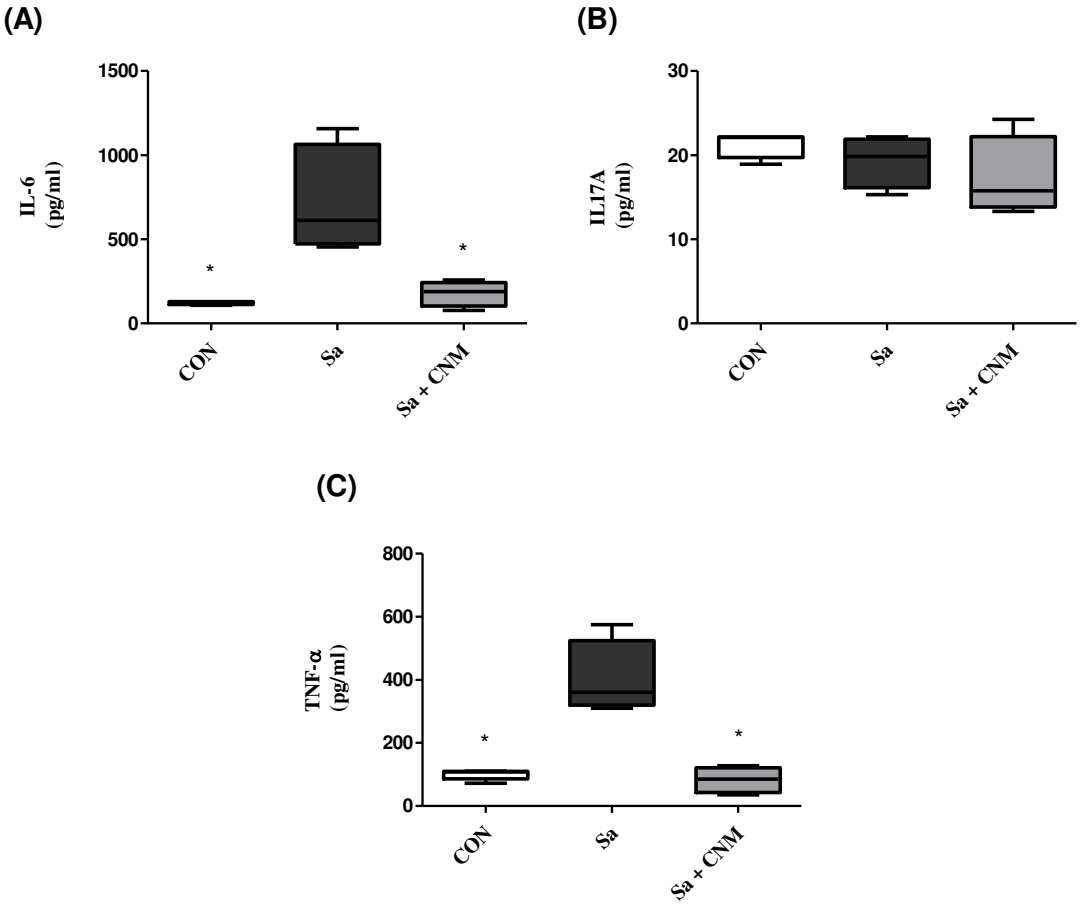


**Figura 6**. Efeito do tratamento tópico com Cinamaldeído nas concentrações de citocinas presentes no tecido das feridas contaminadas por *Staphylococcus aureus*. (A) Análise da concentração de IL-6 nos tecidos cutâneos dos grupos experimentais. (B) Análise da concentração de TNF-α nos tecidos cutâneos dos grupos experimentais. (C) Análise da concentração de IL-17 nos tecidos cutâneos dos grupos experimentais. CON: animais com lesões cutâneas não infectadas; Sa: animais com lesões cutâneas infectadas com *S. aureus*; Sa + CNM: animais com lesões cutâneas infectadas com *S. aureus*. (* P< 0,05)

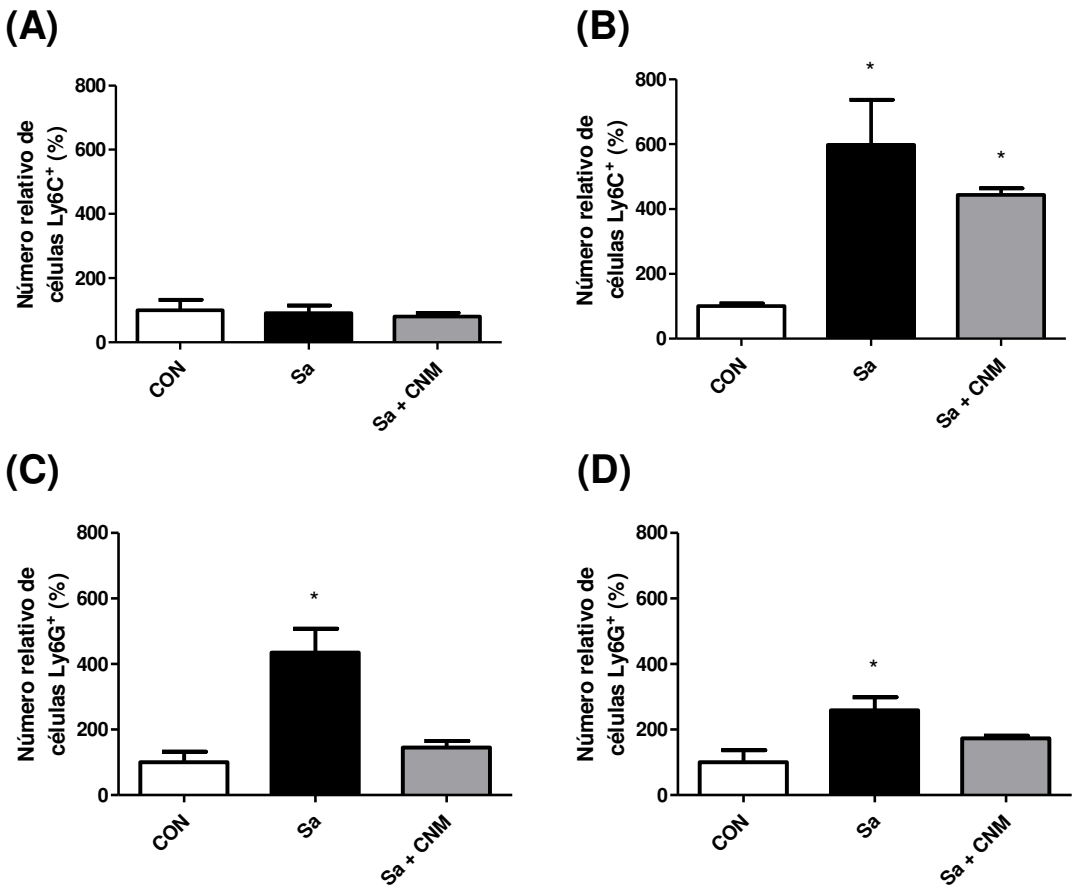


**Figura 7.** Efeito do tratamento tópico com Cinamaldeído na quantidade de macrófagos ($Ly6C^+$) e neutrófilos ($Ly6G^+$) na pele e sangue de animais submetidos a lesões contaminadas por *Staphylococcus aureus*. (A) Quantidade relativa de células $Ly6C^+$ na pele dos diferentes grupos experimentais; (B) Quantidade relativa de células $Ly6C^+$ no sangue dos diferentes grupos experimentais; (C) Quantidade relativa de células $Ly6G^+$ na pele dos diferentes grupos experimentais; (D) Quantidade relativa de células $Ly6G^+$ no sangue dos diferentes grupos experimentais

**Tabela 1.** Escores constituintes da escala de avaliação da lesão induzida em camundongos.

| Características | Interpretação | Valor |
|---|---|---|
| **Área ($cm^2$)** | | |
| | < 0,01 $cm^2$ | 0 |
| | 0,01 a 0,09 $cm^2$ | 1 |
| | 0,1 a 0,2 $cm^2$ | 2 |
| | 0,3 a 0,6 $cm^2$ | 3 |
| | 0,7 a 1 $cm^2$ | 4 |
| | 1,1 a 2 $cm^2$ | 5 |
| | 2,1 a 3 $cm^2$ | 6 |
| | 3,1 a 4 $cm^2$ | 7 |
| **Parâmetros Inflamatórios** | | |
| **Quantidade de Exsudato** | | |
| | Nenhum | 0 |
| | Leve | 1 |
| | Moderado | 2 |
| | Grande | 3 |
| **Tipo de Exsudato** | | |
| | Nenhum | 0 |
| | Sangue | 1 |
| | Serossanguinolento | 2 |
| | Seroso | 3 |
| | Purulento | 4 |
| **Edema** | | |
| | Sem edema | 0 |
| | Leve | 1 |
| | Moderado | 2 |
| | Grave | 3 |
| **Cor ao redor da ferida** | | |
| | Normal | 0 |
| | Vermelho | 1 |
| | Branco ou hipopgmentado | 2 |
| | Vermelho escuro ou roxo | 3 |
| | Preto ou hiperpigmentado | 4 |
| **Parâmetros de Debridamento** | | |
| **Tipo de Tecido** | | |
| | Tecido epitelial | 0 |
| | Tecido de granulação | 1 |
| | Esfacelo | 2 |
| | Escara | 3 |

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# ANEXOS

**ANEXO A:** Artigo Publicado - Himatanthus drasticus Leaves: Chemical Characterization and Evaluation of Their Antimicrobial, Antibiofilm, Antiproliferative Activities

*molecules*

MDPI

*Article*

# *Himatanthus drasticus* Leaves: Chemical Characterization and Evaluation of Their Antimicrobial, Antibiofilm, Antiproliferative Activities

**Cristiane Santos Silva e Silva Figueiredo [1], Joice Castelo Branco Santos [1], José Artur de Aguiar Castro Junior [1], Vinícius Galvão Wakui [2], João F. S. Rodrigues [1], Mariana Oliveira Arruda [1,3], Andrea de Souza Monteiro [1], Valério Monteiro-Neto [1,3], Maria Rosa Quaresma Bomfim [1], Lucília Kato [2], Luís Cláudio Nascimento da Silva [1,*,†] and Marcos Augusto Grigolin Grisotto [1,4,*,†]**

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**Abstract:** Plant-derived products have played a fundamental role in the development of new therapeutic agents. This study aimed to analyze antimicrobial, antibiofilm, cytotoxicity and antiproliferative potentials of the extract and fractions from leaves of *Himatanthus drasticus*, a plant from the Apocynaceae family. After harvesting, *H. drasticus* leaves were macerated and a hydroalcoholic extract (HDHE) and fractions were prepared. Antimicrobial tests, such as agar-diffusion, Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were carried out against several bacterial species. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Klebsiella pneumoniae* were inhibited by at least one extract or fraction in the agar-diffusion assay (inhibition halos from 12 mm to 30 mm). However, the lowest MIC value was found for HDHE against *K. pneumoniae*. In addition, HDHE and its fractions were able to inhibit biofilm formation at sub-inhibitory concentrations (780 µg/mL and 1.56 µg/mL). As the best activities were found for HDHE, we selected it for further assays. HDHE was able to increase ciprofloxacin (CIP) activity against *K. pneumoniae*, displaying synergistic (initial concentration CIP + HDHE: 2 µg/mL + 600 µg/mL and 2.5 µg/mL + 500 µg/mL) and additive effects (CIP + HDHE: 3 µg/mL + 400 µg/mL). This action seems to be associated with the alteration in bacterial membrane permeability induced by HDHE (as seen by propidium iodide labeling). This extract was non-toxic for red blood cell or human peripheral blood mononuclear cells (PBMCs). Additionally, it inhibited the lipopolysaccharide-induced proliferation of PBMCs. The following compounds were detected in HDHE using HPLC-ESI-MS analysis: plumieride, plumericin or isoplumericin, rutin, quercetin and derivatives, and chlorogenic acid. Based on these results we suggest that compounds from *H. drasticus* have antimicrobial and antibiofilm activities against *K. pneumoniae* and display low cytotoxicity and anti-proliferative action in PBMC stimulated with lipopolysaccharide.

