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POTENCIAL TERAPÊUTICO DE CINAMALDEÍDO NO TRATAMENTO DE FERIDAS CUTÂNEAS INFECTADAS

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São Luís
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Orientador: Elizabeth Soares Fernandes
Co-orientador: Valério Monteiro Neto

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RESUMO

Atualmente há uma grande procura por produtos extraídos de plantas que apresentam atividade antimicrobiana e que também beneficiam o processo de cicatrização. Isto se deve principalmente, ao aumento da prevalência de isolados resistentes aos diferentes tipos de antibióticos comercializados. Feridas infectadas são um problema sério de saúde pública no mundo, sendo comuns em pacientes com queimadura e úlceras. O cinamaldeído, composto majoritário do extrato da canela, vem sendo estudado por suas propriedades antimicrobianas e imunomoduladoras. Entretanto, pouco se sabe acerca de seus efeitos em bactérias presentes em feridas infectadas como *Pseudomonas aeruginosa*, *Enterococcus faecalis*, e *Staphylococcus aureus,* nem sobre suas propriedades cicatrizantes. Assim, este estudo tem como objetivo avaliar o efeito do cinamaldeído na virulência e viabilidade de *P. aeruginosa*, *E. faecalis* e *S. aureus in vitro,* bem como seus efeitos *in vivo* na infecção e cicatrização. Inicialmente, foram realizados experimentos de microdiluição para a avaliação da concentração inibitória mínima e bactericida mínima. Em seguida, o efeito de concentrações subinibitórias do cinamaldeído foram testadas na produção de fatores de virulência por estas bactérias. Os efeitos do cinamaldeído foram avaliados *in vivo*, em modelo de infecção sistêmica induzida por *S. aureus* em *Galleria mellonella* e em modelo de cicatrização de feridas infectadas por *P. aeruginosa* em camundongos**.** Observou-se que o cinamaldeído apresenta satisfatória atividade antimicrobiana contra as três espécies de bactérias avaliadas, sendo capaz de interferir com o crescimento, viabilidade para as três espécies analisadas e produção de fatores de virulência como produção de biofilme, atividade hemolítica, adesão, metabolismo e diminuição da resistência ao soro nos isolados de *Staphylococcus aureus* e *Pseudomonas aeruginosa*, sem causar adaptação destes microrganismos. Observou-se ainda, que o tratamento protegeu *G. mellonella* contra a infecção por *S. aureus*, por diminuir a carga bacteriana além de aumentar a sobrevivência das larvas. Quando avaliado em modelo de cicatrização em camundongos, o cinamaldeído reduziu o tempo de cicatrização em feridas infectadas por *P. aeruginosa,* além de reduzir em 50% a população microbiana no leito da lesão. Os efeitos cicatrizantes do cinamaldeído foram revertidos em animais tratados sistemicamente com o HC-030031, antagonista para o receptor de potencial transitório anquirina 1 (TRPA1), alvo de atuação do cinamaldeído *in vivo*. Concluímos que o cinamaldeído, composto majoritário da canela, pode ser recomendado como um novo candidato terapêutico na cicatrização de feridas infectadas.

Palavras-chave: cicatrização; ferida infectada; efeito antimicrobiano; Cinamaldeído, TRPA1

ABSTRACT

Currently, there is a great demand for products extracted from plants that have antimicrobial activity and that also benefit the cicatrization process. This is mainly due to the increase in the prevalence of isolates resistant to the different types of antibiotics marketed. Infected wounds are a serious public health problem in the world, being common in patients with burns and ulcers. Cinnamaldehyde, the major compound of the cinnamon extract, has been studied for its antimicrobial and immunomodulatory properties. However, little is known about its effects on bacteria present in infected wounds such as *Pseudomonas aeruginosa, Enterococcus faecalis, and Staphylococcus aureus*, nor on its healing properties. Thus, this study aims to evaluate the effect of cinnamaldehyde on the virulence and viability of *P. aeruginosa, E. faecalis and S. aureus* in vitro, as well as its in vivo effects on infection and healing. Initially, microdilution experiments were performed to evaluate the minimal inhibitory concentration and minimal bactericide. Next, the effect of subinhibitory concentrations of cinnamaldehyde were tested on the production of virulence factors by these bacteria. The effects of cinnamaldehyde were evaluated in vivo in a model of systemic infection induced by S. aureus in *Galleria mellonella* and in a model of wound healing of *P. aeruginosa* infected in mice. It was observed that cinnamaldehyde presents satisfactory antimicrobial activity against the three species of bacteria evaluated, being able to interfere with the growth, viability for the three species analyzed and production of virulence factors such as biofilm production, hemolytic activity, adhesion, metabolism and Reduction of serum resistance in the isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, without causing adaptation of these microorganisms. It was further observed that the treatment protected *G. mellonella* against *S. aureus* infection by reducing bacterial load and increasing larval survival. When evaluated in a wound healing model in mice, cinamaldehyde reduced healing time in *P. aeruginosa* infected wounds, and reduced the microbial population in the lesion bed by 50%. The healing effects of cinnamaldehyde were reversed in animals treated systemically with HC-030031, antagonist to the transient potential receptor ankyrin 1 (TRPA1), targeting cinamaldehyde in vivo. We conclude that cinnamaldehyde, the major compound of cinnamon, may be recommended as a new therapeutic candidate in the healing of infected wounds.

Keywords: healing; Infected wound; Antimicrobial effect; Cinnamaldehyde

LISTA DE ABREVIATURAS E SIGLAS

15d-PGJ2 – 15-deoxi-delta (12,14)- prostaglandina J2

4-HNE - 4-Hydroxynonenal

AHL - acyl homoserine lactones

AI 2 – Autoindutor 2

ANOVA – análise de variância

AZT – Aztreonan

BHI – caldo de infusão cérebro-coração

CBM – Concentração bactericida mínima

CD11 – grupo de diferenciação 11

CIM – Concentração inibitória mínima

CIPRO – Ciprofloxacina

CLI – Clidamicina

CLSI – *Clinical and Laboratory Standards Institute*

COX – ciclo-oxigenase

CPM – Cefepime

CTZ – Ceftazidima

FGF – Fator de crescimento de fibroblasto

FtsZ – *filamentous temperature-sensitive protein Z*

GEN – Gentamicina

IC 50 – Concentração inibitória de 50%

IL-6 – Interleucina 6

iNOS – Óxido Nítrico sintase induzível

IPM –Imipenem

LB – Luria Bertani

LDL – lipídeo de densidade baixa

LPS – Lipopolissacarídeo

MER – Meropenem

MH – Muller Hinton

MMP's – Metaloproteinases

MRSA – *Staphylococcus aureus* meticilina resistente

MTA - 5'-metiltioadenosina

Multi – Multidroga resistente.

NF-kβ – Fator nuclear kβ

NO – Óxido nítrico

PBS – tampão fosfato

PCR – Reação em cadeia da polimerase

PEN – Penicilina

pH – Potencial de hidrogêniô

POLI – Polimixina B;

QS – Quorum sensing

SAH – S-adenosilhomocisteína

SAM – S-adenosilmetionina

SUT – sulfametaxazol+trimetoprim

TBARS – Ácido tiobarbitúrico

TGF – Fator de crescimento transformante

TNF – Fator de necrose tumoral

TRPA 1 – Receptor de potencial transitório de anquirina 1

TSB – caldo tryptone soya

UFC – unidades formadoras de colônia

VAN – Vancomicina

VEGF – Fator de crescimento do endotélio vascular

VRE – *Enterococcus faecalis* vancomicina resistente

LISTA DE SÍMBOLOS

% - Percentagem

ºC – Graus Celsius

mg/mL – Miligrama por mililitro

µl – Microlitro

Sumário

1. INTRODUÇÃO

Ferida pode ser definida como o rompimento da estrutura e das funções normais desde as camadas mais superficiais até as mais profundas. O tratamento de feridas é relatado desde a era pré-histórica, com o uso dos mais diversos produtos tópicos: leite, plantas, resina, lama entre outros.

O objetivo destes tratamentos é acelerar o processo cicatricial, a partir de reações celulares e bioquímicas, que é didaticamente separado em três fases: processo inflamatório, proliferação celular e remodelação.

As feridas podem ser classificadas de várias maneiras, de acordo com a etiologia, morfologia, grau de contaminação, fase de evolução cicatricial, características do leito, do exsudato, entre outros. A forma mais corriqueira de avalição de uma ferida consiste em distribuí-las em lesões agudas e crônicas, sendo que a última apresenta uma alteração das etapas do processo de cicatrização e reparação tecidual.

Alterações do processo fisiológico de cicatrização ocorrem devido a estímulos extrínsecos e intrínsecos do paciente. Dentre os fatores extrínsecos destaca-se a técnica a ser empregada e o tipo de cobertura. Dentre os estímulos intrínsecos estão as co-morbidades, como diabetes mellitus, úlceras vasculogênicas, sendo a mais importante a de origem venosa, e a infecção do leito da lesão.

A infecção se tornou um dos maiores problemas na atualidade, devido ao surgimento crescente de isolados bacterianas resistentes a um grande número de classes de antibióticos. Este perfil de resistência pode ser devido, principalmente, a produção de enzimas que degradam o antimicrobiano, alteração do sítio de ação do antibiótico ou expressão de bombas de efluxo.

As propriedades de patogenicidade aliadas a produção de outros fatores de virulência, como produção de toxinas e formação de biofilme permitem ao agente infectante uma maior resistência ao sistema imune, culminando, desta forma, na perpetuação da colonização e demora do processo cicatrização pela inibição da proliferação e migração dos queratinócitos, além da deposição de colágeno pelos fibroblastos, devido a altas taxas de citocinas inflamatórias.

Como objetivo de otimizar o processo cicatricial, vários recursos terapêuticos estão sendo estudados para fazer frente a esta problemática. Dentro desta perspectiva, a pesquisa de compostos extraídos de plantas ganhou grande relevância na última década. Assim, esta

pesquisa visa avaliar as propriedades antimicrobianas e cicatrizantes de um composto oriundo do extrato da *Cinnamomun sp*.

Cinnamomum sp é uma especiaria utilizada desde os tempos bíblicos. Seu extrato tem sido utilizado no combate das mais diversos tipos de doenças no oriente, como por exemplo em enfermidades respiratórias e digestivas. As diferentes partes desta planta possuem os mesmos tipos de compostos só que em proporções variadas, com constituintes majoritários como o cinamaldeído (casca), eugenol (folha) e cânfora (raiz).

Dentre estes compostos, destaca-se a ação antimicrobiana e anti-inflamatória do cinamaldeído. Em relação as bactérias, ressalta-se a sua interferência na produção do fatores de virulência mediados pelo auto indutor 2, molécula sinalizadora presente tanto em bactérias Gram-positivas quanto em Gram-negativas; e na alteração da cinética da formação do anel Z, evento mediado pela molécula *filamentous temperature-sensitive protein Z* (FtsZ), comumente conservada entre as bactérias.

Adicionalmente, o cinamaldeído apresenta um importante efeito anti-inflamatório. Este efeito biológico inicia-se após a ligação com o receptor de potencial transitório tipo anquirina 1 (TRPA1), presente tanto em células neurais quanto em não-neurais. Na década passada, este receptor foi considerado relvante para liberação de neuropeptídios na inflamação neurogênica, após ativação por estímulos térmicos, mecânicos e químicos.

No entanto, há poucos relatos de sua ação sobre queratinócitos, fibroblastos e células epiteliais e muito menos sobre a sua influência no processo cicatricial via receptor TRPA1. Desta forma, parece ser provável que o cinamaldeído possa acelerar o processo cicatricial em feridas infectadas, embora não haja evidência dessa ação em modelos de feridas cutâneas.

2. REVISÃO BIBLIOGRÁFICA

2.1 O Processo Inflamatório e a Cicatrização

A inflamação é uma resposta celular e humoral de magnitude variável com repercussões locais ou sistêmicas, caracterizada por diversas alterações bioquímicas e celulares em resposta a lesão tecidual, antígenos ou agentes infecciosos. A inflamação é caracterizada por rubor, calor, formação de edema, dor e perda de função.

O processo inflamatório consiste de diferentes etapas que podem ser classificadas didaticamente em fase aguda, fase crônica e fase de resolução e reparo. envolve a interação de polimorfonucleares, monócitos/macrófagos, linfócitos, mastócitos, plaquetas, fibroblastos, neurônios e células endoteliais (VOLTARELLI, 1994; VALDERRAMAS, 2006).

Após a ruptura da pele, o organismo tenta reaver a homeostasia anterior ao dano. Para isso, é necessário um processo complexo de interação entre a matriz extracelular, mecanismos celulares e moleculares os quais promovem a liberação de mediadores, para remodelação e reparo da lesão (BALBINO, PEREIRIA, CURI, 2005)..

Os eventos da cicatrização iniciam-se imediatamente após a lesão tecidual e são divididos didaticamente, em três fases: inflamatória, proliferativa e de remodelamento. Durante a inflamação aguda, há a participação ativa de células residentes e inflamatórias. Ao mesmo tempo, ocorre a liberação de diversos componentes celulares (histamina, triptase, tripsina, prostaglandinas, dentre outros) e a ativação de sistemas plasmáticos como o complemento, coagulação e cininas. Essas alterações promovem o aparecimento dos primeiros sinais cardinais da inflamação (calor, rubor, edema seguido de migração celular e dor) (TRENGOVE, BIELEFELDT-OHMANN, STACEY, 2000; HELDIN, ERIKSSON, OSTMAN, 2002).

Nas primeiras 24 horas, a ação conjunta de mediadores e matriz provisória que facilita a migração celular nas fases posteriores do processo cicatricial. Portanto ocorre o acúmulo de células inflamatórias (neutrófilos e macrófagos) no sítio inflamatório e são liberados diversos mediadores inflamatórios, como espécies reativas de oxigênio, óxido nítrico, interleucinas (ILs), quimiocinas, fator de necrose tumoral (TNFα), dentre outros; os quais atuam no local e sistemicamente (GERSZTEN et al., 1999).

Os neutrófilos são as mais numerosas das células no leito da ferida e apresentam como função principal a destruição de microrganismos e o debridamento do tecido desvitalizado, preparando o local para a regeneração tecidual (BALBINO et al., 2005). Para

tanto estas células liberam uma grande variedade de substâncias como espécies reativas de oxigênio (ROS), interleucinas, TNFα. Estas duas últimas moléculas estimulam a síntese de fatores como NFκβ e fator transformador de crescimento α (TGFα) e β (TGFβ). O TGFβ promove a diferenciação e proliferação de células epiteliais, fibroblastos, e vasos sanguíneos; em um processo no qual existe o predomínio de intensa mitose celular (HENG, 2011).

Após a eliminação dos fatores que promovem intensa inflamação, são liberadas citocinas anti-inflamatórias e a formação de mediadores lipídicos envolvidos na resolução da inflamação (resolvinas e protectinas). Estas moléculas aumentam a captação de neutrófilos apoptóticos por macrófagos, bem como participam do sequestro de mediadores inflamatórios (MANTOVANI et al., 2011). Ainda na fase de resolução, ocorre a alteração fenotípica de macrófagos que passam a contribuir para o controle do processo inflamatório, por promoverem a diminuição da síntese de interleucina (IL)-12 e TNFα, e o aumento de IL-10, fator de crescimento do endotélio vascular (VEGF) e TGFβ. Todas estas alterações contribuem,para a formação do tecido de granulação e recuperação do leito vascular (a angiogênese) na área lesionada (ENFIELD, LEIBOVICH, 2011; KAJDANIUK et al., 2011; SANTIBANEZ, QUINTANILLA, BERNABEU, 2011)

Assim, o processo de cicatrização inicia-se durante a fase aguda da inflamação, e pode ser classificado em resolução e reparo. Na resolução, com o término do processo inflamatório, existe uma recuperação da estrutura e das funções normais do tecido lesado. Já no reparo, ocorre a substituição de tecido destruído por fibroblasto, sendo a estrutura tecidual devolvida, mas não a função do tecido lesado (ROY, SEN, 2012). A característica básica do processo de cicatrização, é o desenvolvimento do tecido de granulação, composto por capilares e a reconstituição de matriz extracelular, com deposição de colágeno, fibronectina e outros fatores protéicos. Os principais agentes estimulantes para a síntese destes componentes são as células endoteliais, os fibroblastos e os queratinócitos, influenciados pela síntese de TGF-β, Fator de crescimento de fibroblasto (FGF) e VEGF (MARTIN, 1997; LI, CHEN, KIRSNER, 2007)

Na fase proliferativa, logo após o segundo dia do início da lesão, ocorre a re-epitelização, formação de novos vasos sanguíneos, fibroplasia e aproximação das extremidades do leito da feridas por intermédio dos miofibroblastos. Durante a re-epitelização, há migração de queratinócitos para o leito da ferida, que ao entrarem em contato com o colágeno, passam a liberar metaloproteinases do tipo 1 (MMP-1), degradando-o (LI, CHEN, KIRSNER, 2007). A interação entre receptores de integrina e a matriz provisória permite a migração de células para o leito da ferida. Concomitantemente, novos vasos são

formados à partir do 1º dia da lesão, a fim de facilitar o transporte de oxigênio e nutrientes, necessários para manutenção do metabolismo celular do novo tecido a ser formado (TONNESEN, FENG, CLARK, 2000).