**ANEXO B:** Artigo Publicado (Sulforaphane Modulates Joint Inflammation in a Murine Model of Complete Freund's Adjuvant-Induced Mono-Arthritis)

molecules

MDPI

*Article*

# Sulforaphane Modulates Joint Inflammation in a Murine Model of Complete Freund's Adjuvant-Induced Mono-Arthritis

**João Francisco Silva Rodrigues [1], Cristiane Santos Silva e Silva Figueiredo [1], Thayanne França Muniz [1], Alana Fernanda de Aquino [1,2], Larissa Neuza da Silva Nina [1], Nagila Caroline Fialho Sousa [1], Luis Claudio Nascimento da Silva [1], Breno Glaessner Gomes Fernandes de Souza [3], Tatiane Aranha da Penha-Silva [3], Ana Lúcia Abreu-Silva [3], Joicy Cortez de Sá [1], Elizabeth Soares Fernandes [1,†] and Marcos Augusto Grigolin Grisotto [1,2,*,†]**

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**Abstract:** Rheumatoid arthritis (RA) is characterized by inflammation of one or more joints, and affects ~1% of the adult population worldwide. Sulforaphane (SFN) is a natural compound that has been suggested as an antioxidant. Here, SFN's effects were evaluated in a murine mono-arthritis model. Mono-arthritis was induced in mice by a single intra-articular injection of Complete Freund's Adjuvant (CFA-10 µg/joint, in 10 µL) into the ipsilateral joint. The contralateral joint received an equal volume of PBS. On the 4th day post-joint inflammation induction, animals received either SFN (10 mg/kg) or vehicle (3% DMSO in saline), intraperitoneally (i.p.), twice a day for 3 days. Joint swelling and secondary mechanical allodynia and hyperalgesia were evaluated over 7 days post-CFA. After this period, animals were culled and their blood and synovial fluid samples were collected for analysis of cell populations, cytokine release and thioredoxin reductase (TrxR) activity. Knee joint samples were also collected for histology. SFN reduced joint swelling and damage whilst increasing the recruitment of $Ly6C^+$ and $Ly6G^+$ cells to CFA-injected joints. SFN-treated animals presented down-regulation of CD11b and CD62L on synovial fluid $Ly6G^+$ cells. Synovial fluid samples obtained from CFA-injected joints and plasma samples of SFN-treated mice presented higher levels of IL-6 and increased activity of TrxR, in comparison with controls. These results indicate that SFN reduces knee joint damage by modulating cell activation/migration to the joints, cytokine production and increasing the activity of TrxR, and therefore, may represent an alternative treatment to joint inflammation.

**Keywords:** rheumatoid arthritis; sulforaphane; oedema; IL-6; thioredoxin reductase

**ANEXO C**: Artigo Publicado -Óleo essencial da Canela (Cinamaldeído) e suas aplicações biológicas

# Óleo essencial da Canela (Cinamaldeído) e suas aplicações biológicas

*Cinnamon essential oil (cinnamaldehyde) and its applications*

Cristiane Santos Silva e Silva Figueiredo[1], Patrícia Viera de Oliveira[2], Warlison Felipe de Silva Saminez[3], Roseana Muniz Diniz[4], João Francisco Silva Rodrigues[5], Mari Silma Maia da Silva[6], Luís Cláudio Nascimento da Silva[7], Marcos Augusto Grigolin Grisotto[8].

**Resumo:** Cinamaldeído (CND) é o principal componente ativo do óleo essencial da canela (*Cinnamomum* sp) e tem sido amplamente utilizado em atividades biológicas e farmacológicas, tendo sido relatado atividade antimicrobiana, antioxidante, antidiabética, dentre outras. Devido os diversos relatos das propriedades farmacológicas do composto, esse foi escolhido para revisão de literatura. A seleção da bibliografia foi obtida a partir de bases de dados (google scholar, NCBI - National Center for Biotechnology Information, PubMed e Scielo - Scientific Electronic Library Online). A composição química do CND tem compostos terpenóides que têm poderosa atividade antimicrobiana contra fungos, bactérias Gram-positivas e Gram-negativas tais como *Aeromonas hydrophila*, *Enterococcus faecalis*, *Clostridium botulinum*, *Staphylococcus aureus*, *Escherichia coli* O157: H7 e *Salmonella enterica serovar Typhimurium*. A atividade anti-inflamatória induz a apoptose e inibi a proliferação celular, nas respostas imunes mediadas por monócitos/macrófagos, além de diminuir a produção de óxido nítrico induzido por lipopolissacáridos de um modo dependente da dose. CND ao ser administrado por via oral em ratos diabéticos demonstrou melhora no conteúdo de glicogênio muscular e hepático aumentando a liberação de insulina. Além disso CND estimulou a angiogênese *in vivo* e *in vitro*, regulando positivamente o fator de crescimento endotelial vascular. A partir do que foi relatado constatou-se que o CND possui muitas atividades com potencial farmacológico, mas percebe-se que se faz necessário estudos sobre o(s) mecanismos(s) de ação dessas atividades a fim de se proporcionar o uso seguro e eficaz do cinamaldeído.

**Palavras-chave:** *Cinnamomum* sp, cinamaldeído, atividades biológicas.

**Abstract:** Cinnamaldehyde (CND) is the main active component of cinnamon essential oil (Cinnamomum sp) and has been widely used in biological and pharmacological activities, and antimicrobial, antioxidant and antidiabetic activities have been reported. Due to the diverse reports of the pharmacological properties of the compound, it was chosen for literature review. The selection of the bibliography was obtained from databases (google scholar, NCBI - National Center for Biotechnology Information, PubMed and Scielo - Scientific Electronic Library Online). The chemical composition of CND has terpenoid compounds that have potent antimicrobial activity against fungi, Gram-positive and Gram-negative bacteria such as *Aeromonas hydrophila, Enterococcus faecalis, Clostridium botulinum, Staphylococcus aureus, Escherichia coli O157: H7 and Salmonella enterica serovar Typhimurium*. The anti-inflammatory activity induces apoptosis and inhibits cell proliferation in monocyte/macrophage mediated immune responses and decreases the production of nitric oxide induced by lipopolysaccharides in a dose-dependent manner. CND when administered orally in diabetic rats demonstrated an improvement in the muscle and liver glycogen content, increasing the release of insulin. In addition, CND stimulated angiogenesis in vivo and in vitro, regulating vascular endothelial growth factor positively. Based on what has been reported, it has been observed that CND has many activities with pharmacological potential, but it is perceived that it is necessary to study the mechanism (s) of action of these activities in order to provide the use safe and effective use of cinnamaldehyde.

**Key words:** *Cinnamomum* sp, cinnamaldehyde, biological activities.

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[6]Aluna de Pós-graduação do Programa de Mestrado em Biologia Parasitária da Universidade Ceuma.
[7,8] Docentes da Universidade Ceuma.

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**ANEXO D:** Normas de Submissão da Revista Current Medicinal Chemistry

MANUSCRIPT PREPARATION

The manuscript should be written in English in a clear, direct and active style. All pages must be numbered sequentially, facilitating in the reviewing and editing of the manuscript.

MICROSOFT WORD TEMPLATE

It is advisable that authors prepare their manuscript using the template available on the Web, which will assist in preparation of the manuscript according to Journal's Format. Download the Template.

SECTIONS IN MANUSCRIPTS

Manuscripts submitted for research and review articles in the journal should be divided into the following sections

- Title
- Title Page
- Structured Abstract
- Graphical Abstract
- Keywords
- Text Organization
- Conclusion
- List of Abbreviations (if any)
- Consent for Publication
- Conflict of Interest
- Acknowledgements
- References
- Appendices
- Figures/Illustrations (if any)
- Chemical Structures (if any)
- Tables (if any)
- Supportive/Supplementary Material (if any)

Title

The title of the article should be precise and brief and must not be more than 120 characters.

Authors should avoid the use of non-standard abbreviations and question marks in titles. The first letter of each word should be in capital letters except for articles, conjunctions and prepositions.

Authors should also provide a short ‘running title’. Title, running title, byline, correspondent footnote and keywords should be written as presented in original manuscripts.

Title Page

Title page should include paper title, author(s) full name and affiliation, corresponding author(s) names complete affiliation/address, along with phone, fax and email.

Structured Abstract

The abstract of an article should be its clear, concise and accurate summary, having no more than 250 words, and including the explicit sub-headings (as in-line or run-in headings in bold). Use of abbreviations should be avoided and the references should not be cited in the abstract. Ideally, each abstract should include the following sub-headings, but these may vary according to requirements of the article.

- Background
- Objective
- Methods
- Results
- Conclusion

Graphical Abstract

A graphic should be included when possible with each manuscript for use in the Table of Contents (TOC). This must be submitted separately as an electronic file (preferred file types are EPS, PDF, TIFF, Microsoft Word, PowerPoint and CDX etc.). A graphical abstract, not exceeding 30 words along with the illustration, helps to summarize the contents of the manuscript in a concise pictorial form. It is meant as an aid for the rapid viewing of the journals' contents and to help capture the readers’ attention. The graphical abstract may feature a key structure, reaction, equation, etc. that the manuscript elucidates upon. It will be listed along with the manuscript title, authors’ names and affiliations in the contents page, typeset within an area of 5 cm by 17 cm, but it will not appear in the article PDF file or in print.

Graphical Abstracts should be submitted as a separate file (must clearly mention graphical abstract within the file) online via Bentham's Journal Management System.

Keywords

6 to 8 keywords must be provided. Choose important and relevant keywords that researchers in your field will be searching for so that your paper will appear in a database search.

Text Organization

The main text should begin on a separate page and should be divided into title page, abstract and the main text. The text may be subdivided further according to the areas to be discussed, which should be followed by the List of Abbreviations, Conflict of Interest, Acknowledgements and Reference section. For Review, the manuscript should be divided into title page, abstract and the main text. The text may be subdivided further according to the areas to be discussed, which should be followed by the Acknowledgements and Reference section. The review article should mention any previous important reviews in the field and contain a comprehensive discussion starting with the general background of the field. It should then go on to discuss the salient features of recent developments. The authors should avoid presenting material which has already been published in a previous review. The authors are advised to present and discuss their observations in brief.

The manuscript style must be uniform throughout the text and 10 pt Times New Roman font should be used. The full term for an abbreviation should precede its first appearance in the text unless it is a standard unit of measurement. The reference numbers should be given in square brackets in the text. Italics should be used for Binomial names of organisms (Genus and Species), for emphasis and for unfamiliar words or phrases. Non-assimilated words from Latin or other languages should also be italicized e.g in vivo, in vitro, per se, et al. etc.

SECTION HEADINGS

Section headings should be numbered sequentially, left aligned and have the first letter capitalized, starting with the introduction. Sub-section headings however, should be in lower-case and italicized with their initials capitalized. They should be numbered as 1.1, 1.2, etc.

INTRODUCTION

The Introduction section should include the background and aims of the research in a comprehensive manner.

MATERIALS AND METHODS

This section provides details of the methodology used along with information on any previous efforts with corresponding references. Any details for further modifications and research should be included.

EXPERIMENTAL

Repeated information should not be reported in the text of an article. A calculation section must include experimental data, facts and practical development from a theoretical perspective.

RESULTS

Results should be precise.

DISCUSSION

This should explore the significance of the results of the work, present a reproducible procedure and emphasis the importance of the article in the light of recent developments in the field.

Extensive citations and discussion of published literature should be avoided.

The Results and Discussion may be presented together under one heading of “Results and Discussion”. Alternatively, they may be presented under two separate sections (“Results” section and “Discussion” Sections). Short sub- headings may be added in each section if required.

CONCLUSION

A small paragraph summarizing the contents of the article, presenting the final outcome of the research or proposing further study on the subject, may be given at the end of the article under the Conclusion section.

Greek Symbols and Special Characters

Greek symbols and special characters often undergo formatting changes and get corrupted or lost during preparation of manuscript for publication. To ensure that all special characters used are embedded in the text, these special characters should be inserted as a symbol but should not be a result of any format styling (Symbol font face) otherwise they will be lost during conversion to PDF/XML.

Authors are encouraged to consult reporting guidelines. These guidelines provide a set of recommendations comprising a list of items relevant to their specific research design. Chemical equations, chemical names, mathematical usage, unit of measurements, chemical and physical quantity & units must conform to SI and Chemical Abstracts or IUPAC.

All kinds of measurements should be reported only in International System of Units (SI).

Appendices

In case there is a need to present lengthy, but essential methodological details, appendices must be used, which can be a part of the article. An appendix must not exceed three pages (Times New Roman, 10 point fonts, 900 max. words per page).The information should be provided in a condensed form, ruling out the need of full sentences. A single appendix should be titled APPENDIX, while more than one can be titled APPENDIX A, APPENDIX B, and so on.

Supportive/Supplementary Material

We do encourage to append supportive material, for example a PowerPoint file containing a talk about the study, a PowerPoint file containing additional screenshots, a Word, RTF, or PDF document showing the original instrument(s) used, a video, or the original data (SAS/SPSS files, Excel files, Access Db files etc.) provided it is inevitable or endorsed by the journal's Editor.

Supportive/Supplementary material intended for publication must be numbered and referred to in the manuscript but should not be a part of the submitted paper. In-text citations as well as a section with the heading "Supportive/Supplementary Material" before the "References" section should be provided. Here, list all Supportive/Supplementary Material and include a brief caption line for each file describing its contents.

Any additional files will be linked to the final published article in the form supplied by the

author, but will not be displayed within the paper. They will be made available in exactly the same form as originally provided only on our Web site. Please also make sure that each additional file is a single table, figure or movie (please do not upload linked worksheets or PDF files larger than one sheet). Supportive/ Supplementary material must be provided in a single zipped file not larger than 4 MB.

Authors must clearly indicate if these files are not for publication but meant for the reviewers'/editors' perusal only.

List of Abbreviations

If abbreviations are used in the text either they should be defined in the text where first used, or a list of abbreviations can be provided.

**ANEXO E:** Normas da revista Frontiers in Microbiology

Author Guidelines

1. Summary Table

Please view the table below for a summary on currently accepted article types and general manuscript style guidelines. Article types may vary depending on journal.

| | Abstract (max. length) | Running title (5 words) | Figures and/or tables (combined) | Manuscript (max. length) | Peer review | Author fees | Submitted to PubMed Central or other indexing databases |
|---|---|---|---|---|---|---|---|
| Original Research | 350 words | ✔ | 15 | 12'000 words | ✔ | ✔ | ✔ |
| Review | 350 words | ✔ | 15 | 12'000 words | ✔ | ✔ | ✔ |
| Book Review | ✘ | ✘ | 1 | 1'000 words | ✔ | ✘ | ✔ |
| Brief Research Report | 250 words | ✔ | 4 | 4'000 words | ✔ | ✔ | ✔ |
| Classification | 250 words | ✔ | 10 | 2'000 words | ✔ | ✔ | ✔ |
| Case Report | 350 words | ✔ | 4 | 3'000 words | ✔ | ✔ | ✔ |
| Clinical Study Protocol | 350 words | ✔ | 15 | 12'000 words | ✔ | ✔ | ✔ |
| Clinical Trial | 350 words | ✔ | 15 | 12'000 words | ✔ | ✔ | ✔ |
| Code | 250 words | ✔ | 3 | 3'000 words | ✔ | ✔ | ✔ |
| Community Case Study | 350 words | ✔ | 5 | 5'000 words | ✔ | ✔ | ✔ |
| Conceptual Analysis | 350 words | ✔ | 10 | 8'000 words | ✔ | ✔ | ✔ |
| CPC | 250 words | ✔ | 6 | 2'500 words | ✔ | ✔ | ✔ |
| Curriculum, Instruction, and Pedagogy | 350 words | ✔ | 5 | 5'000 words | ✔ | ✔ | ✔ |
| Data Report | ✘ | ✔ | 2 | 3'000 words | ✔ | ✔ | ✔ |
| Editorial | ✘ | ✘ | 0 | 1'000 words* | ✔ | ✘ | ✔ |
| Empirical Study | 350 words | ✔ | 10 | 8'000 words | ✔ | ✔ | ✔ |
| Evaluation | 350 words | ✔ | 5 | 6'000 words | ✔ | ✔ | ✔ |
| Field Grand Challenge | ✘ | ✔ | 1 | 2'000 words | ✔ | ✘ | ✔ |
| Focused Review [1] | 350 words | ✔ | 5 | 5'000 words | ✔ | ✘ | ✔ |
| Frontiers Commentary [1] | ✘ | ✘ | 1 | 1'000 words | ✔ | ✘ | ✔ |
| General Commentary | ✘ | ✘ | 1 | 1'000 words | ✔ | ✔ | ✔ |
| Hypothesis and Theory | 350 words | ✔ | 15 | 12'000 words | ✔ | ✔ | ✔ |
| Methods | 350 words | ✔ | 15 | 12'000 words | ✔ | ✔ | ✔ |
| Mini Review | 250 words | ✔ | 2 | 3'000 words | ✔ | ✔ | ✔ |
| Opinion | ✘ | ✔ | 1 | 2'000 words | ✔ | ✔ | ✔ |
| Policy & Practice Reviews | 350 words | ✔ | 15 | 12'000 words | ✔ | ✔ | ✔ |
| Policy Briefs | 125 words | ✔ | 5 | 3'000 words | ✔ | ✔ | ✔ |
| Protocols | 350 words | ✔ | 15 | 12'000 words | ✔ | ✔ | ✔ |
| Perspective | 250 words | ✔ | 2 | 3'000 words | ✔ | ✔ | ✔ |
| Registered Report | 350 words | ✔ | 15 | 12,000 words | ✔ | ✔ | ✔ |
| Research Snapshot | 50 words | ✔ | 1 | 500 words | ✔ | ✔ | ✔ |
| Specialty Grand Challenge | ✘ | ✔ | 1 | 2'000 words | ✔ | ✘ | ✔ |
| Systematic Reviews | 350 words | ✔ | 15 | 12'000 words | ✔ | ✔ | ✔ |
| Technology Report | 350 words | ✔ | 15 | 12'000 words | ✔ | ✔ | ✔ |

(1) Tier 2 article - field level article reserved to authors of selected Tier 1 articles.

* Editorials for Research Topics with 5 to 10 published articles have a maximum of 1'000 words, for Research Topics with more than 10 published articles the following applies: 1'100 words for 11 articles, 1'200 for 12 articles, 1'300 for 13 articles etc. up to maximum 5'000 words, for 50 or more papers.

Appendices and footnotes will be considered in the total length and word count of the article.

2. Manuscript Guidelines

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Correct attribution of the original source in repositories or pre-print servers must be included on submission, or added at re-submission if the deposition is done during the review process.

If the article is published, authors are then strongly encouraged to link from the preprint server to the Frontiers publication to enable readers to find, access and cite the final peer-reviewed version. Please note that we cannot consider for publication content that has been previously published, or is already under review, within a scientific journal, book or similar entity.

2.2. Registration with Frontiers

Please note that the corresponding and all submitting authors MUST register with Frontiers

before submitting an article. You must be logged in to your personal Frontiers Account to submit an article.

For any co-author who would like his/her name on the article abstract page and PDF to be linked to a Frontiers profile on the Loop network, please ensure to register before the final publication of the paper.

2.3. Manuscript Requirements and Style Guide

2.3.1. General standards

Word Files

If working with Word please use Frontiers Word templates.

LaTeX Files

If you wish to submit your article as LaTeX, we recommend our Frontiers LaTeX templates. These templates are meant as a guide, you are of course welcome to use any style or formatting and Frontiers journal style will be applied during typesetting.

Experiments

Authors are required to specifically state in their legends how many times experiments were performed (in general we require n=3 as a minimum) and what specific statistical analysis was performed.

2.3.1.1. Article Type

Frontiers requires authors to carefully select the appropriate article type for their manuscript, and to comply with the article-type descriptions defined in the journal's "Article Types", which can be seen from the "For Authors" menu on any Frontiers journal page. Please note that not all articles types are available for all journals/specialties. Please contact us if you have any questions. Please pay close attention to the word count limits.

Focused Reviews, Frontiers Commentaries and Grand Challenge articles are invited by the chief editor and cannot be part of any Frontiers Research Topic. Unless you were contacted by the chief editor or the editorial office regarding the submission of a paper selected for tier 2 promotion, do not submit a Focused Review or a Frontiers Commentary - instead, submit a Review or a General Commentary.

Please see Additional Requirements for specific article types including Focused Reviews, General Commentaries, Protocols and Data Reports.

2.3.1.2. Manuscript Length

Frontiers encourages its authors to closely follow the article word count lengths given in the Summary Table. The manuscript length includes only the main body of the text, footnotes and all citations within it, and excludes abstract, section titles, figure and table captions, funding statements, acknowledgments and references in the bibliography. Please indicate the number of

words and the number of figures included in your manuscript on the first page.

2.3.1.3. Language Editing

Frontiers requires manuscripts submitted to meet international standards for English language to be considered for publication.