Na fase de remodelamento, que ocorre entre o final de primeira semana e se estende por até 21 dias, ocorre a apoptose dos fibroblastos, miofibroblastos, células endoteliais e macrófagos. Durante este período há intensa deposição e reabsorção do colágeno principalmente através da ação das MMPs, no intuito de remodelar e fortalecer a ferida (LI et al., 2007). Além da degradação de colágeno, as MMPs promovem a clivagem de receptores para fatores de crescimento, dentre outras funções (GILL, PARKS, 2008)

Na ausência da remoção eficiente do agente agressor (patógeno, isquemia, estase vascular), a inflamação perpetua em uma fase crônica caracterizada por densa infiltração de macrófagos e linfócitos; e proliferação de vasos sanguíneos e tecido conjuntivo (KOLLURU, BIR, KEVIL, 2012). A intensa destruição tecidual, decorrente da migração de células inflamatórias e liberação de mediadores inflamatórios no tecido, pode acarretar em necrose e perda de função tecidual (LASKIN et al., 2011).

2.2 Feridas crônicas e colonização microbiana

Existem diferentes tipos de lesões que podem ser classificadas como crônicas, como a úlcera do pé diabético, úlcera por pressão, úlceras traumáticas e úlceras venosas, sendo a primeira e a última responsáveis pela maioria dos casos, liderando os casos de morbidade e mortalidade hospitalar (PHILLIPS, DOVER, 1991; JONES, 2009).

O elo comum entre estes tipos de lesão é a colonização do leito da ferida por microrganismos que circundam a área afetada. Normalmente, a pele e as membranas mucosas são colonizadas por diversos grupos de microrganismos, os quais podem ser divididos em flora residente e transitória. A primeira consiste de microrganismos não-patogênicos ou potencialmente patogênicos e a segunda inclui aqueles relacionados à colonização da pele e mucosas por patógenos provenientes do ambiente hospitalar, devido à estadia prolongada do paciente. Este segundo grupo de microrganismos é mais agressivos devido a habilidade destes em produzirem fatores de virulência e de serem resistentes aos antimicrobianos comercializados.

Dentre os fatores de virulência mais importantes está a produção de biofilme, que também contribui ativamente para a cronificação das feridas. O biofilme consiste em uma comunidade séssil de múltiplas espécies bacterianas embebida numa matrix rica em polímeros de carboidratos que as tornam resistentes a antimicrobianos e à resposta imune. Outros produtos bacterianos que contribuem para a virulência bacteriana são endotoxinas e exotoxinas, capazes de promover lesão tecidual e amplificar a resposta inflamatória, contribuindo para o declínio do processo de reparação tecidual (KANE, 2007; DOWD et al 2008). Adicionalmente, características inerentes aos pacientes como baixa perfusão tecidual, idade, imunossupressão, medicação e doenças concomitantes; também influenciam o processo fisiológico de reparação tecidual (ZHAO et al, 2016)

Um estudo realizado com 168 pacientes demonstrou que o microorganismo mais comum encontrado em ulcerações, é o *Staphylococcus aureus,* equanto que *Pseudomonas aeruginosa* é o patógeno mais frequentemente associado à infecções nas camadas mais profundas da área lesionada (SCHIMIDT *et al*, 2000; HOWELL-JONES, KEH, 2005). De forma geral, 338 tipos distintos de bactérias foram isolados de feridas crônicas, com uma média de 6.9 tipos de bactéria por ferida. As técnicas de identificação molecular mostraram que dentre as 20 bactérias mais presentes em feridas, 9 são anaeróbicas com grande prevalência de *Bacteroides, Peptoniphilus, Finegoldia, Anaerococcus e Clostridium* (RHOADS et al., 2012). Outros estudos realizados em amostras de lesões crônicas

demonstraram que bactérias anaeróbicas representam a principal população nas feridas crônicas, chegando a compor cerca de 49% da microbiota da lesão (BOWLER, DAVIES, 1999; DOWD et al., 2008).

Estima-se que 1% da população adulta, independentemente da idade, desenvolverá úlceras venosas (KURZ et al., 1999). O custo efetivo para o tratamento das lesões crônicas varia, segundo o tipo de abordagem terapêutica empregada. O custo global com assistência médico-hospitalar fica em torno de U$ 630,00 a U$ 4900,00, segundo estudo realizado por HANKIN e colaboradores em 2012. Grande parte deste custo elevado é proporcionado pelo longo tempo de tratamento dos pacientes, uma vez que na maioria dos casos a duração do mesmo excede 6 meses.

Assim, a cada ano, as úlceras crônicas custam ao sistema de saúde dos Estados Unidos, cerca de 1,5 a 3,0 bilhões de dólares (ONGENAE, PHILLIPS, 1993). Embora esta morbidade possa ocorrer em qualquer idade, os idosos apresentam maior risco de desenvolver esta enfermidade. Com o aumento da expectativa de vida da sociedade atual, estes custos com o serviço de saúde só tendem a aumentar.

No Brasil, um estudo realizado em Sorocaba, avaliou o período de internação e o custo efetivo do tratamento de pacientes com esta patologia. Relatou-se que pacientes com lesão tecidual apresentam tempo de internação variável (4 a 61 dias), podendo o custo médio com os procedimentos hospitalares variar entre R$ 1.000,00 à R$ 7.000,00 (MILMAN et al., 2001).

As opções de tratamento para feridas cutâneas crônicas são amplas, pois, como visto anteriormente, estas lesões apresentam etiologia e características diversas, o que torna o tratamento dispendioso e com grande diversidade de propostas terapêuticas. Estas incluem administração medicamentosa de antimicrobianos e anti-inflamatórios, aplicação de curativos com diversos tipos de cobertura e, até mesmo, cirurgia (FRYKBERG, R. G.; BANKS, 2015). De forma geral, a meta de qualquer um destes tratamentos é a cura da ferida e a prevenção da sua recorrência.

2.3 O Potencial terapêutico do Cinamaldeído e os receptores TRPA1

As plantas medicinais e seus constituintes vêm acompanhando há séculos a evolução terapêutica para o tratamento sistêmico ou tópico de diversas doenças, incluindo enfermidades infecciosas e lesões cutâneas. Atualmente estima-se que aproximadamente 80% da população

mundial faz uso terapêutico de plantas medicinais, setor este que movimenta cerca de US$ 22 bilhões por ano (PINTO et al., 2002).

O gênero *Cinnamomum* constitui-se de árvores aromáticas pertencentes à família Lauraceae, sendo utilizado para diversas finalidades terapêuticas como para analgesia, febre e dores musculares (SHEN et al., 2012). Os principais constituintes da casca da canela são o eugenol e o cinemaldeido, sendo este último, o componente majoritário. Vários estudos indicam que o cinamaldeído apresenta grande poder antibacteriano, causando a inibição do crescimento de *Clostridium perfringens, Bacteroides fragilis, Escherichia coli, Staphylococcus aureus* e outros microrganismos (KIM, 2004; SENHAJI, FAID, KALALOU, 2007). Dependendo da concentração utilizada, este composto é capaz de atenuar a síntese de fatores de virulência, como a liberação de toxinas e atividade hemolítica de microrganismos via inibição de *quorum sensing* (QS) (SMITH-PALMER, STEWARTT, FYFE, 2002).

O QS compreende um conjunto de moléculas sinalizadoras que são liberadas durante a fase de crescimento microbiano, e que permitem a expressão gênica coordenada de fatores essenciais para a sobrevivência do microrganismo (SOUSA et al., 2007). Existem três tipos prinicpais de QS, classifcadas de acordo com a via ativada: 1) a via da acyl-homoserine-lactone (AHL) em bactérias Gram-negativas, 2) a via de liberação de moléculas sinalizadoras por bactérias Gram-positivas e 3) a via do autoindutor 2, molécula que influencia a expressão gênica tanto de bactérias Gram-positivas quanto Gram-negativas (WILLIAMS, 2007). Adicionalmente, estudos recentes demonstraram a ação imunomodulador do cinamaldeído. KOH et al (2011) demonstraram que o cinamaldeído é capaz de alterar a resposta imune *in vitro*, por promover a diferenciação de linfócitos duplo positivos em CD4+ ou CD8+ positivos. Outro papel importante seria a inibição da ativação do NFκβ, o que culmina na diminuição da atividade da óxido nítrico sintase induzida (iNOS) e da ciclooxigenase do tipo 2 (COX-2). Ainda, o cinamaldeído é capaz de promover a liberação de IL-1β, TNFα, e EROs em monócitos e macrófagos estimulados com lipopolissacarídeo (CHAO et al., 2008) *in vitro*.

Estudos *in vivo* monstraram que o cinamaldeído é capaz de inibir a produção de NO, TNFα e prostaglandina E_2 em modelo de edema de pata em camundongos (LIAO et al., 2012). Segundo Li et al (2011), o tratamento repetido com este composto diminuiu a concentração sérica de IL-2, IL-4 e IL-10 em camundogos BALB/c imunizados com ovoalbumina. Mais recentemente, foi demonstrado que o cinamaldeído tem ação no choque séptico, uma vez que o tratamento com este composto reduziu a resposta inflamatória induzida por LPS (MENDES et al., 2016). O Cinamaldeído apresenta outras funções de

relevância para a cicatrização, como a capacidade de promover a formação de novos vasos sanguíneos e o aumento do fluxo sanguíneo (CHOI et al., 2009, AUBDOOL et al., 2016).

Sugere-se que a ação do cinamaldeído deve-se à ativação de receptores expressos em células neuronais e não neuronais, denominados como receptores de potencial transitório anquirina 1 (TRPA1; do inglês *Transient Receptor Potential Ankyrin 1*), o qual faz parte de uma família de receptores originalmente identificados como expressos em fibras sensoriais do tipo C e Aδ e, quando estimulados, promovem a liberação dos neuropeptídeos substância P (SP) e do peptídeo relacionado ao gene da calcitonina (CGRP; do inglês *Calcitonin Gene-Related Peptide*) (JORDT et al., 2004; YUE et al 2015).

Como característica comum, todos os receptores TRP fazem com que as membranas fiquem permeáveis a cátions, particularmente o Ca^{+2} e podem ser ativados por diversos agonistas endógenos: alterações no pH; estímulos mecânicos; hormônios; EROs; produtos oxidados e outros mediadores inflamatórios, e exógenos: como produtos derivados de plantas, fumaça de cigarro, poluição e substâncias químicas (HUANG, ZHANG, MCNAUGHTON, 2006; FERNANDES, FERNANDES, KEEBLE, 2012; YUE et al 2015).

Os ativadores endógenos do TRPA1 são frequentemente compostos eletrofílicos como EROs e mediadores lipídicos como a 15-deoxy-delta (12,14)-prostaglandina J_2 (15d-PGJ_2) (CRUZ-ORENGO et al., 2008); produzidos durante a infecção. O TRPA1 apresenta um importante papel no estabelecimento de processos dolorosos e inflamatórios como a artrite reumatóide, colite ulcerativa, doença de Chron, choque séptico, dentre outros (FERNANDES et al., 2012; BAUTISTA, PELLEGRINO, TSUNOZAKI 2013). Apesar dos diferentes estudos indicando o TRPA1 como alvo farmacológico do cinamaldeído em mamíferos, sugere-se que este composto é capaz de modular a resposta inflamatória, também por mecanismos independentes da ativação do TRPA1 (MENDES et al., 2016).

2.4 Modelos experimentais de indução de cicatrização em camundongos

Existem vários modelos de cicatrização que são pertinentes a busca de novos compostos com propriedades terapêuticas, bem como no estudo de vias envolvidos neste processo (SALCIDO, POPESCU, AHN, 2007; WILHELM, WILHELM, BIELFELDT 2016). Os modelos de experimentação em animais permitem o estudo de vários aspectos da cicatrização. Dentre estes, o modelo de cicatrização de lesão aguda é o mais simples e mais utilizado, podendo ser induzido por incisão, excisão, queimaduras ou por pressão. Estes modelos, empregados em roedores, permitem a avaliação, de forma relativamente simples, do

processo de cicatrização, além de oferecerem, principalmente em camundongos, a possibilidade de manipulações gênicas, possibilitando assim, a utilização de animais com deleção gênica específica (GONZÁLEZ-SUÁREZ et al 2001; MACK, MAYTIN, 2010). Desta forma, a utilização de modelos experimentais em roedores permite a avaliação de vias envolvidas na patofisiologia da cicatrização e de potenciais alvos terapêuticos. Outra vantagem é que na cicatrização permite a observação completa do processo num período inferior ao observado em humanos, uma vez que estes animais apresentam cicatrização acelerada (DUNN et al 2013). É importante mencionar que, além destas características, roedores não apresentam hipertrofia ou formação de quelóide (Wong et al 2011).

Em relação aos modelos de cicatrização, destaca-se os induzidos pressão, queimaduras e excisão. Há vários modelos de indução de lesão por úlcera de pressão, como o de Hashimoto, Kurose e Kawamata (2008) no qual é realizada uma incisão cirúrgica e implantada uma placa de metal ou um íman de neodímio na região peritoneal, no método por Tsuj et al (2005) é necessário a compressão por 8 h, pela inserção de uma câmara de pressão numa dobra cutânea; e no método de Stadler et al (2004), um modelo não invasivo, que envolve a superposição de dois ímans sobre a pele com duração de 12 horas.

No modelo de queimadura, geralmente utiliza-se camundongos jovens. A área a ser lesionada é imergida em água (60 a 100ºC) por 8 a 12 segundos, para indução da lesão. Os animais são observados quanto aos sinais de dor e desconforto e são tratados com analgésicos. A desvantagem desta abordagem deve-se à diferença do metabolismo entre camundongos e humanos, o que acarreta déficit na compreensão da hipermetabolização pós-queimadura (VARGA et al, 2010). A grande desvantagem deste modelo é o desconforto produzido no animal e nos experimentos com infecção, a diminuição da sobrevida dos animais (KENDRA et al 1999)

O modelo mais comumente empregado para o estudo de fatores que retardam a cicatrização, é o modelo de lesão excisional. Os benefícios deste modelo são técnica e material relativamente simples, reprodutibilidade e praticidade, obtenção adequada de quantidade de amostra e menor agressividade (WOUNG et al, 2010).

Apesar de úteis, modelos de cicatrização em roedores diferem do que ocorre em humanos. De fato, o processo de cicatrização em roedores ocorre, principalmente, por contração, enquanto que em humanos, este pode ocorre por repitelização e formação de tecido de granulação (SEATON, HOCKING, GIBRAN 2015). Adicionalmente, os modelos de indução de úlcera de pressão tem seu manejo mais difícil, pois necessitam da medida precisa

da pressão exercida sobre a pele além de interromper a rede vascular onde a placa de metal é implantada (HASHIMOTO, KUROSE E KAWAMATA 2008).

3. OBJETIVOS

3.1 Objetivo geral:

Avaliar o efeito antimicrobiano e cicatrizante do cinamaldeído em modelos *in vitro* e *in vivo*.

3.2 Objetivos específicos:

- Avaliar a ação antimicrobiana e anti-virulência do cinamaldeído contra bactérias de relevância para feridas cutâneas *in vitro* e *in vivo*;
- Verificar o efeito do cinamaldeído na cicatrização de feridas infectadas por *P. aeruginosa*
- Analisar a associação entre efeitos cicatrizantes do cinamaldeído e a ativação do receptor TRPA1

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5. LISTA DE PUBLICAÇÕES

- **Cinnamaldehyde inhibits *Staphylococcus aureus* virulence factors and protects against infection in a *Galleria mellonella* model; Publicado na *Frontiers in Microbiology* em 2016**

- **Concentrações sub-inibitórias de cinamaldeído atenuam a virulência de *Pseudomonas aeruginosa in vitro* e promovem a cicatrização de feridas cutâneas em camundongos; A ser submetido à *Britsh Journal of Dermatology***

5.1 **ARTIGO I**

Cinnamaldehyde inhibits *Staphylococcus aureus* virulence factors and protects against infection in a *Galleria mellonella* model

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Cinnamaldehyde Inhibits *Staphylococcus aureus* Virulence Factors and Protects against Infection in a *Galleria mellonella* Model

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Bacterial resistance to the available marketed drugs has prompted the search of novel therapies; especially in regards of anti-virulence strategies that aim to make bacteria less pathogenic and/or decrease their probability to become resistant to therapy. Cinnamaldehyde is widely known for its antibacterial properties through mechanisms that include the interaction of this compound with bacterial cell walls. However, only a handful of studies have addressed its effects on bacterial virulence, especially when tested at sub-inhibitory concentrations. Herein, we show for the first time that cinnamaldehyde is bactericidal against *Staphylococcus aureus* and *Enterococcus faecalis* multidrug resistant strains and does not promote bacterial tolerance. Cinnamaldehyde actions were stronger on *S. aureus* as it was able to inhibit its hemolytic activity on human erythrocytes and reduce its adherence to latex. Furthermore, cinnamaldehyde enhanced the serum-dependent lysis of *S. aureus*. *In vivo* testing of cinnamaldehyde in *Galleria mellonella* larvae infected with *S. aureus*, showed this compound improves larvae survival whilst diminishing bacterial load in their hemolymph. We suggest that cinnamaldehyde may represent an alternative therapy to control *S. aureus*-induced bacterial infections as it presents the ability to reduce bacterial virulence/survival without promoting an adaptive phenotype.

Keywords: essential oil, cinnamaldehyde, infection, bacterial virulence, *S. aureus*

INTRODUCTION

Bacterial pathogens have evolved several mechanisms to acquire resistance to drug and hereby survive antibiotic treatment in eukaryotic hosts, including mutations, plasmid acquisition, amongst others (Blair et al., 2015; Lin et al., 2015). In fact, multidrug resistant strains have been observed with increasing frequency and their spreading has been recognized as one of the most

alarming issues for the global health system, resulting in high levels of morbidity and mortality (Wilson et al., 2016). Infections caused by staphylococcal and enterococcal are reported as a major problem in hospitalized patients especially those using indwelling medical devices such as urinary catheters, feeding tubes, and peripherally inserted central catheters (Padmavathy et al., 2015; Tong et al., 2015). In order to cause infection, these pathogens produce a range of virulence factors which in turn, promote host tissue damage and contribute to bacterial evasion from the host's immune response and their subsequent survival in the bloodstream (Bhatty et al., 2015; Thammavongsa et al., 2015; Theilacker et al., 2015). This scenario coupled with a diminished antibiotic pipeline has lead to serious social and economic complications and it has prompted the search of novel compounds and therapies to combat bacterial infections (Barriere, 2015).

The antimicrobial properties of plant-derived products have been tested against several pathogens. Cinnamaldehyde is the predominant active compound found in the cinnamon oil from the stem bark of *Cinnamomum cassia*. It is well-known for its wide spectrum antimicrobial activity at concentrations higher than 500 mg/ml (Chen et al., 2015; Shen et al., 2015; Utchariyakiat et al., 2016). The antimicrobial actions of cinnamaldehyde are related to inhibition of cell division through FtsZ (filamentation temperature sensitive protein Z; Domadia et al., 2007), reduction of energy generation and glucose uptake or expenditure (Gill and Holley, 2004) and effects on bacterial cell membrane permeability and integrity (Gill and Holley, 2004; Shen et al., 2015). Recently, cinnamaldehyde was shown to protect against the systemic inflammatory response syndrome (SIRS) induced by the Gram-negative bacteria cell wall component lipopolysaccharide (LPS) in mice (Mendes et al., 2016). A similar effect was observed when cinnamaldehyde was administered to *Galleria mellonella* infected with *Listeria monocytogenes* (Upadhyay and Venkitanarayanan, 2016).

Previous reports described the antibacterial effects of cinnamaldehyde against *Staphylococcus aureus* (Shen et al., 2015) and *Enterococcus faecalis* (Chang et al., 2001). Although, the ability of this compound to interact with the cell walls of these bacteria is well-studied, little is known of its effects on bacterial virulence, especially when tested at sub-inhibitory concentrations. Anti-virulence strategies have gained attention in the recent years as a novel therapeutic paradigm (Rasko and Sperandio, 2010; Kong et al., 2016). These approaches aim to inhibit the synthesis of bacterial virulence factors that are essential for bacterial survival within the host; thus, making the bacteria less pathogenic and/or decreasing the probability of resistance development rather than targeting bacterial viability (Heras et al., 2015).

Here, we investigated the antimicrobial and anti-virulence properties of cinnamaldehyde against *S. aureus* and *E. faecalis*, including multidrug resistant strains. Additionally, we evaluated the ability of cinnamaldehyde to protect against *S. aureus*-induced infection in *G. mellonella* larvae, an alternative model of bacterial infection.

MATERIALS AND METHODS

Bacterial Strains

All tested bacteria were kindly provided by the bacterial collection sector of the Universidade CEUMA and included: six strains of *S. aureus* (standard strains ATCC 25923 and ATCC 6538; clinical isolates SA01, SA02, SA03, SA04); four strains of *E. faecalis* (standard strain ATCC 19443; clinical isolates EF01, EF02, EF03). Susceptibility to antimicrobials was determined in an automated VITEK® 2 system (BioMérieux Clinical Diagnostics, USA) and data interpretation was performed as recommended by the Clinical Laboratory Standards Institute [CLSI] (2015). The multiple antibiotic resistance (MAR) index was calculated using the formula MAR = x/y, where "x" was the number of antibiotics to which the isolate demonstrated resistance; and "y" was the total number of antibiotics tested. The antibiotic susceptibility profile of each strain is shown at **Table 1**.

TABLE 1 | Antibiotic susceptibility profiles of *Enterococcus faecalis* and *Staphylococcus aureus* strains.

Strain	Antibiotic							MAR
	PEN	VAN	OXA	GEN	CLI	CIP	SUT	
E. faecalis ATCC 19443	S	S	–	S	–	S	–	0
E. faecalis 1	S	S	–	S	–	S	–	0
E. faecalis 2	R	S	–	S	–	S	–	0.25
E. faecalis 3	R	S	–	R	–	R	–	0.75
S. aureus ATCC 25923	S	S	S	S	S	S	S	0
S. aureus ATCC 6538	S	S	S	S	S	S	S	0
S. aureus 1	S	S	S	S	S	S	S	0
S. aureus 2	R	S	R	S	R	S	R	0.50
S. aureus 3	R	S	R	S	R	S	R	0.50
S. aureus 4	R	S	R	S	R	S	S	0.43

Experiments were performed three times in duplicate.
PEN, penicillin; VAN, vancomycin; OXA, oxacillin; GEN, gentamicin; CLI, clindamycin, CIP, ciprofloxacin; SUT, sulfamethoxazole/trimethoprim. Multiple antibiotic resistance (MAR) index.

Antimicrobial Assays

The antimicrobial activity of *trans*-cinnamaldehyde (Sigma-Aldrich®; 99% purity) was determined by the microdilution method (Clinical Laboratory Standards Institute [CLSI], 2015). Briefly, each strain was grown on Müeller-Hinton Agar (MHA) plates at 37°C for 24 h, and suspended in saline solution (~1.5 × 10^8 CFU/ml). For the determination of minimum inhibitory concentrations (MICs), 10 µl of bacterial suspension (approximately 1.5 × 10^8 CFU/ml) were incubated in Müeller-Hinton (MH) broth containing cinnamaldehyde at different concentrations (62.5–2,000 µg/ml). Serial dilutions of ciprofloxacin (0.06–256 µg/ml) were used as positive controls, while sterile dimethyl sulfoxide (DMSO; 2% in phosphate-buffered saline; PBS) was used as negative control. Samples were then, incubated for 24 h at 37°C. The MIC was defined as the lowest concentration at which no bacterial growth was observed. For determining the minimum bactericidal concentrations (MBCs), just after the MIC experiments, the cultures were seeded on MHA and incubated for 24 h at 37°C. The MBC corresponded to the lowest concentration of the compound to which no viable bacteria was observed.

Analysis of Bacterial Tolerance to Drug

In order to investigate whether cinnamaldehyde is able to induce bacterial tolerance to drug, we performed serial passage experiments, using the standard strains of *S. aureus* (ATCC 25923) and *E. faecalis* (ATCC 19443). For this, bacterial suspensions (1 ml, ~1.5 × 10^8 CFU/ml) were added to six-well tissue culture plates containing MH broth and sub-inhibitory concentrations (MIC/2) of cinnamaldehyde or ciprofloxacin (positive control). After 24 h at 37°C, the culture growing at one dilution below the MIC was used to inoculate the subsequent passage, and this process was repeated for a total of 10 passages. The compound concentration range of each new passage was based on the MIC calculated for the previous passage. Vehicle-treated bacteria (2% DMSO in PBS) were used as negative controls.

Anti-biofilm Activity

Biofilm formation was quantified according to the method previously described by Stepanović et al. (2004). For this, 10 µl of bacterial suspension (prepared as described above) were added per well in to a 96-well cell culture plate containing sub-inhibitory concentrations of cinnamaldehyde (MIC/2 and MIC/4) and 200 µl of Luria-Bertani (LB) broth. Vehicle (2% DMSO in PBS)-treated bacteria and broth without bacteria were used as positive and negative controls, respectively. Samples were incubated at 37°C and after 24 h, and then, the wells were washed three times with PBS. Biofilm was stained with 5% crystal violet for 10 min at room temperature, and immediately solubilised with methanol (200 µl, 100%). The absorbance was read at 570 nm. Relative biofilm mass results are expressed as percentage (%) in relation to control (vehicle-treated wells). In a different set of experiments, the effects of cinnamaldehyde on bacterial viability were assessed and calculated by addition of PrestoBlue® reagent (1:10; Life Technologies), according to the manufacturer's instructions. Cell viability is expressed as absorbance in nm.

Studies with Human Samples

Blood samples were collected from three healthy volunteers with no recent history of taking either antibiotic or anti-inflammatory drugs, and/or infectious or inflammatory diseases in the last 3 weeks prior to sample collection; after a written informed consent was obtained. The study was reviewed and approved by the Human Research Ethics Committee of the Universidade CEUMA (CEP-UNICEUMA) and was performed in accordance with the Declaration of Helsinki 1975, as revised in 2008.

Hemolysis Assay

Samples (2.5 ml of blood) were collected in heparinised tubes and the erythrocytes were immediately isolated by centrifugation at 1,500 rpm for 10 min. After removal of plasma, the erythrocytes were washed three times with PBS (pH 7.4) and then suspended in BHI broth. In parallel, bacterial suspensions were obtained as described for MIC determination (~1.5 × 10^8 CFU/ml). Aliquots of 10 µl of each bacterial suspension were added into 200 µl of BHI broth supplemented with human erythrocytes (2%) and incubated with sub-inhibitory concentrations of cinnamaldehyde (MIC/2 and MIC/4) or vehicle (2% DMSO in PBS). After 24 h of incubation at 37°C, the tubes were centrifuged and the supernatant (100 µl/per sample/well) was transferred to a 96-well plate. Absorbance was read at 550 nm and taken as an indicative of hemolytic activity. Results are expressed as percentage (%) in relation to the hemolytic activity of each bacterial strain incubated with vehicle (2% DMSO in PBS; vehicle-controls).

Analysis of Bacterial Survival following Incubation with Human Serum

This assay was performed according to the method previously described by Ismail et al. (1988), modified. Blood samples (2.5 ml) were collected in tubes containing no anticoagulant. Serum was separated by centrifugation at 1,500 rpm for 10 min. Aliquots (10 µl/well) of the bacterial suspensions (~1.5 × 10^8 CFU/ml) were mixed with 140 µl/well of BHI broth containing sub-inhibitory concentrations of cinnamaldehyde (MIC/2 and MIC/4) or vehicle (2% DMSO in PBS) and 60 µl/well of serum. After incubation for 24 h at 37°C, the absorbance was read at 600 nm and taken as bacterial growth index. Results are expressed as percentage (%) in relation to the bacterial growth registered for each bacterial strain incubated with vehicle (2% DMSO in PBS; vehicle-controls).

Bacterial Adherence to Latex

As previously described (Chandra et al., 2008), siliconized latex catheter segments (4 mm) were placed into tubes containing 4.5 ml of LB medium with and without sub-inhibitory concentrations of cinnamaldehyde or vehicle (2% DMSO in PBS). Then, 225 µl of bacterial suspension (~1.5 × 10^8 CFU/ml) were added to each tube. Tubes were incubated at 37°C for 3 h and then, the latex segments were washed three times with PBS and plated on LB Agar. Following incubation for 24 h, at 37°C, plates

were analyzed for CFU counting. The results are expressed as CFU/ml.

S. aureus-Induced Infection in *G. mellonella*

The *in vivo* antimicrobial actions of cinnamaldehyde were evaluated in an *in vivo* model of infection induced by *S. aureus* in *G. mellonella* larvae. Briefly, *G. mellonella* larvae (~200 mg) were randomly distributed in two experimental groups (n = 10/group), and were then infected by injection of 10 µl of bacterial suspension (*S. aureus* ATCC 25923; 1.0×10^5 CFU/ml in PBS) in to the last left proleg. The larvae were incubated at 37°C. After 2 h, the larvae received either cinnamaldehyde at different doses (2.5–5.0 µg /100 mg of larvae) or vehicle (PBS, 5 µl/100 mg), and were incubated at 37°C. Mortality rate was observed over 4 days post-infection.

In order to assess the bacterial load in the hemolymph, in a separate set of experiments, the larvae were infected with *S. aureus* as described above and then received either cinnamaldehyde (5.0 µg/100 g of larvae; n = 5/day) or vehicle (PBS; n = 5/day). Larvae were incubated at 37°C for up to 4 days. Five larvae of each group were culled per day and analyzed for bacterial load. Briefly, at each time point, the larvae were cut through in a cephalocaudal direction with a scalpel blade and squeezed to remove the hemolymph. Serial dilutions (10x) of the hemolymph of each larvae were made in PBS and 4 µl of each dilution were incubated in MHA and cultured for 24 h at 37°C. After this period, the plates were analyzed for CFU counting. The results are expressed as CFU/ml.

Statistical Analysis

Statistical analyses were performed using the software GraphPad Prism version 5.0[1]. Data from were analyzed by two-way analysis of variance (ANOVA) and Tukey test. A p-value of <0.05 was considered as statistically significant. Differences in *G. mellonella* larvae survival were determined using the Kaplan–Meier method to calculate survival fractions and log-rank test was used to compare survival curves.

RESULTS

Cinnamaldehyde Inhibits the Growth of *S. aureus* and *E. faecalis* without Inducing an Adaptive Phenotype

We initially analyzed the antimicrobial effects of cinnamaldehyde against clinical isolates and ATCC standard strains of *S. aureus* and *E. faecalis*. The strains showed different susceptibility profiles to clinically available antibiotics (**Table 1**). Amongst the three tested *E. faecalis* clinical isolates, two were resistant to at least one antibiotic: *E. faecalis* strain 2 (EF02) was resistant to penicillin (MAR index: 0.25) and *E. faecalis* strain 3 (EF03) was resistant to penicillin, gentamicin and ciprofloxacin (MAR index: 0.75). Of the four tested *S. aureus* strains, three were resistant to different antibiotics: *S. aureus* strains 2 (SA02) and 3 (SA03) were resistant to penicillin-oxacillin-clindamycin-sulfamethoxazole/trimethoprim (MAR index: 0.57) and *S. aureus* strain 4 (SA04) was resistant to penicillin-oxacillin-clindamycin (MAR index: 0.43).

Cinnamaldehyde was active against all strains of *E. faecalis* and *S. aureus*, including those with a multidrug resistance phenotype (**Table 2**). MIC values were of 0.25 mg/ml for all tested strains, except for the *S. aureus* standard strain ATCC 25923 (MIC value of 0.5 mg/ml). MBC values were of 1.0 mg/ml to all strains, 2–4-fold higher than each respective MIC, indicating a bactericidal action for cinnamaldehyde (**Table 2**). It is important to highlight that at the used concentration, the vehicle (2% DMSO in PBS) did not affect bacterial growth.

Additionally, when incubated *in vitro* with cinnamaldehyde, neither *S. aureus* (ATCC 25923) nor *E. faecalis* (ATCC 19443) developed adaptive phenotypes even after 10 sequential passages. In contrast, both strains became tolerant to the clinically used antibiotic ciprofloxacin as MIC values increased from 0.0625 to 0.5 µg/ml for *S. aureus*, and from 0.125 to 0.5 µg/ml for *E. faecalis*.

Cinnamaldehyde Sub-inhibitory Concentrations Do Not Affect Biofilm Formation by *E. faecalis*

We attempted to analyze the effects of sub-inhibitory concentrations of cinnamaldehyde (MIC/4 or MIC/2) on the ability of *E. faecalis* and *S. aureus* to form biofilm. As depicted on **Figures 1A** and **2A**, cinnamaldehyde did not diminish biofilm formation by these bacteria at any of the tested concentrations. However, cinnamaldehyde treatment increased biofilm mass for some strains of *S. aureus* (*S. aureus* ATCC 6538, SA01 and SA03). In order to assess whether cinnamaldehyde-induced increase in biofilm formation is due to accumulation of dead cells, we evaluated the viability of the *S. aureus* (ATCC 6538) cells composing the biofilm. We found that cinnamaldehyde (MIC/2) decreases *S. aureus* viability (32.1 ± 3.9%; **Figure 1A**, inset box), indicating reduction in the number of viable cells.

TABLE 2 | Antimicrobial activity of cinnamaldehyde against *Staphylococcus aureus* and *Enterococcus faecalis*.

Strain	MIC[1]	MBC[2]
E. faecalis ATCC 19443	0.25	1
E. faecalis 1	0.25	1
E. faecalis 2	0.25	1
E. faecalis 3	0.25	1
S. aureus ATCC 25923	0.5	1
S. aureus ATCC 6538	0.25	1
S. aureus 1	0.25	1
S. aureus 2	0.25	1
S. aureus 3	0.25	1
S. aureus 4	0.25	1

Experiments were performed three times in duplicate.
[1]Minimum Inhibitory Concentration (MIC) and [2]Minimum Bactericidal Concentration (MBC) are expressed in mg/ml.