For authors who would like their manuscript to receive language editing or proofing to improve the clarity of the manuscript and help highlight their research, Frontiers recommends the language-editing services provided by the following external partners:

Editage

Frontiers is pleased to recommend language-editing service provided by our external partner Editage to authors who believe their manuscripts would benefit from professional editing. These services may be particularly useful for researchers for whom English is not the primary language. They can help to improve the grammar, syntax and flow of your manuscripts prior to submission. Frontiers authors will receive a 10% discount by visiting the following link: http://editage.com/frontiers/

The Charlesworth Group

Frontiers recommends the Charlesworth Group Author Services, who has a long standing track record in language editing and proofing. This is a third-party service for which Frontiers authors will receive a discount by visiting the following link: http://www.charlesworthauthorservices.com/~Frontiers.

Note that sending your manuscript for language editing does not imply or guarantee that it will be accepted for publication by a Frontiers journal. Editorial decisions on the scientific content of a manuscript are independent of whether it has received language editing or proofing by the partner services, or other services.

2.3.1.4. Language Style

The default language style at Frontiers is American English. If you prefer your article to be formatted in British English, please specify this on your manuscript first page. For any questions regarding style Frontiers recommends authors to consult the Chicago Manual of Style.

2.3.1.5. Search Engine Optimization (SEO)

There are a few simple ways to maximize your article’s discoverability. Follow the steps below to improve search results of your article:

Include a few of your article's keywords in the title of the article;

Do not use long article titles;

Pick 5 to 8 keywords using a mix of generic and more specific terms on the article subject(s);

Use the maximum amount of keywords in the first 2 sentences of the abstract;

Use some of the keywords in level 1 headings.

2.3.1.6. Title

The title is written in title case, centred, and in 16 point bold Times New Roman font at the top of page.

The title should be concise, omitting terms that are implicit and, where possible, be a statement of the main result or conclusion presented in the manuscript. Abbreviations should be avoided within the title.

Witty or creative titles are welcome, but only if relevant and within measure. Consider if a title meant to be thought-provoking might be misinterpreted as offensive or alarming. In extreme cases, the editorial office may veto a title and propose an alternative.

Authors should try to avoid, if possible:

Titles that are a mere question without giving the answer.

Unambitious titles, for example starting with "Towards", "A description of", "A characterization of", "Preliminary study on".

Vague titles, for example starting with "Role of...", "Link between...", "Effect of..." that do not specify the role, link, or effect.

Include terms that are out of place, for example the taxonomic affiliation apart from species name.

For Corrigenda, Book Reviews, General Commentaries and Editorials, the title of your manuscript should have the following format:

"Corrigendum: Title of original article"

"Book Review: Title of book"

General Commentaries

"Commentary: Title of original article" (This does not apply to Frontiers Commentaries)

"Response: Commentary: Title of original article"

"Editorial: Title of Research Topic"

For article types requiring it, the running title should be a maximum of 5 words in length. (see Summary Table)

2.3.1.7. Authors and Affiliations

All names are listed together and separated by commas. Provide exact and correct author names as these will be indexed in official archives. Affiliations should be keyed to the author's name with superscript numbers and be listed as follows: Laboratory, Institute, Department, Organization, City, State abbreviation (USA, Canada, Australia), and Country (without detailed

address information such as city zip codes or street names).

Example: Max Maximus, Department of Excellence, International University of Science, New York, NY, USA.

The Corresponding Author(s) should be marked with an asterisk. Provide the exact contact email address of the corresponding author(s) in a separate section.

Correspondence:

Dr. Max Maximus

maximus@gmail.com

If any authors wish to include a change of address, list the present address(es) below the correspondence details using a unique superscript symbol keyed to the author(s) in the author list.

2.3.1.8. Consortium/Group and Collaborative Authors

Consortium/group authorship should be listed in the manuscript with the other author(s). In cases where authorship is retained by the consortium/group, the consortium/group should be listed as an author separated by "," or "and". Consortium/group members can be listed in a separate section at the end of the manuscript.

Example: John Smith, Barbara Smith and The Collaborative Working Group.

In cases where work is presented by the author(s) on behalf of a consortium/group, it should be included in the manuscript author list separated with the wording "for" or "on behalf of". The consortium/group will not retain authorship.

Example: John Smith and Barbara Smith on behalf of The Collaborative Working Group.

2.3.1.9. Headings and Sub-headings

Except for special names (e.g. GABAergic), capitalize only the first letter of headings and subheadings. Headings and subheadings need to be defined in Times New Roman, 12, bold. You may insert up to 5 heading levels into your manuscript (not more than for example: 3.2.2.1.2 Heading title).

2.3.1.10. Abstract

As a primary goal, the abstract should render the general significance and conceptual advance of the work clearly accessible to a broad readership. In the abstract, minimize the use of abbreviations and do not cite references. The text of the abstract section should be in 12 point normal Times New Roman. See Summary Table for abstract requirement and length according to article type.

For Clinical Trial article types, please include the Unique Identifier and the URL of the publicly accessible website on which the trial is registered.

2.3.1.11. Keywords

All article types: you may provide up to 8 keywords; at least 5 are mandatory.

2.3.1.12. Text

The entire document should be single-spaced and must contain page and line numbers in order to facilitate the review process. Your manuscript should be written using either LaTeX or MS-Word.

Templates are available (see above)

2.3.1.13. Nomenclature

The use of abbreviations should be kept to a minimum. Non-standard abbreviations should be avoided unless they appear at least four times, and defined upon first use in the main text. Consider also giving a list of non-standard abbreviations at the end, immediately before the Acknowledgments.

Equations should be inserted in editable format from the equation editor.

Italicize Gene symbols and use the approved gene nomenclature where it is available. For human genes, please refer to the HUGO Gene Nomenclature Committee (HGNC). New gene symbols should be submitted here. Common Alternative gene aliases may also be reported, but should not be used alone in place of the HGNC symbol. Nomenclature committees for other species are listed here. Protein products are not italicized.

We encourage the use of Standard International Units in all manuscripts.

Chemical compounds and biomolecules should be referred to using systematic nomenclature, preferably using the recommendations by IUPAC.

Astronomical objects should be referred to using the nomenclature given by the International Astronomical Union provided here.

Life Science Identifiers (LSIDs) for ZOOBANK registered names or nomenclatural acts should be listed in the manuscript before the keywords. An LSID is represented as a uniform resource name (URN) with the following format:

urn:lsid:::[:]

For more information on LSIDs please see Inclusion of Zoological Nomenclature section.

2.3.1.14. Sections

Your manuscript is organized by headings and subheadings. For Original Research Articles, Clinical Trial Articles, and Technology Reports the section headings should be those appropriate for your field and the research itself.

For Original Research Articles, it is recommended to organize your manuscript in the following sections or their equivalents for your field:

Introduction

Succinct, with no subheadings.

Materials and Methods

This section may be divided by subheadings. This section should contain sufficient detail so that when read in conjunction with cited references, all procedures can be repeated. For experiments reporting results on animal or human subject research, an ethics approval statement should be included in this section (for further information, see section Materials and Data Policies)

Results

This section may be divided by subheadings. Footnotes should not be used and have to be transferred into the main text.

Discussion

This section may be divided by subheadings. Discussions should cover the key findings of the study: discuss any prior art related to the subject so to place the novelty of the discovery in the appropriate context; discuss the potential short-comings and limitations on their interpretations; discuss their integration into the current understanding of the problem and how this advances the current views; speculate on the future direction of the research and freely postulate theories that could be tested in the future.

For further information, please see Additional Requirements for specific article types including Focused Reviews, General Commentaries, Case Reports and Data Reports amongst others or you can check the descriptions defined in the journal's "Article Types", which can be seen from the "For Authors" menu on any Frontiers journal page.

2.3.1.15. Acknowledgments

This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

2.3.1.16. Author Contributions Statement

The Author Contributions Statement is mandatory and should represent all the authors. It can be up to several sentences long and should briefly describe the tasks of individual authors. Please list only 2 initials for each author, without full stops, but separated by commas (e.g. JC, JS). In the case of two authors with the same initials, please use their middle initial to differentiate between them (e.g. REW, RSW). The Author Contributions Statement should be included at the end of the manuscript before the References.

2.3.1.17. Conflict of Interest Statement

A Conflict of Interest Statement needs to be included at the end of the manuscript before the references. Here, the authors need to declare whether or not the submitted work was carried out in the presence of any personal, professional or financial relationships that could potentially be construed as a conflict of interest. For more information on conflicts of interest, see our Editorial Policies.

2.3.1.18. Contribution to the Field Statement

When you submit your manuscript, you will be required to briefly summarize in 200 words your manuscript's contribution to, and position in, the existing literature of your field. This should be written avoiding any technical language or non-standard acronyms. The aim should be to convey the meaning and importance of this research to a non-expert. While Frontiers evaluates articles using objective criteria, rather than impact or novelty, your statement should frame the question(s) you have addressed in your work in the context of the current body of knowledge, providing evidence that the findings - whether positive or negative - contribute to progress in your research discipline. This will assist the Chief Editors to determine whether your manuscript fits within the scope of a specialty as defined in its mission statement; a detailed statement will also facilitate the identification of the Editors and Reviewers most appropriate to evaluate your work, ultimately expediting your manuscript's initial consideration.

Example Statement on: Markram K and Markram H (2010) The Intense World Theory – a unifying theory of the neurobiology of autism. Front. Hum. Neurosci. 4:224. doi: 10.3389/fnhum.2010.00224

Autism spectrum disorders are a group of neurodevelopmental disorders that affect up to 1 in 100 individuals. People with autism display an array of symptoms encompassing emotional processing, sociability, perception and memory, and present as uniquely as the individual. No theory has suggested a single underlying neuropathology to account for these diverse symptoms. The Intense World Theory, proposed here, describes a unifying pathology producing the wide spectrum of manifestations observed in autists. This theory focuses on the neocortex, fundamental for higher cognitive functions, and the limbic system, key for processing emotions and social signals. Drawing on discoveries in animal models and neuroimaging studies in individuals with autism, we propose how a combination of genetics, toxin exposure and/or environmental stress could produce hyper-reactivity and hyper-plasticity in the microcircuits involved with perception, attention, memory and emotionality. These hyper-functioning circuits will eventually come to dominate their neighbors, leading to hyper-sensitivity to incoming stimuli, over-specialization in tasks and a hyper-preference syndrome. We make the case that this theory of enhanced brain function in autism explains many of the varied past results and resolves conflicting findings and views and makes some testable experimental predictions.

2.3.2. References

All citations in the text, figures or tables must be in the reference list and vice-versa. The references should only include articles that are published or accepted. Data sets that have been deposited to an online repository should be included in the reference list, include the version and unique identifier when available. For accepted but unpublished works use "in press" instead of page numbers. Unpublished data, submitted manuscripts, or personal communications should be cited within the text only, for the article types that allow such inclusions. Personal communications should be documented by a letter of permission. Website urls should be included as footnotes. Any inclusion of verbatim text must be contained in quotation marks and clearly reference the original source. Preprints can be cited as long as a DOI or archive URL is available, and the citation clearly mentions that the contribution is a preprint. If a peer-reviewed journal

publication for the same preprint exists, the official journal publication is the preferred source.

The following formatting styles are meant as a guide, as long as the full citation is complete and clear, Frontiers referencing style will be applied during typesetting.