[1] www.graphpad.com

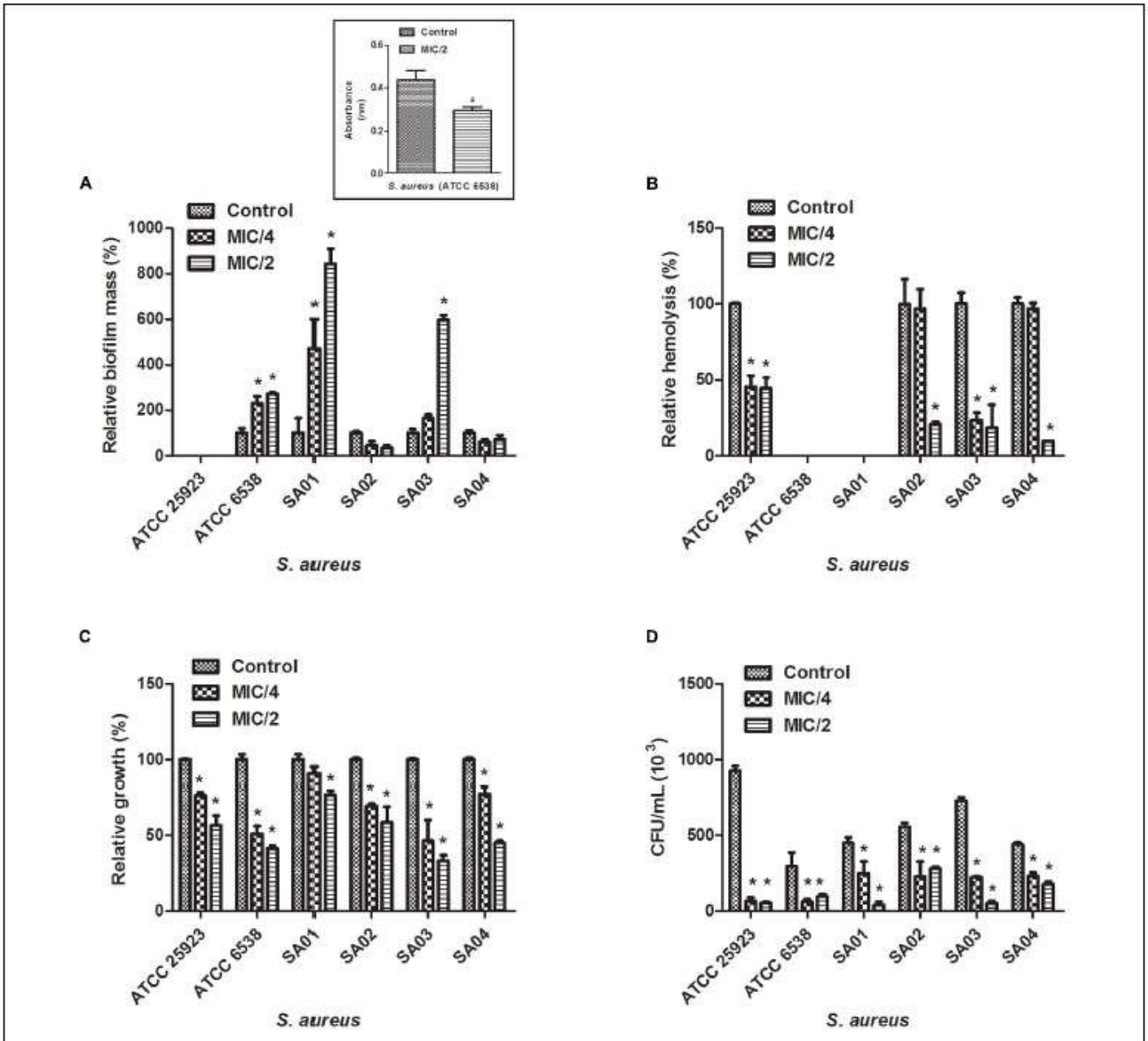

FIGURE 1 | Effect of cinnamaldehyde (MIC/2 and MIC/4) on virulence factors of *Staphylococcus aureus* strains. (A) Biofilm mass production; **(B)** Hemolytic activity; **(C)** Serum resistance; **(D)** Adhesion to latex (catheter). Inset box indicates bacterial viability. *$p < 0.05$, compared with vehicle-treated controls. Experiments were performed three times in duplicate. Each bar represents mean ± SD.

Cinnamaldehyde Sub-inhibitory Concentrations Inhibit the Hemolytic Activity of *S. aureus* but not *E. faecalis*

Three clinical isolates of *S. aureus* (SA02, SA03, SA04) and the standard *S. aureus* strain ATCC25923 were hemolytic. Hemolysis was reduced by cinnamaldehyde when tested at MIC/2 ($p < 0.05$) (**Figure 1B**). Percentage of inhibitions were of 99.9, 81.4, 90.3, and 55.7%, for SA02, SA03, SA04, and ATCC25923; respectively. The strains ATCC25923 and SA03 were also significantly inhibited ($p < 0.05$) by cinnamaldehyde at MIC/4 (54.4 and 76.7%, respectively). On the other hand, although all strains of *E. faecalis* caused hemolysis, only the strain EF02 was inhibited by cinnamaldehyde at the MIC/4 (56.5%; **Figure 2B**).

Cinnamaldehyde Sub-inhibitory Concentrations Decrease *S. aureus* Survival in the Presence of Human Serum

We next determined whether cinnamaldehyde is able to enhance the lysis of *S. aureus* and *E. faecalis* in the presence of human serum. Our results show that when treated with cinnamaldehyde at MIC/2 and MIC/4, *S. aureus* strains were less able to survive

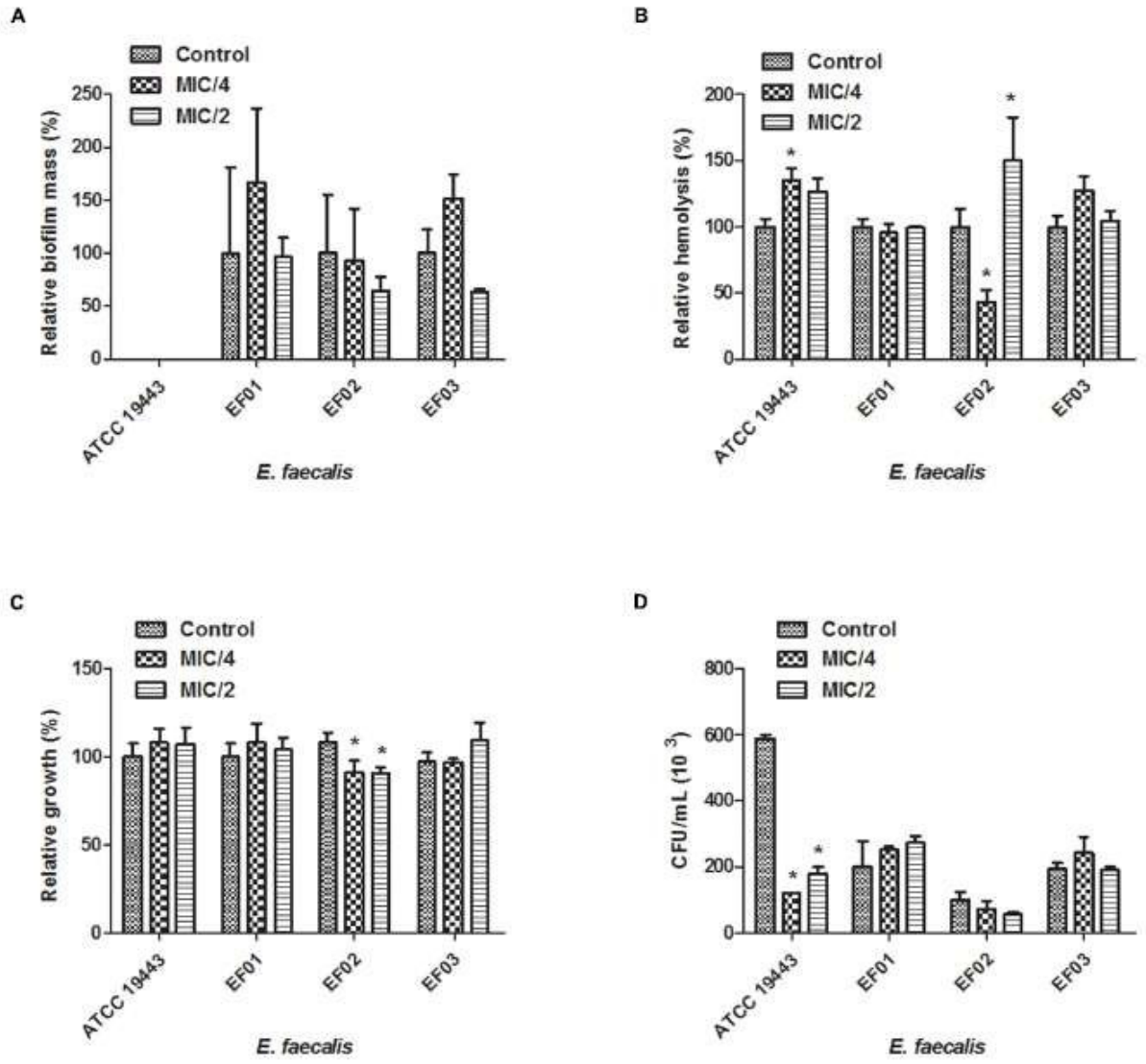

FIGURE 2 | Effect of cinnamaldehyde (MIC/2 and MIC/4) on virulence factors of *Enterococcus faecalis* strains. (A) Biofilm mass production; **(B)** Hemolytic activity; **(C)** Serum resistance; **(D)** Adhesion to latex (catheter). *$p < 0.05$, compared with vehicle-treated controls. Experiments were performed three times in duplicate. Each bar represents mean ± SD.

when incubated with freshly isolated human serum ($p < 0.05$), except SA01 at MIC/4. Inhibitions ranged from 23.5% (SA01) to 66.9% (SA03) for cinnamaldehyde at MIC/2, and from 22.9% (SA01) to 53.5% (SA03) for cinnamaldehyde at MIC/4 (**Figure 1C**). Amongst the *E. faecalis* strains, only EF02 showed a slight reduction on its serum resistance when incubated with cinnamaldehyde (9.1 and 9.4% at MIC/4 and MIC/2, respectively) (**Figure 2C**).

Cinnamaldehyde Sub-inhibitory Concentrations Decrease the Ability of *S. aureus* to Adhere to Latex

We also evaluated whether the sub-inhibitory concentrations of cinnamaldehyde were able to affect bacterial adhesion to latex, using a catheter model. As expected, all tested *S. aureus* and *E. faecalis* strains were able to adhere to latex. The sub-inhibitory concentrations of cinnamaldehyde were able to reduce the adherence to latex by all tested *S. aureus* strains (**Figure 1D**). When tested at MIC/2, cinnamaldehyde maximum inhibitory effects were observed for *S. aureus* ATCC 25923 (94.2%), and the clinical isolates SA03 (93.1%) and SA01 (91.3%). Also importantly, the same concentration of cinnamaldehyde diminished latex adhesion by *S. aureus* ATCC 6538 (67.4%), SA04 (59.6%), and SA02 (48.7%). The adhesion of *S. aureus* ATCC 25923 was also the most reduced by cinnamaldehyde at MIC/4 (93.0%), followed by SA01 (79.6%), SA03 (69.0%), SA02 (58.6%), SA04 (46.7%), and SA01 (44. 7%). On the other hand, this compound only affected the adherence to latex of *E. faecalis* ATCC 19443 with reductions of 79.7 and 69.8% by cinnamaldehyde at MIC/2 and MIC/4, respectively (**Figure 2D**).

Cinnamaldehyde Increases *G. mellonella* Larvae Survival and Reduces Bacterial Load in the Hemolymph

As cinnamaldehyde exhibited stronger actions on *S. aureus*, we performed an *in vivo* infection assay using *G. mellonella* larvae. Cinnamaldehyde or PBS (vehicle) did not induce any toxicity on larvae. Cinnamaldehyde (2.5–5.0 μg/100 mg of larvae) effects were compared to those of vehicle-treated larvae infected with *S. aureus* ATCC 25923. At 2 days post-infection, 80% of the vehicle-treated larvae had died, with no survivals on the third day.

In contrast, cinnamaldehyde enhanced *G. mellonella* larvae survival, as ≥50% remained alive on day 4 ($p < 0.05$; **Figure 3A**). This effect was more pronounced for the highest dose of the compound (5.0 μg/100 mg of larvae). By analyzing the bacterial load, it was observed that cinnamaldehyde significantly reduces the number of *S. aureus* in *G. mellonella* hemolymph samples in comparison with vehicle-treated larvae, as indicated by CFU counting (4-log reduction in bacterial survival). This effect was noted from 48 h post-infection and remained significant throughout the rest of the assay (**Figure 3B**).

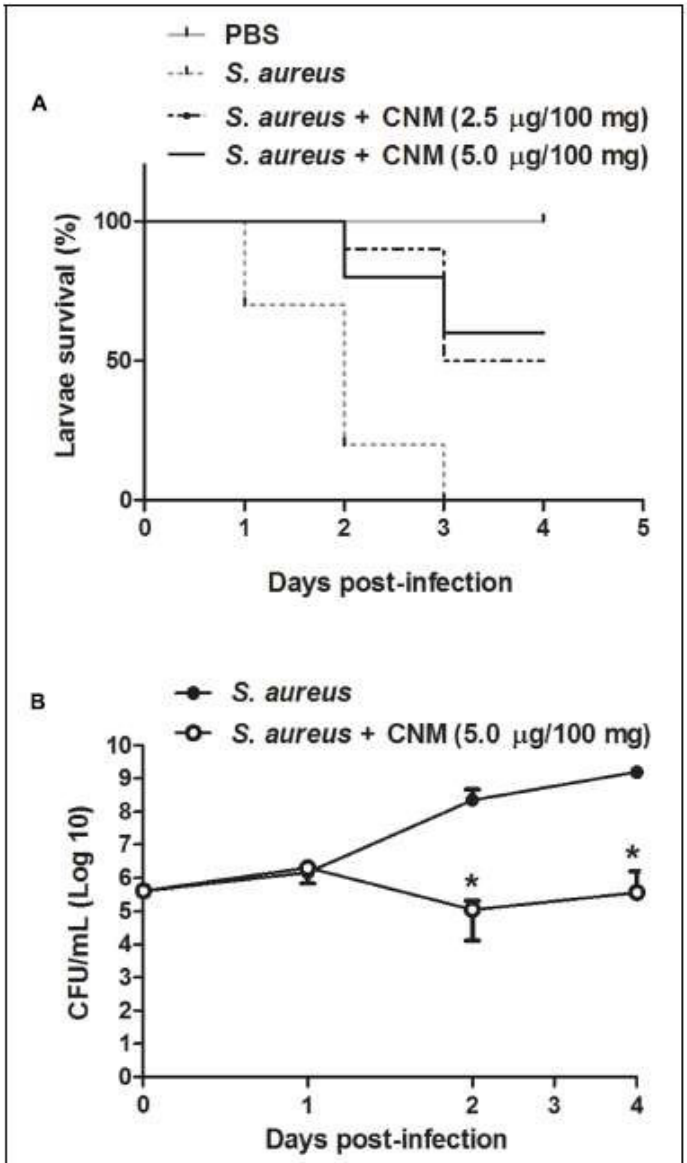

FIGURE 3 | Effect of cinnamaldehyde on survival (A) and hemolymph bacterial load **(B)** of *Galleria mellonella* larvae infected with *S. aureus*. *G. mellonella* received either cinnamaldehyde (CNM; 2.5–5.0 μg/100 mg of larvae) or vehicle (PBS, 5 μl/100 mg) and were evaluated for 4-days post-infection. $n = 10$/group for survival experiments; $n = 5$/group/day for bacterial load quantification. Data for bacterial load is represented as mean ± SD. *$p < 0.05$, compared with vehicle-treated larvae. Experiments were performed twice in duplicate.

DISCUSSION

Cinnamaldehyde Inhibits the Growth of *S. aureus* and *E. faecalis* without Inducing an Adaptive Phenotype

Cinnamaldehyde presented with antimicrobial actions on clinical isolates of *S. aureus* and *E. faecalis*, in addition to ATCC standard strains. This compound was effective on all strains of *E. faecalis* and *S. aureus*, including those with a multidrug resistance phenotype. The antimicrobial properties of cinnamaldehyde have been demonstrated against a range of Gram-positive and Gram-negative pathogens including *S. aureus* and *E. faecalis* (Cox and Markham, 2007; Shen et al., 2015; Upadhyay and Venkitanarayanan, 2016). Cinnamaldehyde actions against these pathogens are related to changes in their cell membrane polarity and permeability (Hammer and Heel, 2012). Importantly, we show for the first time that although becoming tolerant to ciprofloxacin, neither *S. aureus* (ATCC 25923) or *E. faecalis* (ATCC 19443) develop an adaptive phenotype when incubated with cinnamaldehyde *in vitro*.

Cinnamaldehyde Sub-inhibitory Concentrations Do Not Affect Biofilm Formation by *E. faecalis*

Biofilm formation is an important virulence factor involved in staphylococcal and enterococcal infections (Claessen et al., 2014). In a recent report, cinnamaldehyde was shown to inhibit the expression of *sarA* (a positive regulator of biofilm formation) in *S. aureus* at sub-inhibitory concentrations (Jia et al., 2011). Surprisingly, cinnamaldehyde not only had no effects on the ability of *E. faecalis* to form biofilm, but enhanced biofilm formation by some strains of *S. aureus*. These results oppose to those of previously published reports in that this compound was suggested to reduce biofilm formation by these bacteria. Recently, Budri et al. (2015) showed that cinnamaldehyde strongly diminishes biofilm formation by *S. aureus* ATCC 35983 on both polystyrene and stainless steel surfaces, when tested at 0.199 mg/ml. This effect was also observed when cinnamaldehyde was associated with either biodegradable polymers or nanoparticles (Zodrow et al., 2012; Duncan et al., 2015). Similarly, a *Cinnamomum zeylanicum* essential oil, rich in cinnamaldehyde, was shown to reduce

biofilm formation by *E. faecalis* (Abbaszadegan et al., 2016). It is possible that the discrepancies found between our results and the above discussed are due to differences on the strains tested which may present different virulence patterns.

Additionally, different antimicrobial agents may be able to induce biofilm formation at sub-inhibitory concentrations (Kaplan et al., 2012; Schilcher et al., 2016). This effect is suggested to be strain-specific and related to the induction of stress pathways in *S. aureus* that in turn, lead to the expression of biofilm-associated genes (Schilcher et al., 2016). Herein, as in other reports (Kaplan et al., 2012; Schilcher et al., 2016), biofilm formation was evaluated by the crystal violet assay. This method is widely used for this purpose, however, crystal violet stains both viable and dead cells, in addition to the extracellular matrix (Xu et al., 2016). In order to determine whether biofilm formation by *S. aureus* was associated with cell survival, we evaluated the viability of biofilm-forming *S. aureus* (ATCC 6538) cells incubated with cinnamaldehyde at MIC/2. We found that at this concentration, cinnamaldehyde reduces *S. aureus* metabolic activity, indicating loss of viable cells. These results allow us to suggest that the increased biofilm mass observed in cinnamaldehyde-treated *S. aureus* is due to the accumulation of dead cells rather than increase in *S. aureus* virulence. They also support the applicability of cinnamaldehyde in the treatment of *S. aureus*-induced infections.

Cinnamaldehyde Sub-inhibitory Concentrations Inhibit the Hemolytic Activity of *S. aureus* but not *E. faecalis*

Hemolytic toxins are secreted virulence factors expressed by some strains of *S. aureus* and *E. faecalis* which increase pathogenicity (Van Tyne et al., 2013; Tabor et al., 2016). Our data show that cinnamaldehyde diminishes *S. aureus*-induced hemolysis, but is only able to inhibit this parameter in one of the tested *E. faecalis* strains. The effects of cinnamaldehyde on cell survival have been widely studied in different cells lines, including immune cells (Roth-Walter et al., 2014), neurones (Pyo et al., 2013), erythrocytes (Theurer et al., 2013), amongst others. Of importance, this compound was shown to cause hemolysis *per se* when incubated for 48 h with human erythrocytes (Theurer et al., 2013). On the other hand, we show that the hemolysis caused by *S. aureus* is markedly inhibited by sub-inhibitory concentrations of cinnamaldehyde. It is possible that in this experimental setting, cinnamaldehyde targets bacteria rather than erythrocytes. Similarly, Amalaradjou et al. (2014) reported a protective effect for cinnamaldehyde in *Cronobacter sakazakii*-induced intestinal epithelial cell death.

Cinnamaldehyde Sub-inhibitory Concentrations Decrease *S. aureus* Survival in the Presence of Human Serum

Staphylococcus aureus and *E. faecalis* are common etiological agents of bacteraemia which often lead to septic shock and endocarditis (Dahl et al., 2016; Yahav et al., 2016). The survival of these pathogens in the bloodstream is due to their ability to express different virulence factors that target components of the host's immune system (Foster et al., 2014; Hall et al., 2015; Richards et al., 2015). We show that cinnamaldehyde enhances *S. aureus* but not *E. faecalis* killing when incubated with freshly isolated human serum. To the best of our knowledge, this study presents the first evidence on that cinnamaldehyde impairs *S. aureus* resistance to human serum. This may represent an additional mechanism by which cinnamaldehyde confers protection to infection *in vivo*.

Cinnamaldehyde Sub-inhibitory Concentrations Decrease the Ability of *S. aureus* to Adhere to Latex

Catheterization is a potential risk factor for bacterial colonization and infection (Padmavathy et al., 2015; Tong et al., 2015). *S. aureus* and *E. faecalis* are both capable of adhering to abiotic surfaces (such as catheter) due to the expression of surface proteins (Foster et al., 2014), such as the *S. aureus* protein A (SpA) and the enterococcal surface protein (Esp) (Elhadidy and Elsayyad, 2013; Zapotoczna et al., 2016). Cinnamaldehyde strongly inhibited *S. aureus* adherence to latex, an effect that was observed when this compound was tested at MIC/2 and MIC/4 on all strains. When tested on *E. faecalis* strains, cinnamaldehyde only diminished latex adherence by the standard strain ATCC 19443.

The use of essential oils or other plant-derived material to prevent bacterial adhesion to catheters and other medical devices has been pointed as an interesting approach for the medical field (Silva et al., 2016). These strategies involve the modification of the surface by the incorporation of the anti-adhesive compounds, resulting in functionalized surfaces with improved resistance to microbial colonization (Grumezescu, 2013; Trentin et al., 2015). Our results show that cinnamaldehyde may be useful for the development of surface-modified materials in order to prevent *S. aureus* adhesion.

Cinnamaldehyde Increases the *G. mellonella* Larvae Survival and Reduce Bacterial Load in the Hemolymph

Overall, cinnamaldehyde *in vitro* antimicrobial actions were more pronounced on *S. aureus*. *In vivo*-testing of cinnamaldehyde in *G. mellonella* larvae infected with *S. aureus* showed this compound augments larvae survival whilst reducing bacterial load in their hemolymph. *In vivo* protection of infection by cinnamaldehyde has been previously reported. Recently, cinnamaldehyde was shown to protect *G. mellonella* larvae against *L. monocytogenes*-induced infection (Upadhyay and Venkitanarayanan, 2016). This action was attributed to its ability to up-regulate the expression of antimicrobial peptide genes in *G. mellonella* (Upadhyay and Venkitanarayanan, 2016). The immunomodulatory properties of cinnamaldehyde were also evaluated in a mouse model of LPS-induced SIRS. The authors showed that cinnamaldehyde protection is related to the ability of this compound in modulating the immune response through

transient receptor potential ankyrin 1 (TRPA1)-dependent and independent mechanisms (Mendes et al., 2016).

These results are rather promising as cinnamaldehyde is effective against bacteria and also improves the immune response to infection by these pathogens. Here, cinnamaldehyde protected against *S. aureus* infection in *G. mellonella*, in doses equivalent to 25–50 mg/kg. On the other hand, further studies are necessary in order to establish cinnamaldehyde safety and effectiveness in humans when given by oral route. Indeed, reports suggest that cinnamaldehyde presents both genotoxic and irritative effects, although these are noted when this compound is administered at much higher concentrations/doses than the ones investigated in our study, such as $\geq$500 mg/kg (systemically) or $\geq$3% (topically applied to the skin) (for review see, Bickers et al., 2005). Data obtained from animal studies suggest cinnamaldehyde is safe by oral route when administered as either a single dose (2,220 mg/kg) or repeatedly for even over 2 years (up to 550 mg/kg/day). Importantly, cinnamaldehyde excretion rate at 24 h after administration varies between 70 and 98% in rodents, depending on the route of administration; and reaches 100% within 8 h when given orally to healthy human volunteers (for review see, Bickers et al., 2005; Cocchiara et al., 2005). Thus, implementation of dose schemes may also consider the excretion rate of cinnamaldehyde.

Overall, we suggest that cinnamaldehyde may represent an alternative therapy to control *S. aureus*-induced bacterial infections as it presents the ability to diminish bacterial virulence/survival in addition to improve the host's immune response to infection.

AUTHOR CONTRIBUTIONS

TF, JA, BdSP, JdS, ES, BdS, VC, TN, CF, CS, JC, VM-N, LdS, and EF contributed to conception, design, data acquisition, analysis, and interpretation, drafted 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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5.2 **ARTIGO II**

Concentrações sub-inibitórias de cinamaldeído atenuam a virulência de *Pseudomonas aeruginosa in vitro* e promovem a cicatrização de feridas cutâneas infectadas em camundongos

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RESUMO

Introdução: Infecção de feridas cutâneas retarda a cicatrização por promover um ambiente inflamatório sustentado, tanto pela ação dos microrganismos quanto pela ação da resposta imunológica. *Pseudomonas aeruginosa* é um dos patógenos mais comuns na infecção no leito da lesão, é extremamente refratário a terapia antimicrobiana e a resposta imune. Em trabalho anterior, nós demonstramos que o cinamaldeído apresenta um importante papel na regulação da resposta imunológica em camundongos com sepse via TRPA1 e é capaz de controlar a produção de fatores de virulência em outras espécies bacterianas.
Objetivos: o objetivo deste trabalho foi verificar a atividade antimicrobiana e antivirulência do cinamaldeído contra *Pseudomonas aeruginosa*, bem como sua ação na cicatrização de feridas infectadas via ativação de TRPA1.
Métodos: Para atividade antimicrobiana foi empregado a metodologia de microdiluição. A análise da interferência das concentrações subinibitórias do cinamaldeído na atividade hemolítica e na produção de biofilme foi realizado em BHI adicionado com papa de hemácias humanas a 2% e meio Luria Bertani respectivamente. *In vivo* foi realizado modelo de cicatrização em camundongos por excisão e utilizado o composto HC030031 para bloquear o receptor TRPA1.
Resultados: verificou-se que o cinamaldeído apresenta efeito inibidor na produção dos fatores de virulência da *Pseudomonas aeruginosa*, diminuído em 98% a produção do biofilme na maior concentração subinibitória, e redução da atividade hemolítica entre 43% a 80%. Constatamos também que os camundongos infectados e tratados com cinamaldeído apresenta um perfil inflamatório e de contração da lesão similar ao grupo controle, porém diferente, nestes quesitos, do grupo infectado tratado com DMSO, HC030031, e cinamaldeído+HC030031, principalmente entre o terceiro e quinto dia de tratamento.
Conclusão: As propriedades antipatogênicas junto com a ação do reparo tecidual fazem do cinamaldeído uma opção terapêutica atrativa para o tratamento de feridas infectadas.

Palavras-chave: ferida infectada, cinamaldeído, TRPA1, HC 030031, cicatrização, *Pseudomonas aeruginosa*

Introdução

O cinamaldeído é o composto majoritário extraído principalmente da casca de *Cinnamomum* sp, sendo utilizado por séculos na medicina tradicional chinesa devido ao seu efeito antimético, anti-diabético, anti-inflamatório e anti-cancerígeno[1,2]. Adicionalmente, vários estudos apontam que este composto apresenta atividade antimicrobiana contra os mais variados tipos de patógenos, inclusive por interferir com a virulência de bactérias gram-positivas e gram-negativas [3,4,5]. Ainda, evidências obtidas de estudos *in vitro* e *in vivo* têm atribuído uma atividade imunomoduladora para este composto[6,7]. O cinamaldeído regula a liberação de vários mediadores inflamatórios, como TNF-α, IL-6, IL-10, IL-1β e óxido nítrico em modelos de sepse, de alergia e de isquemia cerebral [8,9,10].

Recentemente, o alvo farmacológico do cinamaldeído foi identificado em mamíferos, sendo este, o receptor de potencial transitório anquirina 1 (TRPA1)[11]. O TRPA1 é um canal não seletivo para o Ca^{+2} expresso em células neuronais e não neuronais como células da vasculatura, queratinócitos e células do sistema imune[12]. Identificado como um mediador importante da transdução de processos dolorosos, sua relevância componente chave de processos inflamatórios têm sido cada vez mais estudada[13,14]. De fato, a ativação do TRPA1 participa da transdução da dor na artrite reumatóide e osteoartrite[15], na resposta inflamatória à antígenos bacterianos[9], na doença inflamatória intestinal[16], no prurido de origem inflamatória [17], dentre outras.

Apesar destas evidências, não existem estudos acerca do potencial cicatrizante do cinamaldeído, nem sobre a relevância do TRPA1 no processo de cicatrização. Aqui, investigou-se os efeitos do cinamaldeído sobre *Pseudomonas aeruginosa*, uma das bactérias mais comum encontrada em feridas infectadas[18]. Ainda, avaliou-se o efeito cicatrizante deste composto em modelo de ferida cutânea induzido por excisão da pele, seguida por infecção tópica com *P. aeruginosa*. A relevância do TRPA1 no potencial cicatrizante do cinamaldeído foi investigado em animais tratados com o antagonista seletivo para o TRPA1, HC-030031.

Materiais e métodos

Ensaios *in vitro*

Linhagem bacteriana

Utilizou-se a cepa padrão de *Pseudomonas aeruginosa* ATCC 27853, gentilmente cedida pela bacterioteca da Universidade CEUMA. Foi realizado um repique da cepa em BHI com 20% de glicerol e armazenada a -20ºC. Para os experimentos a bactéria foi incubada em caldo BHI a 37ºC por 24 horas para posterior passagem em agar BHI nas mesmas condições.

Ensaio antimicrobiano

Análise de tolerância bacteriana à droga

.

Atividade antibiofilme

Ensaio de hemólise

Ensaio *in vivo*

Animais

Foram utilizados camundongos *Swiss* (fêmeas, 3-5 meses de idade). Os animais foram obtidos do biotério da Universidade CEUMA. Os animais foram colocados em gaiolas de policarbonato em salas aeradas com clico de 12h de luz/12 horas de escuro a 25ºC ±2ºC, alimentação e água *ad libitum*. Todos os procedimentos foram conduzidos de acordo com a lei 11.794/2008 que regulamenta procedimentos científicos em animais e com parecer aprovado pelo Comitê de ética em animais da Universidade Ceuma (protocolo nº 100/13).

Indução de lesão cutânea

Para indução da lesão cutânea, os camundongos foram anestesiados pela injeção intraperitoneal da mistura de quetamina (50 mg/kg) e xilasina (2 mg/kg) e logo após, o dorso foi tricotomizado. A lesão por excisão foi realizada cirurgicamente com diâmetro de 0,8cm.

Em seguida, um inóculo de *P. aeruginosa* foi adicionado à lesão (30 µl, 1,5x 10^8UFC/ml). Animais tratados com veículo foram utilizados como controle.

Tratamento da lesão cutânea

Após a indução da lesão, os animais foram distribuídos randomicamente em 5 grupos (*n*= 5/grupo): i) não-infectado,tratado com topicamente com veículo (30 µl, 2% DMSO em salina; 1 x ao dia), ii) infectado tratado com veículo; iii) infectado tratado com cinamaldeído 0,5mg/mL (1 x ao dia); iv) infectado tratado com cinamaldeído 0,5mg/ml e o antagonista seletivo do receptor TRPA1 (HC 030031, 30 mg/kg, i.p., 1 x ao dia) e v) infectado tratado com HC-030031. Todos os tratamentos foram iniciados 24 h após indução da lesão e o tratamento com HC-030031 precedeu o tratamento com cinamaldeído em 1h.

Avaliação clínica da lesão

As feridas foram avaliadas 24 horas após a infecção da lesão e diariamente por 7 dias, observando-se na ferida e na área circunscrita 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. Todas as feridas foram fotografadas e medidas em seus diâmetros maior e menor, no momento da produção da falha cutânea e no 7º dia pós infecção precedendo as biópsias. Para a mensuração da área da lesão foi utilizado a seguinte a equação: $A = \pi . R . r$, onde A representa a área (cm2); "R", o raio maior e "r", o raio menor. O grau de contração foi expresso em percentual, e determinado pela equação: 100X (Wo –Wi)/Wo = % de contração. Wo área inicial da ferida e Wi = área no dia da biópsia.

Foi desenvolvido um método para análise da cicatrização baseado no *Pressure Ulcer Scale for Healing e Pressure Sore Status Tool*. A escala foi composta pelos parâmetros: área, parâmetros inflamatórios e parâmetros de debridamento, como mostra a tabela abaixo. Após a análise das feridas somou-se os valores descritos da tabela 1.

Contagem de bactérias do leito da lesão

Os camundongos foram mortos por deslocamento cervical, randomicamente, nos dias 7 de tratamento. O leito da lesão será retirada assepticamente e colocado em tubos com 1mL de solução salina. O tecido foi pesado, homogeneizado e diluído. A quantidade de bactérias no leito da lesão foi determinada por plaqueamento em ágar sangue e ágar McConkey e quantificado como UFC/grama de tecido.

Análise estatística:

Os resultados foram expressos como a média ±erro padrão da média. As porcentagens de inibição foram calculadas como a média das inibições obtidas para cada experimento individual. A avaliação estatística dos resultados foi realizada por meio de análise de variância (ANOVA), seguida pelo teste de Boferroni ou Kruskal-Wallis quando apropriado e por regressão linear simples. Foi adotado o nível de significância igual a 0.05.

Resultados

Determinação das concentrações subinibitórias e da concentração bactericida mínima (MBC)

O MBC do cinamaldeído foi igual a 2mg/ml. As duas maiores concentrações subinibitórias (0,25mg/ml e 0,5mg/ml) não reduziram significativamente o crescimento da *Pseudomonas aeruginosa* ATCC 27853 quando comparada ao controle ($p>0,05$). Isto confirma que estas concentrações (0,25mg/ml e 0,5mg/ml) não diminuíram a produção de fatores de virulência devido a diminuição da população microbiana.

Aumento da resistência antimicrobiana

Realizamos estudo sobre indução de resistência ao cinamaldeído e usamos como controle ciprofloxacino. Após 10 passagens em concentrações subinibitórias do cinamaldeído, observou-se que a concentração inibitória mínima permaneceu a mesma, no entanto o perfil de resistência da *Pseudomonas aeruginosa* passou de 0,0625µg/ml para 1µg/ml, aumento de 16 vezes.

Concentrações subinibitórias do cinamaldeído inibe o metabolismo

O efeito das concentrações subinibitórias do cinamaldeído na *Pseudomonas aeruginosa* ATCC 27853 está demonstrado na figura 1. Nas amostras do controle, o metabolismo foi de 137% ±1,4, após 24 horas de incubação. Contudo, nas placas incubadas com *Pseudomonas aeruginosa* tratadas com 0,25 mg/ml e 0,5mg/ml, o metabolismo foi reduzido significamente ($p<0,05$) em torno de 27%.