SCIENCE, ENGINEERING, and HUMANITIES: For articles submitted in the domains of SCIENCE, ENGINEERING and HUMANITIES please apply Author-Year system for in-text citations.

Reference list: provide the names of the first six authors followed by et al. and doi when available.

In-text citations should be called according to the surname of the first author, followed by the year. For works by 2 authors include both surnames, followed by the year. For works by more than 2 authors include only the surname of the first author, followed by et al., followed by the year. For Humanities and Social Sciences articles please include page numbers in the in-text citations.

Article in a print journal:

Sondheimer, N., and Lindquist, S. (2000). Rnq1: an epigenetic modifier of protein function in yeast. Mol. Cell. 5, 163-172.

Article in an online journal:

Tahimic, C.G.T., Wang, Y., Bikle, D.D. (2013). Anabolic effects of IGF-1 signaling on the skeleton. Front. Endocrinol. 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book:

Sorenson, P. W., and Caprio, J. C. (1998). "Chemoreception," in The Physiology of Fishes, ed. D. H. Evans (Boca Raton, FL: CRC Press), 375-405.

Book:

Cowan, W. M., Jessell, T. M., and Zipursky, S. L. (1997). Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press.

Abstract:

Hendricks, J., Applebaum, R., and Kunkel, S. (2010). A world apart? Bridging the gap between theory and applied social gerontology. Gerontologist 50, 284-293. Abstract retrieved from Abstracts in Social Gerontology database. (Accession No. 50360869)

Patent:

Marshall, S. P. (2000). Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity. U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

Data:

Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and Dissertations:

Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

Preprint:

Smith, J. (2008). Title of the document. Preprint repository name [Preprint]. Available at: https://persistent-url (Accessed March 15, 2018).

For examples of citing other documents and general questions regarding reference style, please refer to the Chicago Manual of Style.

Frontiers Science Endnote Style

Frontiers Science, Engineering and Humanities Bibstyle

HEALTH, PHYSICS AND MATHEMATICS: For articles submitted in the domain of HEALTH or the journal Frontiers in Physics and Frontiers in Applied Mathematics and Statistics please apply the Vancouver system for in-text citations.

Reference list: provide the names of the first six authors followed by et al. and doi when available.

In-text citations should be numbered consecutively in order of appearance in the text – identified by Arabic numerals in the parenthesis for Health articles, and in square brackets for Physics and Mathematics articles.

Reference examples

Article in a print journal:

Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell (2000) 5:163-72.

Article in an online journal:

Tahimic CGT, Wang Y, Bikle DD. Anabolic effects of IGF-1 signaling on the skeleton. Front Endocrinol (2013) 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book:

Sorenson PW, Caprio JC. "Chemoreception,". In: Evans DH, editor. The Physiology of Fishes. Boca Raton, FL: CRC Press (1998). p. 375-405.

Book:

Cowan WM, Jessell TM, Zipursky SL. Molecular and Cellular Approaches to Neural

Development. New York: Oxford University Press (1997). 345 p.

Abstract:

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, editor. Genetic Programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3–5; Kinsdale, Ireland. Berlin: Springer (2002). p. 182–91.

Patent:

Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible Endoscopic Grasping and Cutting Device and Positioning Tool Assembly. United States patent US 20020103498 (2002).

Data:

Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and Dissertations:

Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master’s thesis]. [Chicago (IL)]: University of Chicago

Preprint:

Smith, J. Title of the document. Preprint repository name [Preprint] (2008). Available at: https://persistent-url (Accessed March 15, 2018).

For examples of citing other documents and general questions regarding reference style, please refer to Citing Medicine.

Frontiers Health Endnote Style

Frontiers Health and Physics Bibstyle

2.3.3. Disclaimer

Any necessary disclaimers which must be included in the published article should be clearly indicated in the manuscript.

2.3.4. Supplementary Material

Frontiers journals do not support pushing important results and information into supplementary sections. However, data that are not of primary importance to the text, or which cannot be included in the article because it is too large or the current format does not permit it (such as movies, raw data traces, power point presentations, etc.) can be uploaded during the submission procedure and will be displayed along with the published article. All supplementary files are deposited to FigShare for permanent storage, during the publication stage of the article, and receive a DOI.

The Supplementary Material can be uploaded as Data Sheet (word, excel, csv, cdx, fasta, pdf or zip files), Presentation (power point, pdf or zip files), Supplementary Image (cdx, eps, jpeg, pdf, png or tif), Supplementary Table (word, excel, csv or pdf), Audio (mp3, wav or wma) or Video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv).

Supplementary material is not typeset so please ensure that all information is clearly presented, the appropriate caption is included in the file and not in the manuscript, and that the style conforms to the rest of the article. To avoid discrepancies between the published article and the supplementary material, please do not add the title, author list, affiliations or correspondence in the supplementary files. For Supplementary Material templates (LaTex and Word) see Supplementary Material for Frontiers.

Suggested Fonts

The title is written in title case, centred, and in 16 point bold Times New Roman font at the top of page.

Headings and subheadings need to be defined in Times New Roman, 12, bold.

The text of the abstract section should be in 12 point normal Times New Roman.

The body text is in 12 point normal Times New Roman.

2.3.5. File Requirements

For Latex Files, when submitting your article please ensure to upload all relevant manuscript files including:

tex file

PDF

.bib file (if the bibliography is not already included in the .tex file)

Figures should be included in the provided pdf. In case of acceptance, our Production Office might require high resolution files of the figures included in the manuscript in eps, jpg or tif format. In order to be able to upload more than one figure at a time, save the figures (labeled in order of appearance in the manuscript) in a zip file, and upload them as ‘Supplementary Material Presentation’.

To facilitate the review process, please include a Word Count at the beginning of your manuscript, one option is teXcount which also has an online interface.

During the Interactive Review, authors are encouraged to upload versions using ‘Track Changes’. Editors and Reviewers can only download the PDF file of the submitted manuscript .

2.3.6. Additional Requirements per article types

2.3.6.1. CrossMark Policy

CrossMark is a multi-publisher initiative to provide a standard way for readers to locate the

current version of a piece of content. By applying the CrossMark logo Frontiers is committing to maintaining the content it publishes and to alerting readers to changes if and when they occur. Clicking on the CrossMark logo will tell you the current status of a document and may also give you additional publication record information about the document.

2.3.6.2. Commentaries on Articles

For General Commentaries, the title of your manuscript must have the following format: "Commentary: Title of the original article". At the beginning of your Commentary, please provide the complete citation of the article commented on. Authors commenting on a Frontiers article must submit their commentary for consideration to the same Journal and Specialty as the original article.

Rebuttals may be submitted in response to Commentaries; our limit in place is one commentary and one response. Rebuttals should be submitted as General Commentary articles and the title should have the following format: "Response: Commentary: Title of original article".

2.3.6.3. Book Reviews

The title of a book review needs to follow the format "Book Review: Title of book". For book Reviews, you must also provide the full book details at the beginning of the article in this format: "Book Review: Full book reference"

2.3.6.4. Focused Reviews

For Tier 2 invited Focused Reviews, to shape the paper on the importance of the research to the field, we recommend structuring the Review to discuss the paper's Introduction, Materials and Methods, Results and Discussion. In addition the authors must submit a short biography of the corresponding author(s). This short biography has a maximum of 600 characters, including spaces

A picture (5 x 5 cm, in *.tif or *.jpg, min 300 dpi) must be submitted along with the biography in the manuscript and separately during figure upload.

Focused Reviews highlight and explain key concepts of your work. Please highlight a minimum of four and a maximum of ten key concepts in bold in your manuscript and provide the definitions/explanations at the end of your manuscript under “Key Concepts”. Each definition has a maximum of 400 characters, including spaces.

2.3.6.5 Systematic Reviews

For Systematic Reviews, the following article structure applies.

Title: include systematic review/meta-synthesis/meta-analysis as appropriate in the title

Each of the sections should include specific sub-sections as follows

Abstract

Background

Methods

Results

Conclusions

Introduction

Rationale

Objectives

Research question

Methods

Study design

Participants, interventions, comparators

Systematic review protocol

Search strategy

Data sources, studies sections and data extraction

Data analysis

Results

Provide a flow diagram of the studies retrieved for the review

Study selection and characteristics

Synthesized findings

Risk of bias

Discussion

Summary of main findings

Limitations

Conclusions

2.3.6.6. Data Reports

For Data Reports, please make sure to follow these additional specific guidelines.

1. The data sets (defined as a collection of data that contains individual data units organized in a standardized reusable format, including pre-processed or raw data) must be deposited in a public repository for long-term data preservation prior to submission of the Data Report. The data set(s) is to be fixed and made publicly available upon publication of the Data Report.

2. Our data sharing policy also requires that the dataset be made available to the Frontiers editors and reviewers during the review process of the manuscript. Prior to submission of your Data Report manuscript, please ensure that the repository you have selected supports confidential peer-review. If it does not, we recommend that the authors deposit the datasets to figshare or Dryad Digital Repository for the peer-review process. The data set(s) can then be transferred to another relevant repository before final publication, should the article be accepted for publication at Frontiers.

Note that it is the authors' responsibility to maintain the data sets after publication of the Data Report. Any published Frontiers Data Report article will be considered for retraction should the data be removed from the final selected repository after publication or the access become restricted.

3. The submitted manuscript must include the following details:

Detailed statement of contribution of the data report to the field

Name of the data set

Name of the database/repository where the data set has been submitted

Link to the data set for confidential peer-review (which can be updated after acceptance, prior to publication once the data is made public)

Description of how the data was acquired, data collection period

Filters applied to the data

Overview of the data files and their formats

Reference to and/or description of the protocols or methods used to collect the data

Information on how readers may interpret the data set and reuse the data

All these elements will be peer-reviewed and are required for the publication of the Data Report.

Any future updates to the data set(s) should be deposited as independent versions in a repository and the relevant information may be published as General Commentaries linked on the Frontiers website to the initial Data Report.

Any detailed analyses or new scientific insights relating to the Data Report can be submitted as independent research articles which can also be linked on the Frontiers website to the Data Report article. The protocols and methodology used to collect the data can also be submitted as Methods articles.

2.3.6.7. Case Reports

Case Reports should include the following:

Background

Case Presentation

For human patients: age, sex and occupation of the patient, presenting symptoms, the patient's history and any relevant family or social history, and relevant clinical findings

For animal patients: age, sex, and breed of the animal, presenting problems, the animal's history, and relevant clinical findings.

Description of laboratory investigations and diagnostic tests.

Discussion of the underlying pathophysiology and the novelty or significance of the case. Authors are required to obtain written informed consent from the patients (or their legal representatives) for the publication.

2.3.6.8. Policy & Practice Reviews

For Policy and Practice Reviews, the following article structure applies:

Abstract

Introduction

Sections on assessment of policy/guidelines options and implications

Actionable Recommendations and Conclusions

2.3.6.9. Policy Briefs

For Policy Briefs, the following article structure applies:

Abstract (bullet point format)

Introduction

Sections on Policy Options and Implications

Section on Actionable Recommendations

Conclusions

2.3.6.10. Protocols

For Protocols articles, please make sure to follow these additional specific guidelines.

The submitted manuscript must include the following sections:

An Abstract.

An Introduction outlining the protocol and summarizing its possible applications.

A Materials and Equipment section providing a list of reagents or other materials and/or equipment required to carry out the protocol. For basic-science protocols, the formulation of any solutions, e.g. buffers, should be clearly indicated in the Materials and Equipment section.

A Stepwise Procedures section listing, stepwise, the stages of the protocol. The timing of each step or related series of steps should be indicated, as should points at which it is possible to pause

or halt the procedure without adversely influencing the outcome. For steps requiring repeated measurements, details of precision and accuracy should be presented. Limits of detection or quantification should also be stipulated where appropriate.

An Anticipated Results section describing, and illustrating with figures, where possible, the expected outcome of the protocol. Any analytical software or methods should be presented in detail in this section, as should possible pitfalls and artifacts of the procedure and any troubleshooting measures to counteract them. These last may also be described in an optional Notes section.

Code or training data sets referenced by the protocol and useful in its execution should be hosted in an online repository; their accession numbers or other stable identifiers should be referenced in the Anticipated Results.

The significance of the protocol and any advance represented by the method compared with other, similar methods should be presented in the contribution to the field statement accompanying your manuscript.

2.3.6.11. Code

The code should be novel and presented in human-readable format, adhere to the standard conventions of the language used (variable names, indentation, style and grammar), be well documented (comments in source), be provided with an example data set to show efficacy, be compilable or executable free of errors (stating configuration of system used).

The code should only call standard (freely accessible) libraries or include required libraries, and include a detailed description of the use-scenarios, expected outcomes from the code and known limitations of the code.

Please therefore make sure to provide access to the following upon submission:

Abstract explicitly including the language of code

Keywords including the language of the code in the following format:"code:language"” e.g.: "code:matlab"

Contribution to the field statement including the utility of the code and its language

Main Text including:

code description

application and utility of the code

link to an accessible online code repository where the most recent source code version is stored and curated (with an associated DOI for retrieval after review)

access to test data and readme files

methods used

example of use

known issues

licensing information (Open Source licenses recommended)

Compressed Archive (.zip) of the reviewed version of the code as supplementary material (.zip archives are currently available under the “Presentation” dropdown menu).

2.3.6.12. Registered Report

Registered Reports are empirical research articles outlining a proposed methodology and analyses which are peer-reviewed and pre-registered before data collection. Registered Reports should include an Introduction, Methods and preliminary results from any pilot experiments (if applicable). If the Registered Report is endorsed following peer-review and the research is conducted according to the approved methodology, the manuscript will be given In Principle Acceptance. Following data collection, the authors should submit a complete manuscript containing the peer-reviewed sections included in the Registered Report, as well as the Results and Discussion sections. If the Results include unregistered analysis, these should be indicated separately as ‘Exploratory Analysis’. Authors have 1 year after their registered report is accepted to submit a full manuscript. The format is appropriate for any hypothesis-driven research, including both original studies and replications.

Registered Reports have a maximum word count of 3,000 and may include 2 Figures/Tables. Following data collection, the completed version of the manuscript should follow the guidelines for an Original Research article with a maximum word count of 12,000. Registered Reports incur a A-type article fee, charged after the acceptance of the completed manuscript.

2.4. Figure and Table Guidelines

2.4.1. CC-BY Licence

All figures, tables, and images will be published under a Creative Commons CC-BY licence and permission must be obtained for use of copyrighted material from other sources (including re-published/adapted/modified/partial figures and images from the internet). It is the responsibility of the authors to acquire the licenses, to follow any citation instructions requested by third-party rights holders, and cover any supplementary charges.

2.4.2. General Style Guidelines for Figures

The maximum number of figures and tables for all article types are shown in the Summary Table. Frontiers requires figures to be submitted individually, in the same order as they are referred to in the manuscript, the figures will then be automatically embedded at the end of the submitted manuscript. Kindly ensure that each table and figure is mentioned in the text and in numerical order.

For graphs, there must be a self-explanatory label (including units) along each axis. For figures with more than one panel, panels should be clearly indicated using labels (A), (B), (C), (D), etc. However, do not embed the part labels over any part of the image, these labels will be added

during typesetting according to Frontiers journal style. Please note that figures which are not according to the guidelines will cause substantial delay during the production process.

Permissions may be necessary in the following scenarios:

Republishing

Modifying/adapting

Partial Figures

It is the responsibility of the authors to acquire the licenses, to follow any citation instructions requested by third-party rights holders, and cover any supplementary charges.

2.4.3. General Style Guidelines for Tables

Tables should be inserted at the end of the manuscript. If you use a word processor, build your table in word. If you use a LaTeX processor, build your table in LaTeX. An empty line should be left before and after the table.

Please note that large tables covering several pages cannot be included in the final PDF for formatting reasons. These tables will be published as supplementary material on the online article abstract page at the time of acceptance. The author will notified during the typesetting of the final article if this is the case. A link in the final PDF will direct to the online material.

For additional information, please see our Editorial Policies: 3.5 Image Manipulation.

2.4.4. Figure and Table Requirements

Legends

Legends should be preceded by the appropriate label, for example "Figure 1" or "Table 4". Figure legends should be placed at the end of the manuscript (for supplementary images you must include the caption with the figure, uploaded as a separate file). Table legends must be placed immediately before the table. Please use only a single paragraph for the legend. Figure panels are referred to by bold capital letters in brackets: (A), (B), (C), (D), etc.

Image Size

Figure images should be prepared with the PDF layout in mind, individual figures should not be longer than one page and with a width that corresponds to 1 column or 2 columns.

All articles are prepared using the 2 column layout: 2 column articles can contain images 85 mm or 180 mm wide.

2.4.5. Format

The following formats are accepted:

TIFF (.tif) TIFF files should be saved using LZW compression or any other non-lossy compression method.

JPEG (.jpg)

EPS (.eps) EPS files can be uploaded upon acceptance

Color Image Mode

Images must be submitted in the color mode RGB.

Resolution Requirements

All images must be uploaded separately in the submission procedure and have a resolution of 300 dpi at final size. Check the resolution of your figure by enlarging it to 150%. If the resolution is too low, the image will appear blurry, jagged or have a stair-stepped effect.

Please note saving a figure directly as an image file (JPEG, TIF) can greatly affect the resolution of your image. To avoid this, one option is to export the file as PDF, then convert into TIFF or EPS using a graphics software. EPS files can be uploaded upon acceptance.

Chemical Structures

Chemical structures should be prepared using ChemDraw or a similar program. If working with ChemDraw please use Frontiers ChemDraw Template, if working with another program please follow the guidelines given below:

Drawing settings: chain angle, 120° bond spacing, 18% of width; fixed length, 14.4 pt; bold width, 2.0 pt; line width, 0.6 pt; margin width 1.6 pt; hash spacing 2.5 pt. Scale 100% Atom Label settings: font, Arial; size, 8 pt.

Assign all chemical compounds a bold, Arabic numeral in the order in which the compounds are presented in the manuscript text. Figures containing chemical structures should be submitted in a size appropriate for incorporation into the manuscript.

Legibility

Figures must be legible. Check the following:

The smallest visible text is no less than 8 points in height, when viewed at actual size.

Solid lines are not broken up.

Image areas are not pixilated or stair stepped.

Text is legible and of high quality.

Any lines in the graphic are no smaller than 2 points width.

2.5. Funding disclosure

Details of all funding sources must be provided in the funding section of the manuscript including grant numbers, if applicable. All Frontiers articles are published with open access under the CC-BY Creative Commons attribution license. Articles published with Frontiers automatically fulfil or exceed the requirements for open access mandated by many institutions and funding bodies,

including the National Institutes of Health, the Medical Research Council, Research Councils UK, and the Wellcome Trust. Frontiers submits funding data to the Open Funder Registry which is a funder identification service from CrossRef resulting from collaboration between scholarly publishers and funding agencies.

### 2.6. Materials and Data Policies

Frontiers is committed to open science and open data, and we strongly encourage authors to maximize the availability of data included in their articles by making generated data publicly available where possible, and ensuring that published data sets are cited in accordance with our data citation guidelines. We aim to achieve the best community standards regarding data availability, ensuring increased levels of transparency and reproducibility in our published articles.

Our policies on data availability are informed by community-driven standards, which Frontiers endorses, such as the Transparency and Openness (TOP) guidelines, and the joint declaration of data citation principles produced by FORCE 11.

#### 2.6.1. Availability of Materials

Authors are strongly encouraged to make all materials used to conduct their research available to other researchers. Research materials necessary to enable the reproduction of an experiment should be clearly indicated in the Materials and Methods section. Relevant materials such as protocols, analytic methods, and study material should preferably be uploaded to an online repository providing a global persistent link/identifier. If this is not possible, authors are strongly encouraged to make this material available upon request to interested researchers, and this should be stated in the manuscript.

Resource Identification Initiative

Authors wishing to participate in the Resource Identification Initiative should cite antibodies, genetically modified organisms, software tools, data, databases, and services using the corresponding catalog number and RRID in your current manuscript. For more information about the project and for steps on how to search for an RRID, please click here.

#### 2.6.2. Availability of Data

Frontiers requires that authors make all data relevant to the conclusions of the manuscript available to editors and reviewers during peer-review to enable complete and objective evaluation of the work described.

We strongly encourage authors to make the raw data supporting the conclusions of the manuscript available in publicly accessible repositories. To comply with best practice in their field of research, authors are required to make certain types of data available to readers at time of publication in specific stable, community-supported repositories such as those listed below. Authors are encouraged to contact our data availability office at datapolicy@frontiersin.org prior to submission with any queries concerning data reporting.

2.6.3. Data Citation Guidelines

Authors are encouraged to cite all datasets generated or analyzed in the study. Where datasets are cited, they should be included in the references list to maximize future usability. The following format should be used:

[Dataset] Author names. (year) Data Title. Repository name. Version. Persistant identifier

2.6.4. Data Availability Statements

Data availability statements are required for all manuscripts published with Frontiers. During the submission process, authors will be asked to detail the location of the raw data underlying the conclusions made in the manuscript, and whether it will be made available to other researchers following publication. Authors will also be asked for the details of any existing datasets that have been analysed in the manuscript. These datasets should be cited in accordance with our data citation guidelines.

A statement will be automatically generated using the information provided in the submission form; however, manuscripts containing incomplete or incorrect statements will be prevented from entering the review process.

Examples of acceptable statements

Datasets are in a publicly accessible repository:

The datasets [GENERATED/ANALYZED] for this study can be found in the [NAME OF REPOSITORY] [LINK]

Datasets are available on request:

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

All relevant data is contained within the manuscript:

All datasets [GENERATED/ANALYZED] for this study are included in the manuscript and the supplementary files.

Restrictions apply to the datasets:

The datasets for this manuscript are not publicly available because: [VALID REASON]. Requests to access the datasets should be directed to [NAME, EMAIL].

Data has been obtained from a third party:

The data analyzed in this study was obtained from [SOURCE], the following licenses/restrictions apply [RESTRICTIONS]. Requests to access these datasets should be directed to [NAME, EMAIL].