Concentrações subinibitórias do cinamaldeído inibem a atividade hemolítica

Os efeitos das concentrações subinibitórias do cinamaldeído da atividade hemolítica estão apresentados na figura 2. Foi observado que as concentrações subinibitórias apresentam uma certa atividade hemolítica em 24 horas de incubação de maneira dose dependente ($p<0,05$). Da mesma forma, a incubação do microrganismo com concentrações subinibitórias do cinamaldeído promoveram uma redução na atividade hemolítica em cerca de 43% a 90%, para a concentração maior e menor respectivamente.

Concentrações subinibitórias diminuem a produção de biofilme

As duas concentrações subinibitórias do cinamaldeído diminuíra ($p<0,05$) a formação do biofilme em microplaca a 37ºC (figura 3). Pode-se notar, também, que esta diminuição foi dose dependente, na qual a redução da menor e da maior concentração foi cerca de 83% e 98% respectivamente.

Avaliação da cicatrização de feridas em camundongos

Avaliação macroscópica das lesões foi realizada em 7 dias. A cicatrização da incisão cutânea foi determinada pelo percentual da contração da lesão devido a regeneração da epiderme como ilustra a figura. Não houve diferença estatística entra a cicatrização dos camundongos infectados com *Pseudomonas aeruginosa* e os camundongos não infectados tratados com DMSO ($p>0,05$), apresentando contração da ferida em 59% e 60% respectivamente. No entanto, os camundongos infectados e tratados com DMSO apresentaram diferença na contração da lesão entre o terceiro e quinto dia de tratamento quando comparado com os tratados com cinamaldeído e do segundo ao quinto dia quando comparado com o grupo não infectado, com o total de contração da lesão de 54% para o grupo controle infectado. Interessantemente os grupos tratados com HC030031, com ou sem cinamaldeído, apresentaram diferença estatística com apenas no sexto e sétimo dia de tratamento quando comparado com o controle não infectado e a partir do 3º com os camundongos infectados e tratados com cinamaldeído. A ferida apenas regrediu em 36% nos camundongos tratados com HC030031. Em relação ao escore de inflamação pode-se constatar o grupo não infectado tratado com DMSO apresentou o mesmo perfil inflamatório que os camundongos tratados somente com cinamaldeído ($p<0,05$) e que este teve um menor perfil inflamatório que os outros três grupos no terceiro e quarto dia de tratamento ($p<0,05$). Isto comprova que o TRPA1 está envolvido no processo de cicatrização, influenciando a inflamação e na contração da lesão.

Contagem de *Pseudomonas aeruginosa* no leito da lesão

No sétimo dia de observação e sexto de tratamento, foi feito a incisão do leito da lesão para verificação da quantidade de *Pseudomonas aeruginosa*. Pode-se observar que o tratamento com cinamaldeído reduziu em 52% a quantidade bactérias no leito da lesão, quando comparado com o controle infectado. Os grupos tratado com cinamaldeído + HC030031 e HC 030031 apresentaram uma redução em torno de 42%. Apenas o grupo com cinamaldeído apresentou uma maior redução quando comparado com os outros grupos ($p<0,05$).

Discussão

A pele é o maior órgão do ser humano e constitui uma parte integral do sistema imune inato, servido como a primeira linha de defesa contra a infecção por prevenir a adesão e invasão microbiana[22]. Contudo, quando a lesão ocorre a estrutura anatômica da pele, uma infecção pela flora residente ou transitória pode ocorrer, se as condições homeostáticas não forem rapidamente restauradas[23].

O processo de reparação tecidual apresenta atividade imunológica e não requer muito auxílio, mas vários fatores de risco como infecção e imunossupressão prejudicam este processo[24]. Dentre os diversos tipos de patógenos, *Pseudomonas aeruginosa* é considerada uma das mais comuns bactérias que causam infecção persistentes em feridas[25]. Estudos recentes têm relatado a formação de biofilme de *P. aeruginosa* na pele humana[26,27], além de sua multidroga resistência[28 -30], dificultando seu tratamento.

A busca de novos tratamento para esta problemática está em alta nos dias atuais. Dentre os diversos tipos de compostos extraídos de plantas, o cinamaldeído apresenta efeitos promissores. Esta molécula é um composto diterpeno volátil, majoritário encontrado no extrato de *Cinamomum*[31] e exibe propriedades farmacológicas como ação anti-inflamatória, anti-tumor, antibacteriano, antidiabético, anti-obesidade e neuroproteção [2,32,33].

Nossos dados indicam que o cinamaldeído acelera o processo de cicatrização em feridas infectadas, em um modelo de lesão induzida em camundongo, por reduzir os parâmetros inflamatório, aumentar a velocidade de contração da lesão além de reduzir a produção dos fatores de virulência do isolado de *Pseudomonas aeruginosa,* quando administrado topicamente uma vez ao dia durante sete dias.

Todos os parâmetros estudados acerca da atividade cicatrizante do tratamento tópico do cinamaldeído foram afetados significativamente. Ainda, demonstrou-se que este composto

apresenta atividade antimicrobiana contra *P. aeruginosa*, além de interferir com sua virulência, sem causar adaptação nesta bactéria. Há duas explicações para a diminuição do crescimento das cepas analisadas, a primeira é que os óleos essensiais apresenta propriedade lipolifílica que podem causar danos à membrana celular, o que afeta a homeostase e o equilíbrio de pH e de íons orgânicos. As concentrações bactericidas de cinamaldeído, assim como outras concentrações subinibitórias, aumentam a permeabilidade da membrana plasmática em *Pseudomonas aeruginosa*, aumentando o influxo de íons inorgânicos[34]. A outra explicação seria a sua ação no *filamentous temperature-sensitive protein Z* (FtsZ), uma molécula comumente conservada no reino bacteriana. O FtsZ tem um importante papel na divisão bacteriana e um atrativo alvo para o desenvolvimento antimicrobiano para resistência bacteriana. O cinamaldeído inibe a taxa de hidrólise de GTP e liga ao FtsZ, alterando a cinética da formação do anel Z, o que diminui a taxa de crescimento[35].

Como mencionado anteriormente, o tratamento *in vitro* com concentrações subinibitórias de cinamaldeído diminui a produção de fatores de virulência importantes para o processo de cronicidade da lesão (biofilme e atividade hemolítica). Isto é um fator preponderante para o tratamento de feridas infectadas já que o isolado bacteriano testado apresenta a habilidade de formar biofilme, tornando-o resistente a ação da resposta imune bem como a intervenção antimicrobiana[36]. Biofilme bacteriano é um importante fator que contribui para a cronicidade das feridas infectadas[37], já que mais da metade dos pacientes hospitalizados apresentam biofilme no leito da lesão[38]. Adicionalmente, as concentrações subinibitórias do cinamaldeído inibiram a atividade hemolítica de *P. aeruginosa*, bem como sua atividade metabólica. Um efeito similar foi recentemente demostrado para *Staphylococcus aureus*, outra bactéria relevante em feridas infectadas[5.] Segundo Tkaczyk e colaboradores[39] a inibição deste fator de virulência em *Staphylococcus aureus* reduz a área de dermonecrose em camundongos, o que possibilitaria um efeito parecido com infecções causadas por *Pseudomonas aeruginosa*. Estas ações, teoricamente, permitiria um efeito benéfico para a cicatrização de feridas. Já em relação ao metabolismo, foi comprovado que o cinamaldeído diminui a quantidade intracelular de ATP por inibir a ATPase, o que provoca o efeito encontrado nesta pesquisa[40].

O problema de se utilizar doses subinibitórias para o tratamento de infecções é o surgimento de cepas com perfil alterado de sensibilidade ao antimicrobiano, por isso que a eficácia do tratamento de antibióticos para *P. aeruginosa* está ficando limitado devido à grande prevalência de cepas consideradas multidroga resistentes[28]. Contudo estas concentrações não induziram a presença de isolados com alteração em tal perfil de sensibilidade, diferentemente do que ocorreu com o uso de subdoses de ciprofloxacina. Importantemente, o cinamaldeído

também não induziu um perfil adaptativo quando incubado com *S. aureus*[5], reforçando seu potencial terapêutico contra infecções bacterianas.

Além do potencial antimicrobiano *in vitro*, o cinamaldeído reduziu o número de bactérias no leito da ferida *in vivo*. A redução do número de bactérias da ferida pode contribuir parcialmente para sua eficácia no processo de cicatrização. De fato, o cinamaldeído acelerou o processo de cicatrização. A síntese de colágeno, que reflete o aumento da proliferação celular, não apenas confere força e integridade a matriz tecidual, mas também apresenta papel importante na homeostasia e no processo de epitelização[41,42]. Os resultados demonstraram uma potente capacidade de cicatrização como evidência da contração da lesão; com aumento da força tênsil da lesão nos grupos tratados com cinamaldeído. Sabe-se que o cinamaldeído sugerindo um efeito proliferativo e anti-inflamatório para este composto. O cinamaldeído regula a liberação de vários mediadores inflamatórios, como TNF-α, IL-6, IL-10, IL-1β e óxido nítrico *in vivo*[8,9,43]. Estes mediadores contribuem para o processo de cicatrização, atuando sobre a angiogênese[44] e a formação do colágeno, o qual é essencial para a fase proliferativa da cicatrização[45].

Recentemente, os efeitos do cinamaldeído sobre o fluxo sanguíneo da pele, bem como sua atividade proliferativa em queratinócitos foram demonstrados[46]. Parte dos efeitos imunomoduladores deste composto, bem como suas ações sobre o leito vascular se devem pelo menos parcialmente, à ativação do receptor TRPA1[9,47]. Aqui, demonstrou-se que o efeito cicatrizante do cinamaldeído é revertido em animais tratados com o antagonista seletivo para o TRPA1, HC-030031; sugerindo que a ativação deste receptor é essencial ao processo de cicatrização induzido pelo cinamaldeído. Entretanto, não se sabe ao certo em qual etapa do processo de cicatrização o TRPA1 participa, nem a relevância individual de sua expressão neuronal e não neuronal para esta resposta.

Em resumo, nós demostramos que o cinamaldeído apresenta boa atividade antimicrobiana contra *P. aeruginosa.* Ainda, observou-se que concentrações subinibitórias deste composto diminuem a produção de fatores de virulência importantes para a perpetuação da colonização de *P. aeruginosa* no leito da ferida. Demonstrou-se também que concentrações subinibitórias de cinamaldeído promovem cicatrização de feridas cutâneas infectadas de forma dependente da ativação do receptor TRPA1.

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Apoio financeiro

FAPEMA, CAPES e CNPQ.

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

Características	Interpretação	Valor
1. Área (contração em relação ao 1º dia de tratamento)	0 a 20%	0
	21 a 40%	1
	41 a 60%	2
	61 a 80%	3
	81 a 100%	4
	101 a 120%	5
	121 a 140%	6
	141 a 160%	7
	Acima de 160%	8
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 pele da ferida	Normal para o animal	0
	Vermelho	1
	Branco ou hipopigmentado	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
		0
Quantidade de tecido necrótico	Nenhum	1
	< 25% do leito da ferida	2
	25 a 49% do leito da ferida	3
	50 a 75% do leito da ferida	4
	76 a 100% do leito da ferida	

Legendas das Figuras

Figura 1. Efeito do HC 030031 e das concentrações subinibitórias do cinamaldeído na viabilidade a cepa padrão de *Pseudomonas aeruginosa*. MIC – concentração inibitória mínima.

Figura 2. Efeito do HC030031 das concentrações subinibitórias do cinamaldeído na padrão taxa metabólica de cepa padrão de *Pseudomonas aeruginosa.* MIC – concentração inibitória mínima. * - $p<0,05$

Figura 3. Efeito das concentrações subinibitórias do cinamaldeído na produção de biofilme de cepa padrão de *Pseudomonas aeruginosa*. MIC – concentração inibitória mínima. * - $p<0,05$

Figura 4. Efeito das concentrações subinibitórias do cinamaldeído na atividade hemolítica de cepa padrão de *Pseudomonas aeruginosa.* MIC – concentração inibitória mínima. * - $p<0,05$

Figura 5. Tratamento tópico com cinamaldeído (0,5mg/ml) em feridas infectadas com cepa padrão de *Pseudomonas aeruginosa* em camundongos *swiss,* a lesão foi monitorada por 7 dias. (A) Avaliação diária da contração da lesão (percentual) durante 7 dias de tratamento. * - $p<0,05$ em relação ao controle negativo; # - $p< 0,05$ em relação ao cinamaldeído. (B) Avaliação da quantidade de *Pseudomonas aeruginosa*(UFC/g de tecido) no leito da lesão após 7 dias de tratamento. * - $p<0,05$ em relação ao controle negativo; # - $p< 0,05$ em relação ao cinamaldeído (C) Tamanho da população de *Pseudomonas aeruginosa* (UFC/g de tecido) após 7 dias de tratamento. * - $p<0,05$.

FIGURA 1

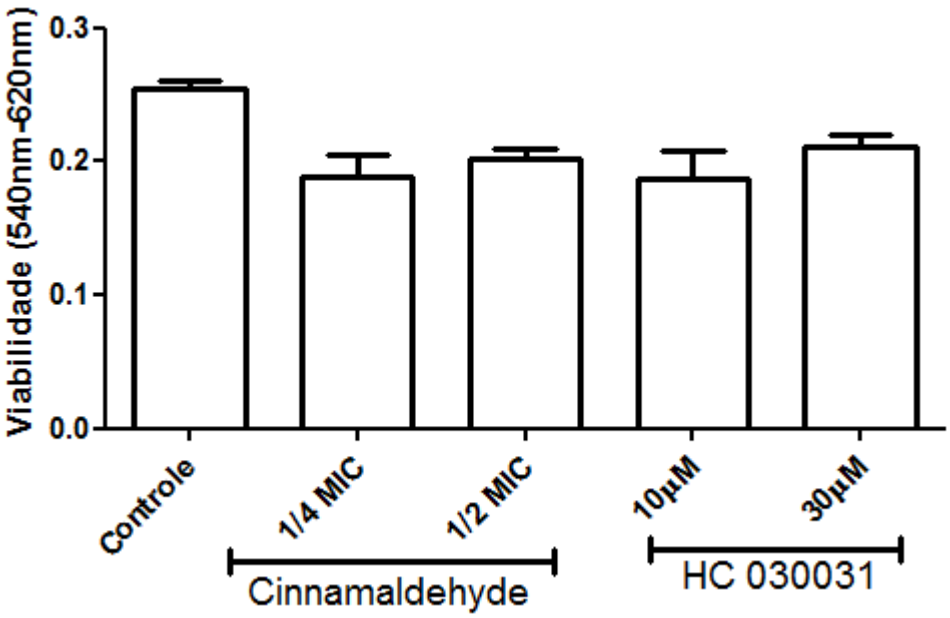

Viabilidade (540nm-620nm)
0.3
0.2
0.1
0.0
Controle
1/4 MIC
1/2 MIC
10µM
30µM
Cinnamaldehyde
HC 030031

FIGURA 2

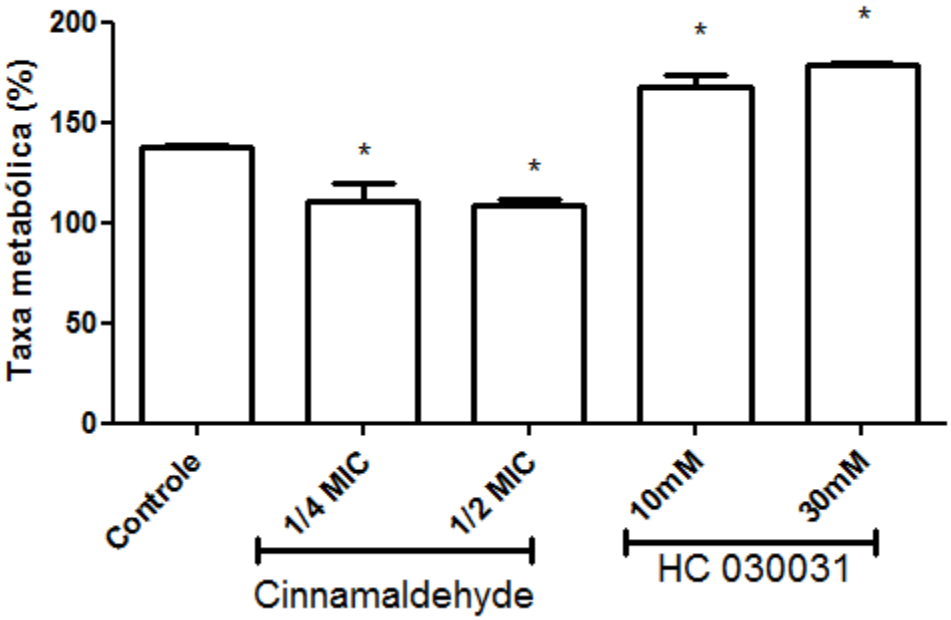

Taxa metabólica (%)
200
150
100
50
0
*
*
*
*
Controle
1/4 MIC
1/2 MIC
10mM
30mM
Cinnamaldehyde
HC 030031