No datasets were generated for this study

2.6.5. Recommended and Required Repositories

Authors are required to deposit the following data-types in public, community-supported repositories, such as those listed below, prior to publication of an associated Frontiers manuscript:

Data-type Recommended Repositories Metadata Standard

Genetic and genomic sequence (DNA/ RNA)^ GenBank

DNA Data Bank of Japan

(DDBJ)

European Nucleotide Archive

(ENA) MiXS

Metagenomic sequence EBI Metagenomics MiXS

DNA and RNA trace or short-read sequencing data NCBI Trace Archive NCBI Sequence Read Archive MiXS

Genetic polymorphism data, including SNP and CNV data dbSNP

dbVar

European Variation Archive

DGVa MiXS

Gene expression data; chromatin immunoprecipitation data (deep-sequencing or microarray) ArrayExpress

Gene Expression Omnibus (GEO) MIAME / MINSEQE

Data linking genotype to phenotype dbGaP

Protein sequence data UniProt

Proteome profiling data PRIDE

PeptideAtlas

ProteomeXchange MIAPE

Small molecule, protein, protein complex data structural data: Crystallography Open Database

Cambridge Structural Database

wwPDB (Protein DataBank)

Electron Microscopy Databank CIF

Taxonomy data Zoobank

^ Genetic sequence variants should be annotated according to the guidelines established by the

Human Variome Project.

Authors are encouraged to consider deposition in public, community-supported repositories of the data-types listed below:

Data-type Recommended Repositories Metadata Standard

Protein-protein interaction data Database of Interacting Proteins (DIP) MIMIx

Metabolite and metabolome profiling data MetaboLights

Human Metabolome Database MSI

Small-molecule screening data, chemical compound data PubChem CIF

Flow cytometry data Flow Repository

Brain Imaging data / Neuroimaging data OpenNeuro

INDI

NITRC

NeuroVault [Statistical maps] BIDS

Trait data TRY database

Phenology data National Phenology Network

Any data FigShare

Dryad Digital Repository None

2.6.6. Inclusion of Zoological Nomenclature

The International Code of Zoological Nomenclature, in a recent 2012 amendment to the 1999 Zoological Code, allows all electronic-only papers, such as those published by the Frontiers journals, to have valid new taxon names and nomenclatural acts. However, these new names or nomenclatural acts must be registered in ZOOBANK and have associated Life Science Identifiers (LSIDs). Registration must be done by the authors before publication. Should your manuscript include any zoological new taxon names and/or nomenclatural acts, please ensure that they are registered prior to final publication.

2.6.7. Inclusion of RNAseq Data

Studies employing RNASeq for comparative transcriptomic analyses must contain at least 3 biological replicates (unless otherwise justified). Each biological replicate should be represented in an independent library, each with a unique barcode if libraries are multiplexed for sequencing. Validation on a number of key transcripts highlighted in the study is also highly recommended.

Full data accompanying these experiments must be made available to reviewers at the time of submission in a freely accessible resource e.g the sequence read archive (SRA) or European

Nucleotide Archive (ENA). Depending on the question addressed in a manuscript, de novo assemblies of transcriptomes may also require multiple replicates and assembled sequences together with sequence annotation must be made freely available e.g figshare or dryad.

2.6.8 Inclusion of Proteomics Data

Authors should provide relevant information relating to how peptide/protein matches were undertaken, including methods used to process and analyse data, false discovery rates (FDR) for large-scale studies and threshold or cut-off rates for peptide and protein matches. Further information should include software used, mass spectrometer type, sequence database and version, number of sequences in database, processing methods, mass tolerances used for matching, variable/fixed modifications, allowable missed cleavages, etc.

Authors should provide as supplementary material information used to identify proteins and/or peptides. This should include information such as accession numbers, observed mass (m/z), charge, delta mass, matched mass, peptide/protein scores, peptide modification, miscleavages, peptide sequence, match rank, matched species (for cross-species matching), number of peptide matches, etc. Ambiguous protein/peptide matches should be indicated.

For quantitative proteomics analyses, authors should provide information to justify the statistical significance, including biological replicates, statistical methods, estimates of uncertainty, and the methods used for calculating error.

For peptide matches with biologically relevant post-translational modifications (PTMs) and for any protein match that has occurred using a single mass spectrum, authors should include this information as raw data or annotated spectra, or submit data to an online repository (recommended option; see table below).

Raw or matched data and 2-DE images should be submitted to public proteomics repositories such as those participating in ProteomeXchange. Submission codes and/or links to data should be provided within the manuscript.

2.7. Statistics

Frontiers requires that all statements concerning quantitative differences should be based on quantitative data and statistical testing. For example, if a quantitative statement is made regarding the abundance of a certain protein based on a western blot, we request that the blot be scanned and the abundance assessed quantitatively using the correct analytic software (e.g. ImageJ) and statistics in order to support that statement.

Statistics should/must be applied for independent experiments. The number of independent samples and the deviation parameters (e.g. Standard Error of the Mean, Standard Deviation, Confidence Intervals) should be clearly stated in the Methods or the Figure legends. In general, technical replicates within a single experiment are not considered to be independent samples. Where multiple comparisons are employed (e.g. microarray data or Genome-wide association studies), any analysis should correct for false positive results. Descriptions of statistical procedures should include the software and analysis used, and must be sufficiently detailed to be

reproduced.

3. Editorial Policies and Publication Ethics

Frontiers' ethical policies are a fundamental element of our commitment to the scholarly community. These policies apply to all the Frontiers in journal series. Frontiers has been a member of the Committee of Publication Ethics since January 2015 and follows COPE guidelines where applicable.

3.1. Authorship and Author Responsibilities

Frontiers follows the International Committee of Medical Journal Editors guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

Substantial contributions to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work;

Drafting the work or revising it critically for important intellectual content;

Provide approval for publication of the content;

Agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Contributors, who do not meet these criteria, but nonetheless provided important contributions to the final manuscript should be included in the acknowledgements section. It is the authors responsibility to get written approval by persons named in the acknowledgement section. In order to provide appropriate credit to all authors, as well as assigning responsibility and accountability for published work, individual contributions should be specified as an Author Contributions statement. This should be included at the end of the manuscript, before the References. The statement should specify the contributions of all authors. You may consult the Frontiers manuscript guidelines for formatting instructions. Please see an example here:

AB, CDE and FG contributed conception and design of the study; AB organized the database; CDE performed the statistical analysis; FG wrote the first draft of the manuscript; HIJ, KL, AB, CDE and FG wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

The corresponding author takes primary responsibility for communication with the journal and editorial office during the submission process, throughout peer review and during publication. The corresponding author is also responsible for ensuring that the submission adheres to all journal requirements including, but not exclusive to, details of authorship, study ethics and ethics approval, clinical trial registration documents and conflict of interest declaration. The corresponding author should also be available post-publication to respond to any queries or critiques.

Requests to modify the authors list after submission should be made to the editorial office using the authorship changes form.

3.2. Research Integrity

Material submitted to Frontiers must comply with the following policies to ensure ethical publication of academic work:

Original content and duplicate publication: Frontiers only publishes original content. Authors confirm the submission of original content in the Terms & Conditions upon submission. Manuscripts submitted to Frontiers must not have been previously published or be under consideration for publication elsewhere, either in whole or in part. If an article has been previously submitted for publication elsewhere, Frontiers will only consider publication if the article has been definitively rejected by the other publisher(s) at the point of submission to Frontiers.

Redundant publication: Frontiers considers the submission and publication of very similar articles based on the same experiment or study to be unethical.

Fabrication and falsification: Frontiers opposes both the fabrication of data or images (i.e. fake or made up data) and the falsification of data or images (i.e. the intentional misrepresentation or deceptive manipulation of data).

Plagiarism: Plagiarism occurs when an author attempts to present previously published work as original content. Every manuscript submitted to Frontiers is screened for textual overlap by the software CrossCheck, powered by iThenticate. Manuscripts found to contain textual overlap are not considered for publication by Frontiers. For more details on what constitutes plagiarism, please see here.

We reserve the right to contact the affiliated institutions of authors, who have not acted according to good research and publication practices.

3.3. Translations

Frontiers accepts manuscript submissions that are exact translations of previously published work. This should be clearly stated in the manuscript upon submission. Permission from the original publisher and authors needs to be sought and also stated in the manuscript, and the relevant documents should be provided as supplementary data for verification by the Editor and the editorial office. The original work from which the manuscript has been translated should be clearly referenced.

"This is a (‘language’) language translation/reprint of (‘insert title here’) originally published in (‘insert name here’). (‘Insert name here’) prepared this translation with support from (insert name of funding source, if any). Permission was granted by (‘Insert name here’).”

Please note that Frontiers may request copies of related publications if there are any concerns about overlap or possible redundancy.

3.4. Plagiarism and Duplication

Frontiers checks all submitted manuscripts for plagiarism and duplication, and publishes only original content. Those manuscripts where plagiarism or duplication is shown to have occurred

will not be considered for publication in a Frontiers journal. It is required that all submissions must consist as far as possible of content that has not been published previously. In accordance with COPE guidelines, we expect that "original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations." This condition also applies to an author's own work.

For submissions adapted from theses, dissertations, conference abstracts or proceedings papers, please see the following sections for more information.

Theses and Dissertations

Frontiers allows the inclusion of content which first appeared in an author's thesis so long as this is the only form in which it has appeared, is in line with the author's university policy, and can be accessed online. If the thesis is not archived online, it is considered as original unpublished data and thus is subject to the unpublished data restrictions of some of our article types. This inclusion should be noted in the Acknowledgements section of the manuscript and the thesis should be cited and referenced accordingly in the Reference list. For some examples, please check our in Manuscript Requirements and Style Guide at 2.3.1

Conferences, Proceedings and Abstracts

Manuscripts that first appeared as conference papers must be expanded upon if they are to be considered as original work. You are required to add a substantial amount of original content in the form of new raw material (experiments, data) or new treatment of old data sets which lead to original discussion and/or conclusions, providing value that significantly exceeds the original conference version. As a rule of thumb, at least 30% of content must be original. Authors submitting such work are required to:

- Seek permission for reuse of the published conference paper if the author does not hold the copyright (proof of permission should be submitted as supplementary material or sent to editorial.office@frontiersin.org with the manuscript ID upon submission).

- Cite the conference in the Acknowledgements section, or the references section if applicable.

Blogs

Although permissible, extended manuscript content which previously appeared online in non-academic media, e.g. blogs, should be declared at the time of submission in the acknowledgements section of the manuscript.

3.5. Image Manipulation

Frontiers takes concerns regarding image manipulation seriously. We request that no individual features within an image are modified (eg. enhanced, obscured, moved, recycled, removed or added). Image processing methods (e.g. changes to the brightness, contrast or color balance) must be applied to every pixel in the image and the changes should not alter the information illustrated in the figure. Where cropped images of blots are shown in figures, a full scan of the entire original gel(s) must be submitted as part of the supplementary material. Where control images are

re-used for illustrative purposes, this must be clearly declared in the figure legend. If any form of image processing is legitimately required for the interpretation of the data, the software and the enhancement technique must be declared in the methods section of the manuscript. Image grouping and splicing must be clearly stated in the manuscript and the figure text. Any concerns raised over undeclared image modifications will be investigated and the authors will be asked to provide the original images.

3.6. Conflicts of Interest

A conflict of interest can be anything potentially interfering with, or that could reasonably be perceived as interfering with, full and objective peer review, decision-making or publication of articles submitted to Frontiers. Personal, financial and professional affiliations or relationships can be perceived as conflicts of interest.

All authors and members of Frontiers Editorial Boards are required to disclose any actual and potential conflicts of interest at submission or upon accepting an editorial or review assignment.

The Frontiers review system is designed to guarantee the most transparent and objective editorial and review process, and because handling editor and reviewers' names are made public upon the publication of articles, conflicts of interest will be widely apparent.

Failure to declare competing interests can result in the rejection of a manuscript. If an undisclosed competing interest comes to light after publication, Frontiers will take action in accordance with internal policies and Committee on Publication Ethics guidelines.

What Should I Disclose?

As an author, disclosure of any potential conflicts of interest should be done during the submission process. Consider the following questions and make sure you disclose any positive answers:

Did you or your institution at any time receive payment or services from a third party for any aspect of the submitted work?

Do you have financial relationships with entities that could be perceived to influence, or that give the appearance of potentially influencing, what you wrote in the submitted work?

Do you have any patents and copyrights, whether pending, issued, licensed and/or receiving royalties related to the research?

Do you have other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?

If you failed to disclose any of the potential conflicts of interest above during submission, or in case of doubt, please contact as soon as possible the Frontiers Editorial Office at editorial.office@frontiersin.org with the details of the potential conflicts.

Example statement: “Author xxx was employed by company xxxx. All other authors declare no competing interests.”

The handling editors and reviewers will be asked to consider the following potential conflicts of interest before accepting any editing or review assignment:

FAMILY 1. Are any of the authors a spouse or significant other, a member of the same family or a very close personal friend? Review Editors should also not be a member of the same family as the handling editor.

COLLABORATIONS 2. Are you currently hosting or have hosted a Frontiers Research Topic with any of the authors within the past 2 years? Are you currently hosting a Frontiers Research Topic with the Editor?

3. Are you currently collaborating or have you collaborated on a research project or a publication with any of the authors within the past 2 years?

4. Are you currently collaborating or have you collaborated with any of the authors as an advisor or in any other direct supervisory capacity in the past five years?

5. Are you currently collaborating or have you collaborated with any of the authors as a student or in any other direct subordinate capacity in the past five years?

Note: Review Editors should not accept assignments if they have a close professional relationship with the handling editor, which in their view could affect the objectivity of the review.

AFFILIATION 6. Are you affiliated with the same institution as the editor? Are you affiliated with the same institution as any of the authors? If so, has this resulted in interactions, collaborations, or mutual interests with the authors that would compromise your impartiality in conducting this review?

7. Are you a current member of a committee or department that coincides with an affiliation with the editor or any of the authors?

FINANCIAL 8. Do you have a business or professional partnership with any author?

9. Do you have financial interests or business relations with any organization involved in this research or in the preparation of the manuscript?

10. Do you have any financial interest or competing interests in the content of the manuscript that might affect your ability to perform an objective review?

3.7. Bioethics

All research submitted to Frontiers for consideration must have been conducted in accordance with Frontiers guidelines on study ethics. In accordance with COPE guidelines, Frontiers reserves the right to reject any manuscript that editors believe does not uphold high ethical standards, even if authors have obtained ethical approval or if ethical approval is not required.

3.7.1. Studies involving animal subjects

All research involving regulated animals (i.e. all live vertebrates and higher invertebrates) must be performed in accordance with relevant institutional and national guidelines and regulations.

Frontiers follows International Association of Veterinary Editors guidelines for publication of studies including animal research. Approval of research involving regulated animals must be obtained from the relevant institutional review board or ethics committee prior to commencing the study. Confirmation of this approval is required upon submission of a manuscript to Frontiers; authors must provide a statement identifying the full name of the ethics committee that approved the study. For most article types, this statement should appear in the Materials and Methods section. An example ethics statement:

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee].

Should the study be exempt from ethics approval, authors need to clearly state the reasons in the declaration statement and in the manuscript. Studies involving privately owned animals should demonstrate the best practice veterinary care and confirm that informed consent has been granted by the owner/s, or the legal representative of the owner/s. Frontiers supports and encourages authors to follow the ARRIVE guidelines for the design, analysis and reporting of scientific research.

Humane Endpoints

All manuscripts describing studies where death is an endpoint will be subject to additional ethical considerations. Frontiers reserves the right to reject any manuscripts lacking in appropriate justification.

3.7.2. Studies involving human subjects

Research involving human subjects is expected to have been conducted in accordance with the World Medical Association’s Declaration of Helsinki. Studies involving human participants must be performed in accordance with relevant institutional and national guidelines, with the appropriate institutional ethics committee's prior approval and informed written consent from all human subjects involved in the study including for publication of the results. Conformation of this approval is required upon submission of a manuscript to Frontiers; authors must provide a statement identifying the full name of the ethics committee that approved the work and confirm that study subjects (or when appropriate, parent or guardian) have given written informed consent. For most article types, this statement should appear in the Materials and Methods section. An example ethics statement:

This study was carried out in accordance with the recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee]. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Should the study be exempt from ethics approval, authors need to clearly state the reasons in the declaration statement and in the manuscript. In order to protect subject anonymity, identifying information should not be included in the manuscript unless such information is absolutely necessary for scientific purposes AND explicit approval has been granted by the subjects.

3.7.3. Inclusion of identifiable human data

Frontiers follows the ICMJE recommendations on the protection of research participants, which state that patients have a right to privacy that should not be violated without informed consent. We require non-essential identifiable details to be omitted from all manuscripts, and written informed consent will be required if there is any doubt that anonymity can be maintained.

It is the responsibility of the researchers and authors to ensure that these principles are complied with, including the obtaining of written, informed consent.

Written informed consent can be documented on a form provided by an institution or ethics committee, and it must clearly state how the identifiable data will be used. Frontiers also makes available its own form , which may be used for this purpose, but use of the Frontiers form is not required if a suitable alternative form of consent, meeting the ICMJE recommendations, is used. We consider it to be the author' duty to encourage participants or patients whose consent for publication is required to read and understand the ICMJE guidelines, for their information prior to completing the consent form. Participants should also be encouraged to ask any questions and to ensure they are comfortable before they sign the consent form.

The completed consent forms should be stored by authors or their respective institutions, in accordance with institutional policies. Frontiers does not need to view the completed form, and this should not be included with the submission. The completed form should be made available on request from the editor or editorial office, both during the review process and post-publication.

The determination of what constitutes identifiable data lies with our editors and editorial office staff, and manuscripts may be rejected if the required consent documents cannot be provided. Please note that written informed consent for publication is required for all case report articles where the patient or subject is identified or identifiable.

3.7.4. Clinical Trials

The World Health Organization defines a clinical trial as "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes." In accordance with the Clinical Trial Registration Statement from the International Committee of Medical Journal Editors (ICMEJ), all clinical trials must be registered in a public trials registry at or before the onset of participant enrolment. This requirement applies to all clinical trials that begin enrolment after July 1, 2005. To meet the requirements of the ICMJE, and Frontiers', clinical trials can be registered with any Primary Registry in the WHO Registry Network or an ICMJE approved registry.

Clinical trial reports should be compliant with the Consolidated Standards of Reporting Trials (CONSORT) both in terms of including a flow diagram presenting the enrolment, intervention allocation, follow-up, and data analysis with number of subjects for each and taking into account the CONSORT Checklist of items to include when reporting a randomized clinical trial.

The information on the clinical trial registration (Unique Identifier and URL) must be included in the abstract.

3.8. Corrections

Frontiers recognizes our responsibility to correct errors in previously published articles. If it is necessary to communicate important, scientifically relevant errors or missing information, and compelling evidence can be shown that a major claim of the original article was incorrect, a Correction should be submitted detailing the reason(s) for and location(s) of the change(s) needed using the below template. Corrections can be submitted if a small portion of an otherwise reliable publication proves to be misleading, e.g. an error in a figure that does not alter conclusions OR an error in statistical data not altering conclusions OR mislabeled figures OR wrong slide of microscopy provided, or if the author / contributor list is incorrect when a deserving author has been omitted or somebody who does not meet authorship criteria has been included. The contribution to the field statement should be used to clearly state the reason for the Correction. Please note, a correction is not intended to replace the original manuscript.

The title of the submission should have the following format: "Corrigendum: Title of original article". It is advised to use the corrigendum Word and LaTeX templates.

If the error was introduced during the publishing process, the Frontiers Production Office should be contacted.

3.9. Retractions

As a member of the Committee on Publication Ethics (COPE), Frontiers abides by their guidelines and recommendations in cases of potential retraction.

Frontiers also abides by two other key principles, as recommended by COPE:

Retractions are not about punishing authors.

Retraction statements should be public and linked to the original, retracted article.

While all potential retractions are subject to an internal investigation and will be judged on their own merits, Frontiers considers the following reasons as giving cause for concern and potential retraction:

Clear evidence that findings are unreliable, either as a result of misconduct (e.g. data fabrication) or honest error (e.g. miscalculation or experimental error)

Findings have previously been published elsewhere without proper attribution, permission or justification (i.e. cases of redundant publication)

Major plagiarism

The reporting of unethical research, the publication of an article that did not have the required ethics committee approval

Legal issues pertaining to the content of the article e.g. libellous content

Major authorship issues i.e. proven or strongly suspected cases of ghostwriting or sold (‘gift’) authorship

Politically-motivated articles where objectivity is a serious concern

The singling out of individuals or organizations for attack

Faith issues (e.g. intelligent design)

Papers that have made extraordinary claims without concomitant scientific or statistical evidence (e.g. pseudoscience)

Readers who would like to draw the editors' attention to published work that might require retraction should contact the authors of the article and write to the journal, making sure to include copies of all correspondence with authors.

Please find more details on our comments and complaints policy here

3.10. Support and Ethical concerns

In our commitment to continuously improve our website, we welcome your feedback, questions and suggestions. Please visit our Help Center to find guidance on our platform or contact us at support@frontiersin.org.

For any ethical concerns, please contact us at editorial.office@frontiersin.org.

## CONSIDERAÇÕES FINAIS

As feridas agudas no presente trabalho demonstrou que algumas plantas da família Asteraceae tem relevância medicinal, por suas características anti-inflamatórias e ação no processo de cicatrização. Avaliou-se também a importância do uso dos óleos essenciais no contexto da cicatrização, principalmente em feridas infectadas, por *S. aureus*.

O óleo essencial, cinamaldeído, mostrou-se um potente agente cicatrizante pois, a partir dos seus efeitos antimicrobianos de amplo espectro e a capacidade de reduzir a expressão de vários fatores de virulência, possibilitou a aceleração da cicatrização em feridas infectadas. Além disso CNM apresentou efeitos imunomodulador melhorando a resposta de camundongos e auxiliando no processo de cicatrização, reduzindo a infiltração de neutrófilos nos tecidos cutâneos infectados por *S. aureus* reduzindo a concentração de citocinas inflamatórias. Juntos, estes efeitos levaram a diminuição da gravidade da infecçãoe e a  melhora no reparo tecidual.