FIGURA 3

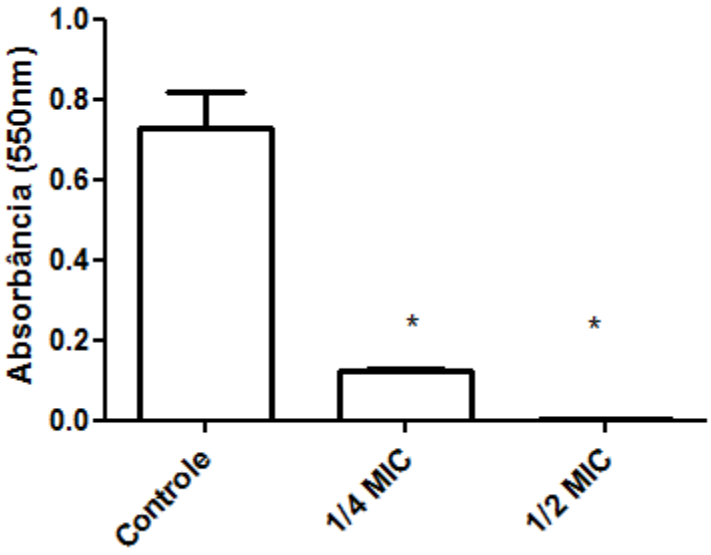


FIGURA 4

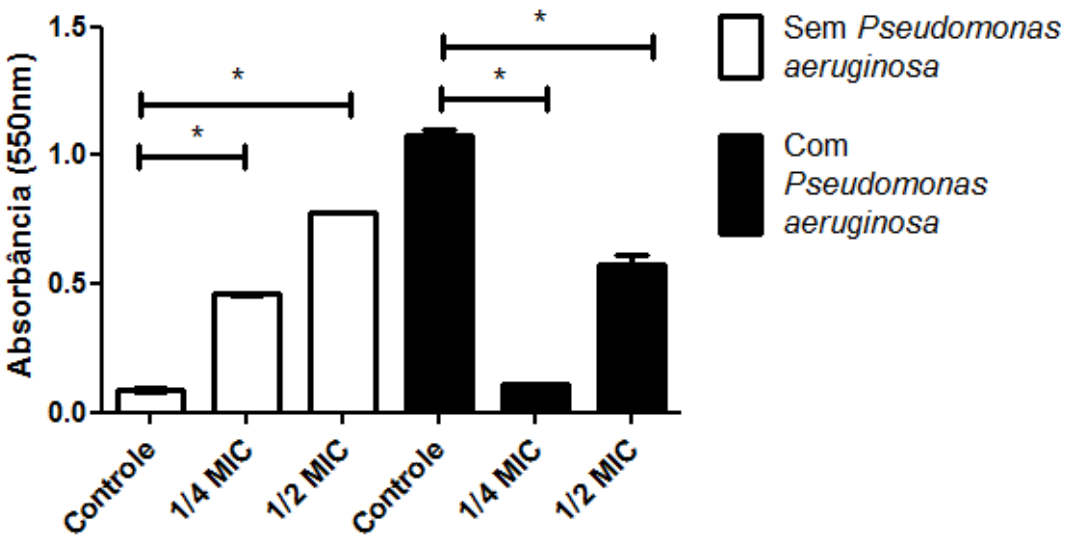
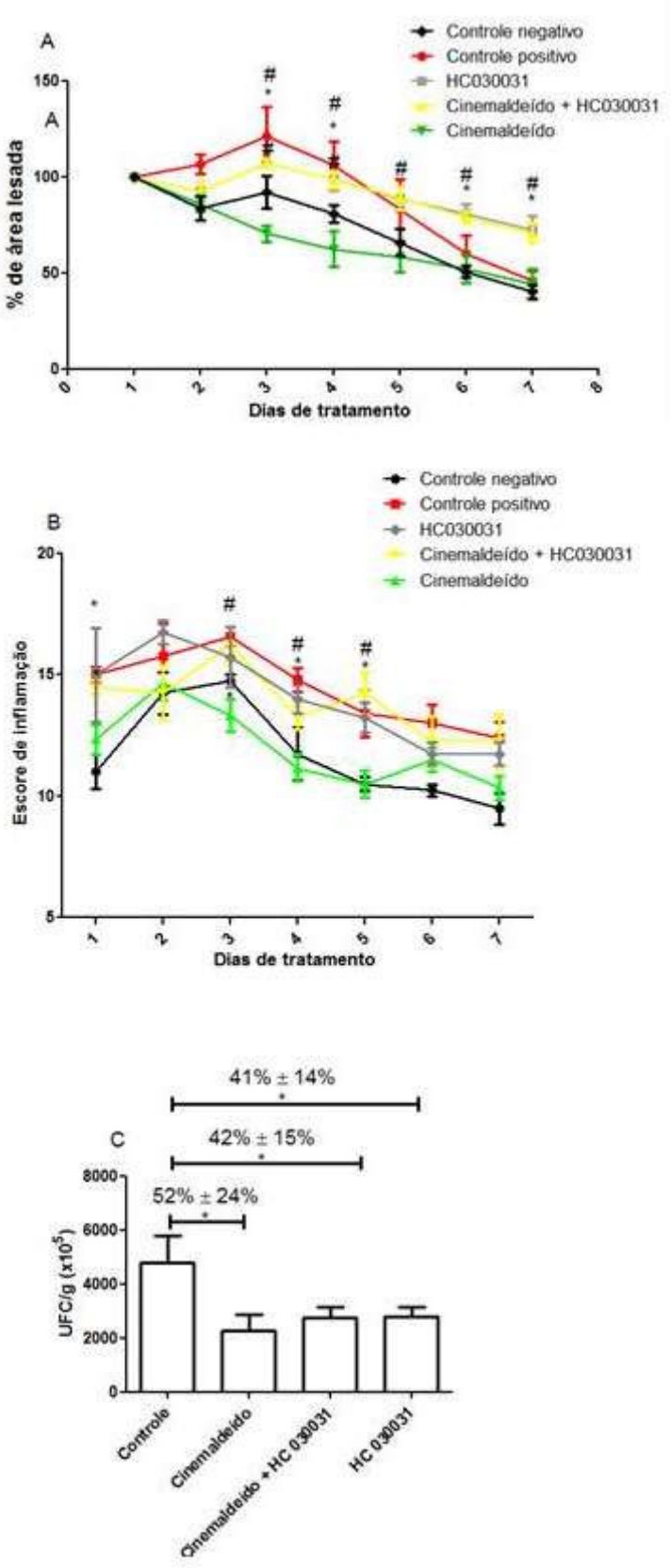

Absorbância (550nm)
1.5
1.0
0.5
0.0
*
*
*
*
Controle
1/4 MIC
1/2 MIC
Controle
1/4 MIC
1/2 MIC
Sem Pseudomonas aeruginosa
Com Pseudomonas aeruginosa

FIGURA 5


A
Controle negativo
Controle positivo
HC030031
Cinemaldeído + HC030031
Cinemaldeído
% de área lesada
Dias de tratamento
B
Escore de inflamação
Dias de tratamento
C
41% ± 14%
42% ± 15%
52% ± 24%
UFC/g (x10^5)
Controle
Cinemaldeído
Cinemaldeído + HC 030031
HC 030031

6. CONSIDERAÇÕES FINAIS

Pode-se comprovar que o cinamaldeído apresenta ótima ação antimicrobiana tantos i*n vitro* quanto *in vivo*. O isolado Gram negativo apresentou maior concentração inibitória mínima e bactericida mínima que os isolados de bactérias Gram positivas. Interessante relatar que as concentrações subinibitórias do cinamaldeído reduziram a produção de virulência dos isolados de *Staphylococcus aureus* e de *Pseudomonas aeruginosa*, porém não alteraram a expressão de determinantes de patogenicidade de *Enterococcus faecalis*.

Adicionalmente, o tratamento com cinamaldeído em larvas de *G. mellonella* infectadas com *Staphylococcus aureus* aumentou a taxa de sobrevência e reduzindo o número de bactérias. No outro experimento *in vivo*, a aplicação tópica diária do cinamaldeído, para avaliação do processo de regeneração tecidual em camundongos, mostrou que em nenhum período observado houve prejuízo clínico aos camundongos e que a aplicação diária de cinamaldeído reduziu os escores de inflamação, população de P. *aeruginosa* no leito da ferida, bem como acelerou o processo de contração da lesão quando comparados com os grupos infectados tratados com veículo e com HC030031. Estes dados mostram que o processo de cicatrização de lesões infectadas é mediado diretamente pelo receptor TRPA1 e que o cinamaldeído é uma boa alternativa terapêutica para esta problemática.

Anexo

Guia aos autores

British Journaul of Dermatology

*A*uthor **Guidelines**

1. ABOUT THE *BJD*

Aims and scope

The *BJD* strives to publish the highest-quality dermatological research. In so doing, the Journal aims to advance the understanding, management and treatment of skin disease in order to improve the outcomes of patient care. The main focus of the *BJD* (described in a position statement published in the June 2015 issue) is to publish the highest-quality research encompassing the following:

- Clinical trials that are registered before recruitment starts and report fully according to the CONSORT guidelines.
- Clinical studies that include epidemiology, qualitative research and mixed methods that conform to the STROBE and SRQR reporting guidelines.
- Translational research that describes basic and applied science of potential clinical relevance.
- Systematic reviews following the PRISMA statement (http://www.prisma-statement.org/).

The *BJD* invites submissions under a broad scope of topics relevant to clinical and experimental research as described in the position statement. The Journal publishes original articles, Reviews, Clinical Guidelines, Research Letters, Perspectives, Case Reports and items of correspondence.

The *BJD* is an official organ of the British Association of Dermatologists, but attracts contributions from dermatology researchers throughout the world. The *BJD* editorial board and editorial team include outstanding clinical academics, scientists and clinicians from across the globe. The circulation and readership of the *BJD* are equally international. The overriding criteria for publication are scientific merit, originality and interest to a multidisciplinary audience.

Journal content and further information – including author guidelines and submission details – can be found online (www.brjdermatol.org). The current Impact Factor is 4.275.

Mission and values

Founded in 1888, the *BJD* has evolved into one of the world's leading general dermatology journals.

The *BJD* brand is underpinned by the provision of trusted information. From this solid foundation, the Journal aims to serve the needs of its multiple stakeholders.

- For **authors**, the *BJD* aims to provide timely, high-quality, constructive peer review, which is focused on improving the quality of accepted manuscripts. Article production should be quick and accurate and free of any charges.
- The *BJD* recognizes the invaluable role of **reviewers** in safeguarding the integrity of the content, and aims to acknowledge fully their contribution. Our editorial team includes section editors for qualitative research, epidemiology, clinical trials, translational research, case reports and correspondence. Peer reviewers are chosen by the large team of associate editors appointed as subject experts. Statistical review is provided, when needed, by the *BJD*statistics editor.
- For **readers**, the *BJD* aims to deliver the highest-quality clinical and experimental research spanning the continuum of dermatological disciplines. Content should be readily discoverable and user friendly. Online innovation should add value to the content, promote understanding and facilitate clinical decision making.
- For **patients**, the *BJD* publishes Plain Language Summaries. These concise summaries of original research articles are intended to open up *BJD* content to patients with skin disease worldwide.
- For **subscribers**, the *BJD* aims to offer flexible purchasing options – from individual article downloads to multiyear, multisite licences – at reasonable prices.
- For **sponsors**, the *BJD* aims to offer ethical marketing solutions that yield measurable returns on investment.

2. MANUSCRIPT CATEGORIES

The *BJD* invites the following types of submission.

Review articles

The Journal aims to publish concise, high-quality review articles of recent advances in laboratory

or clinical research. Review articles may be solicited by the Editor or may be submitted by authors for publication subject to peer review. Review articles must include an unstructured abstract (maximum 250 words) and should not exceed 3000 words of body text. Use of illustrations and figures is encouraged. Review articles must include bulleted statements (maximum 70 words per question) in answer to the following questions: What's already known about this topic? and What does this study add?

NEW Systematic reviews **must** follow the PRISMA-statement to assure completeness. In order to facilitate manuscript review, a completed PRISMA checklist (add link to checklist here) should be submitted at the same time as the manuscript, identified as a 'Supplementary file for review'

Original articles

Original articles are the Journal's primary mode of communication. Original articles must include a structured abstract (maximum 250 words), and should not exceed 3000 words of body text. Original articles must include bulleted statements (maximum 70 words per question) in answer to the following questions: What's already known about this topic? and What does this study add? Please refer to the specific category section for any bulleted statements that must be included.

The categories for original articles within the Journal are as follows:

- Clinical Trials
- Epidemiology
- Translational Research
- Qualitative Research
- Medical Dermatology
- Surgical Dermatology
- Paediatric Dermatology
- General Dermatology

Clinical Trials

- The *BJD* welcomes the submission of manuscripts reporting the findings of clinical trials.
- In general, we recommend that clinical trial manuscripts are prepared in original article format, but for small studies it may be more appropriate to consider submission as a Research Letter. However, if authors choose the latter format, they should still aim to follow the instructions given below.
- All clinical trials must conform to appropriate ethical standards and must have been approved by the relevant ethical committees and authors' and institutional review board(s). A statement to this effect with the exact name of the approving institutional review board(s) should be included in the Materials and methods section. All clinical investigations must be conducted according to the principles expressed in the Declaration of Helsinki.
- From October 2015, any clinical trial submitted to the *BJD* must have been **prospectively registered on a suitable trial registry**. For further details see this editorial. The *BJD* is unlikely to consider manuscripts that do not follow this requirement. If there are good reasons why the trial was not registered, please provide justification when submitting the manuscript. The trial registation number (e.g. NCT012345678) should be given at the end of the abstract
- Submissions reporting industry-sponsored clinical trials are welcomed. The *BJD* recommends that all authors, particularly those reporting industry-sponsored clinical trials, take account of the guidelines of Mansi *et al.* when preparing their manuscript (Mansi BA, Clark J, David FS *et al.* Ten recommendations for closing the credibility gap in reporting industry-sponsored clinical research: a joint journal and pharmaceutical industry perspective. *Mayo Clin Proc* 2012; **87**:424–9).
- The *BJD* is particularly interested in promoting investigator-initiated clinical trials. Unlike industry-sponsored studies these are usually not written by professional medical writers.
- The editorial team has found that providing the study protocol facilitates acceptance of the paper if it is available. Therefore, the *BJD* encourages submission of the protocol at the time of manuscript submission, with the protocol identified as a 'Supplementary file for review'. Submission of the trial protocol is also strongly encouraged for industry-sponsored trials.
- Reporting of randomized controlled trials **must** follow the CONSORT statement to assure completeness. In order to facilitate manuscript review, a completed CONSORT checklist should be submitted at the same time as the manuscript, identified as a 'Supplementary file for review'. For randomized controlled trials with

specific designs, data or interventions, please refer to the extensions of the CONSORT statement. For pilot and feasibility studies, please refer to the CONSORT extension for these studies.

- Even for nonrandomized trials the CONSORT statement, or another appropriate EQUATOR guideline, should be followed as closely as possible. The reporting of systematic reviews and meta-analyses should follow the PRISMA statement. Reporting of meta-analyses and systematic reviews on observational studies should follow the MOOSE consensus statement (see attachment).
- The *BJD* often receives manuscripts reporting some, but not all, outcomes of a particular trial. Separate papers are then sent at a later stage, reporting secondary outcomes from the trial. The *BJD* does not support this approach and prefers to receive a single manuscript reporting **all** outcomes for the trial. Space limitations can be addressed by the appropriate use of online Supporting Information. Authors should note that manuscripts containing incomplete reporting of trial outcomes are likely to be rejected without peer review. The *BJD* may, in exceptional cases, consider manuscripts that do not include certain secondary outcomes (e.g. trials with very long-term follow-up). However, in this situation authors are strongly encouraged to seek advice from the *BJD* editorial team prior to submission, giving a clear explanation of which outcomes are to be reported separately, and the reason(s) for this. The editorial team will then advise whether or not the manuscript will be considered.

Epidemiology

- The *BJD* welcomes the submission of manuscripts reporting the findings of epidemiological studies that aim to improve our understanding of skin diseases, have clinical relevance and/or improve patient care or disease prevention (link to editorial Nijsten *et al.* *BJD*).
- We will be looking for epidemiological studies stimulating independent thinking and challenging the status quo. Whether or not the findings are statistically significant is less relevant than the robustness of the data collection and methodology used.
- In general, we recommend that epidemiology manuscripts are prepared in original article format, but for confirmatory studies or less complicated studies it may be more appropriate to consider submission as a Research Letter. However, if authors choose the latter format, they should still aim to follow the instructions given below.
- All epidemiological studies must conform to appropriate ethical standards and must have been approved by the relevant ethical committees and authors' and institutional review board(s). A statement to this effect with the exact name of the approving institutional review board(s) should be included in the Materials and methods section. All clinical investigations must be conducted according to the principles expressed in the Declaration of Helsinki.
- Reporting of observational studies **must** follow the STROBE statement to assure completeness. In order to facilitate manuscript review, the appropriate (cohort, case–control and cross-sectional studies) and completed STROBE checklist should be submitted at the same time as the manuscript, identified as a 'Supplementary file for review'.
- All manuscripts reporting multivariable prediction models for diagnostic or prognostic research should follow the TRIPOD guidelines. A TRIPOD checklist for model development, model validation or model development and validation should be uploaded during manuscript submission using the file designation: 'Supplementary file for review'.
- Specifically for epidemiological studies, we will look for
  -
  - Use of clinically relevant outcomes.
  - Interpretation of the findings in observational studies focusing on the distinction between association and causality. Of the nine Bradford–Hill criteria, temporality and dose–response relationship are pivotal and need careful consideration in the study design.
  - The issue of possible confounding and especially residual confounding. The latter is important in studies using existing data such as routinely collected data, claims data or large population-based cohorts.
  - Careful and motivated use of subgroup analyses to avoid data dredging.
  - Appropriate statistical testing (see below).
- Routinely collected health data, obtained for administrative and clinical purposes without specific a priori research goals, are increasingly used for research. The REporting of studies Conducted using Observational Routinely collected Data (RECORD) statement has been created as an extension to the STROBE statement to address reporting items specific to observational studies using routinely collected health data. RECORD consists of a checklist of 13 items related to the title, abstract, introduction, methods, results and discussion sections of articles, and other information required for inclusion in such research reports. In addition to the checklist, we would recommend that authors read the accompanying explanatory and elaboration information to enhance the use of the checklist. Examples of good reporting for each RECORD checklist item are also included in the elaboration document. If you are interested in finding out more about RECORD, please visit the website and message board (http://www.record-statement.org). Through implementation of RECORD, authors, journals editors and peer reviewers can encourage transparency of research reporting.

Translational research

In addition to the usual bulleted statements for original articles (What's known and What's new), manuscripts describing laboratory-based studies must also include a third bulleted statements (maximum 70 words) in answer to the following question:

- What is the translational message?

General policy

The *BJD* will publish laboratory-based studies only if they are of direct relevance to the understanding of the clinical features, pathogenesis or treatment of skin diseases in humans. Studies performed exclusively with nonprimary or patient-unrelated cell lines, and studies performed on animals will be considered for peer review only if accompanied by data showing their direct relevance to human dermatological conditions (link to Sprecher editorial *BJD*).

Science should be written with a clinical audience in mind. Authors are encouraged to provide one or more summary diagrams to compliment their written text, particularly when an understanding of complex or novel scientific concepts is required.

Publication standards for scientific research papers

- Publications will provide significant insight into dermatology, including (but not restricted to) the pathogenesis of skin diseases, mechanism of action of current/novel treatments of skin disease and a deeper understanding of normal skin physiology.
- Experimental data should be reproducible and robust. Experiments generating data that are essential to support any conclusion of the study must be repeated a sufficient number of times to demonstrate reproducibility. All data should be original and of high quality with adequate controls.
- All human and animal studies must conform to appropriate ethical standards and must have been approved by the relevant ethical committees and authors' and institutional review board(s). A statement to this effect with the exact name of the approving institutional review board(s) should be included in the Materials and methods section. All clinical investigations must be conducted according to the principles expressed in the Declaration of Helsinki.
- The number of animal and human subjects should be adequate to provide robust statistical analysis and should be stated. Data from cell culture studies should indicate the number of independent experiments and the number of independent donors contributing to the experiments performed.
- In addition to data from immortalized cells lines such as HaCaT, data from primary cell cultures should be included wherever possible or, if not, from a cell line that has been immortalized in a different manner.
- Comprehensive studies involving multiple laboratory techniques that support the conclusions made are usually required. Data generated using a single technique are usually not sufficient. Use of multiple relevant model systems (cell lines, animal and *ex vivo* models) is also encouraged to complement *in vivo*/clinical studies. Research Letters should also contain data supported by several lines of evidence and should be of immediate importance and not preliminary data.

Data presentation

- It is recommended that data are displayed in their raw form and not in a way that conceals their distribution. Individual data should be presented as dot plots next to the average for the group with appropriate error bars. The methods should be described in enough detail that the experimental conditions can be repeated in another laboratory. If any equipment or specific reagent used is detailed, provide the name of the manufacturer, city and country.
- Digital images should not be manipulated (e.g. contrast, brightness) unless the manipulation is applied to the whole image and does not modify the information in any way. Where images have been obviously cropped the full image should be available if requested by the editors or reviewers (for example an entire image of a Western blot with molecular-weight markers).
- Any materials generated during the study (e.g. cell lines, animals, plasmids or antibodies) should be made available to other researchers, where this is practicable.
- Novel DNA or amino acid sequences should be submitted to a public database such as GenBank or the European Molecular Biology Laboratory (EMBL) and the accession number quoted.

Some specific examples are as follows

- *Immunohistochemistry/immunofluorescence* All antibody sources should be provided preferably with clone names so that that they can be clearly identified. Details of secondary antibodies and their conjugated fluorochromes or enzymes should also be provided. Negative controls should also be clearly stated and the

data shown. Methodology should include dilutions, incubation conditions, detection methods and method of data analysis. Expression data should be supported by Western blotting and/or reverse-transcriptase PCR.

- *Quantitative PCR data* Please follow the MIQE guidelines (Bustin SA, Benes V, Garson JA *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009; **55**:611–22). Briefly, this should include details on RNA quality, reverse-transcription conditions, PCR conditions including PCR primer sequences, how reference genes were chosen and how the data were analysed.
- *Proteomics and genomics* Data should provide novel insight into the pathogenesis or mechanisms of action of treatment of skin disease and be supported by further evidence of gene expression. The reasons for focusing on specific genes/pathways should be clearly explained.
- *Mutation reports* A mutation report will be considered for peer review under the following conditions:

1. Reports describing from **one to a few** previously reported mutations will be considered only if associated with a phenotype significantly different from previously reported ones (slight deviation from previous phenotypic descriptions will justify rejection).
2. Large (> 20 cases) series of patients carrying **previously reported or novel** mutations in known genes associated with **typical** phenotypes will be considered only if they provide new insights into genotype–phenotype correlation. Novelty will also be considered (i.e. large series will not be considered if such series are already available in the literature).
3. Reports of one to a few **novel** disease-causing mutations in a gene already known to be associated with the same disease will be considered for review only if no more than two mutation reports implicating the same gene in the same disease have already been published and/or the report contains significant functional data (explaining the function of the gene under study and/or the mechanisms of action of the mutations reported).
4. All mutations identified will have to be verified by direct Sanger sequencing and their pathogenicity supported by at least three of the following: (i) proof of segregation, (ii) absence in at least 300 population-matched controls, (iii) bioinformatics prediction of pathogenicity and conservation and (iv) functional assays.

- *Biomarker and association studies* Genetic association studies should include an estimation of effect size and statistical significance, as well as an estimation of the study power, to allow readers to interpret the findings appropriately. Consideration should be given to the issues of multiple testing, which are inherent in genome-wide analyses (including transcriptome analysis). Novel genetic associations should be replicated in an independent collection. Biomarker association studies should follow the REMARK guidelines [McShane LM, Altman DG, Sauerbrei W *et al.* REporting recommendations for tumour MARKer prognostic studies (REMARK). *Br J Cancer* 2005; **93**:387–91].
- *Flow cytometry* Flow cytometry data plots should be included in the body of the text or in the Supporting Information section. Types of plot, labelling of plots, labelling of plot axes, and information required in figure legends are detailed in Alvarez*et al.* (Alvarez DF, Helm K, DeGregori J *et al.* Publishing flow cytometry data. *Am J Physiol Lung Cell Mol Physiol* 2010; **298**:L127–30). The data may be compiled (in addition to plots) into table format for ease of interpretation.

Qualitative studies

In addition to the usual bulleted statements for original articles (What's known and What's new), manuscripts describing qualitative studies must also include a third bulleted statement (maximum 70 words) in answer to the following question:

- What are the clinical implications of the work?

General policy

The *BJD* will publish high-quality studies using qualitative methods (and mixed-methods studies that include qualitative elements) of direct relevance to understanding and managing the impact of skin conditions in humans.(link to Nelson editorial *BJD*).

Publication standards for qualitative research

- *Scope of publications* Publications will provide significant insight into the perspectives and/or experiences of individuals or groups (e.g. patients, carers, clinicians) in relation to the context and process of dermatology or dermatological care. This includes (but is not restricted to) studies on psychological well-being; social functioning; quality of life (including the development of new patient-reported outcome measures); self-management/coping; patient–professional communication; treatment decision making; clinician training; and service content, organization and delivery, including interventional studies where qualitative components may inform the intervention or its implementation or serve to evaluate outcomes and process issues.

- *Human subjects* All studies must conform to appropriate ethical standards and must have been approved by the relevant ethical committees and authors' and institutional review board(s). A statement to this effect with the exact name of the approving institutional review board(s) should be included in the Materials and methods section. All clinical investigations reported must be conducted according to the principles expressed in the Declaration of Helsinki. Authors must take adequate measures to protect participants' privacy in the reporting of any qualitative research submitted to the *BJD*, for example by anonymizing the names of individuals/locations. While the aim in qualitative research is to preserve the richness and context of the data, authors must ensure as far as possible that any quotations/images used do not contain unique contextual features that would enable participants to be identified.

Reporting recommendations

- When preparing manuscripts for submission to the *BJD*, authors should consider and address (where appropriate) the SRQR (Standards for Reporting Qualitative Research)recommendations (O'Brien BC, Harris IB, Beckman TJ *et al.* Standards for reporting qualitative research: a synthesis of recommendations. *Acad Med* 2014; **89**:1245–51). The SRQR tool comprises broad items that aim to make the reporting of qualitative research more transparent and assist authors in manuscript preparation. It is accessible at the EQUATOR Network website. Reviewers and editors will also use these standards as a guide for evaluating manuscripts for the *BJD*.
- The *BJD* acknowledges the diversity and range of qualitative research methods and perspectives, as well as the flexibility that is core to these approaches. It is recognized that not all items in the list will be applicable to all types of study (e.g. a detailed 'reflexivity' section will be less applicable to a content analysis than to a phenomenological study), and authors will not be required to submit a completed checklist against the 21 items in the SRQR tool.
- In addition to **Title and Summary**, authors must demonstrate in the presentation of their manuscripts that due consideration has been given to any particular requirements for SRQR topics relevant to their research from among **Introduction** (problem formulation, purpose of the research); **Methods** (qualitative approach and research perspective, researcher characteristics/reflexivity, context of the research, sampling strategy, ethical issues, data collection methods and instruments, units of study, data processing and analysis, techniques to enhance trustworthiness); **Results** (data synthesis and interpretation, links to empirical data); **Discussion** (integration with prior work, implications, transferability, contribution to the field, limitations) and **Other** (conflicts of interest and funding). Studies using a range of methods (e.g. in-depth interviews, observational research, documentary analysis) and methodological perspectives (e.g. content analysis, grounded theory, discourse analysis) will be considered.

Perspectives

The *BJD* welcomes submissions to the Perspectives section covering a wide variety of topics relevant to contemporary dermatology health care and research. Such pieces can be opinion essays, which are similar in style to editorials, but are not tied to a particular article and clearly represent the views and opinions of the author(s), not the *BJD*. Perspectives articles can also address a range of social aspects of medicine and health care that are relevant to the practice of dermatology throughout the world. Perspectives should be concise, accessible and carefully crafted pieces; they are limited to 800 words. Perspectives can include one figure or table and a maximum of 10 references. Perspectives are usually invited articles, but may also be submitted as unsolicited articles as long as they conform to the above instructions to authors.

Case reports

The *BJD* includes only case reports or case series that can potentially modify the way in which dermatologists work, or raise new hypotheses, being the starting point of further research. Authors should use the CARE guidelines as a guiding framework. Case reports should not exceed 1200 words of body text, with up to 15 references and four tables or figures. They must include an abstract (unstructured, maximum 250 words) and bulleted statements (maximum 70 words) in answer to the following questions: What's already known about this topic? and What does this study add? Please see the Ethics section of these guidelines in Section 5 regarding preservation of patients' privacy.

Correspondence

The correspondence section includes various different types of letters. These include *BJD* Research Letters, Rapid Response to Recently Published Original Articles (RRR-POA) published by the *BJD*, and Opinion Pieces.

The *BJD* regards Research Letters as its most prestigious form of correspondence. These should not exceed 800 words, 10 references, one figure and one table.

- The *BJD* encourages readers' participation through rapid response to recently published articles. These are intended to provide postpublication peer review. They should be sent within 1 month of publication of the original paper and will be published by fast track. As a form of peer review, rapid responses should have the aim of adding to the author's interpretation, enriching the original paper and increasing the value for readers. They should be scholarly, objective, referenced, respectful to other authors, concise and to the point. Authors should declare any competing interests. This type of letter should not exceed 350 words, four references and one figure. The authors of the original paper are strongly encouraged to give an answer in the same format.
- Opinion Pieces as an item of correspondence are also welcome, being an opportunity to publish original ideas, innovations, scholarly opinions, debates and controversies at an early stage of academic development. They should not exceed 400 words, four references and one figure.
- All items of correspondence should be formatted in one continuous section, with no bulleted statements or abstract. They can be published online or printed at the discretion of the editors.
- For all forms of correspondence, authors are advised to seek their own peer review by local scholars (who can be acknowledged) prior to submission.

Cover Image/Image Gallery

Submissions of diagrams, scientific figures and photographs are requested for the *BJD* Cover Image, to be printed on the front cover of the print version of the Journal. The image should be submitted with a concise, scholarly caption of up to 100 words and up to two references. Photographs or images should be visually arresting, of high quality with translational impact/ educational value. A separate figure legend should not be submitted. Images will be selected based on scientific merit, originality, relevance to the Journal readership and value added to our understanding of dermatological science.

Submissions are also invited to the *BJD* Home Page Image Gallery, using the same rubric as above. As well as scientific submissions, clinical images are welcomed, provided the case has been worked up to extend our knowledge of dermatology. 'Textbook' type submissions are discouraged. Composite images with up to three panels are permitted, for example providing histopathology, immunostains or electron microscopy to accompany the clinical image.

Editorials/Commentaries

Editorials and Commentaries are typically commissioned by the Editors. However, suggestions for such articles are welcomed and should be directed to the editorial office.

3. SUBMISSION OF MANUSCRIPTS

All submissions should be made online at the *BJD* ScholarOne Manuscripts site (formerly known as Manuscript Central). New users should first create an account. Once a user is logged onto the site, submissions should be made via the Author Centre.

Submissions must be accompanied by a completed Author Consent Form. Completed forms must be uploaded to ScholarOne Manuscripts at the same time as manuscript submission using the file designation 'Supplementary file not for review'.

4. PREPARATION OF MANUSCRIPTS

- Manuscripts must be written in British English.
- Manuscript text must be saved in Word (.doc or .docx) or rich text format (.rtf). Do not submit text in PDF format (.pdf). Figures must be saved as separate figure files. GIF, JPEG, PICT or BMP files are acceptable for submission, but only TIFF or EPS files are suitable for printing. After acceptance, you will be contacted to provide print-quality figures if you have not already done so. Please note that if you are able to supply figures in PDF format (.pdf) only, they must be distilled using the 'print optimized' option.
- Abbreviations must be defined when first used in the abstract and in the main text, as well as when first used in table and figure captions.
- Manuscripts must be as succinct as possible. Repetition of information or data in different sections of the manuscript must be carefully avoided. Text must comply with the word limits defined in Section 2, and, where appropriate, include the following.

Title page

The first page of all manuscripts should contain the following information:

1. The title of the paper;
2. A running head not exceeding 70 characters (not needed for correspondence items);
3. Manuscript word, table and figure count;
4. Names of authors as initial(s) followed by surnames;
5. Names of the institutions at which the research was conducted, clearly linked to the respective authors using superscript Arabic numbers;
6. Name, address and e-mail address of the corresponding author;
7. A statement of all funding sources that supported the work;
8. Any conflict of interest disclosures (see Section 5);
9. Bulleted statements (maximum 70 words per question) in answer to each of the following questions: What's already known about this topic? and What does this study add? (not applicable to correspondence items).

Summary (abstract)

- Authors submitting original articles should note that structured Summaries are required. The Summary should adopt the format: Background, Objectives, Methods, Results, Conclusions.
- Review Articles and Case Reports require Summaries but they should not be structured.
- Summaries should contain no citations to previously published work.
- Correspondence items do not require Summaries.

Text

The text should in general, but not necessarily, be divided into sections with the headings Summary, Introduction, Materials and methods (or Patients and methods), Results, Discussion, Acknowledgments, References, Supporting Information, figure legends.

Tables and figures

- Tables should not be inserted in the appropriate place in the text but should be included at the end of the manuscript, each on a separate page.
- Figures must be submitted as a separate file or files.
- Tables and figures should be referred to in text as follows: Figure 1, Figures 2–4 (but when given in brackets: Fig. 1, Figs 2–4); Table 1, Tables 2, 3. Each table and/or figure must have a legend that explains its purpose without reference to the text. Where a figure has more than one panel, each panel should be labelled in the top left-hand corner using lower-case letters in parentheses – (a), (b) etc. – and a brief description of each panel given in the figure legend.
- Colour illustrations are welcomed and all colour is published free of charge to the author.
- Authors are themselves responsible for obtaining permission to reproduce previously published figures or tables. When an individual is identifiable in a photograph, written permission must be obtained (see Section 5).

Electronic artwork

Vector graphics (e.g. line artwork) should be saved in encapsulated postscript format (.eps), and bitmap files (e.g. photographs) in tagged image file format (.tif). Line art must be scanned at a minimum of 800 dpi and photographs at a minimum of 300 dpi.

References

References should be in Vancouver format and appear as consecutive, unbracketed superscript numbers in the text, e.g. 'in our previous reports [1,2] and those of Smith *et al.*[3–5]', and should be listed numerically in the reference list at the end of the article.

Format references as below, using standard (Medline) abbreviations for journal titles. If there are more than four authors, include the first three authors followed by *et al.*

1. de Berker DAR, Baran R, Dawber RPR. The nail in dermatological diseases. In: *Baran and Dawber's Diseases of the Nails and Their Management* (Baran R, Dawber RPR, de Berker DAR, Haneke E, Tosti A, eds), 3rd edn. Oxford: Blackwell Science Ltd, 2001; 172–92.
2. Shuster S. The nature and consequence of Karl Marx's skin disease. *Br J Dermatol* 2008; **158**:1–3.
3. Graham-Brown R, Burns T. *Lecture Notes: Dermatology*. Oxford: Wiley-Blackwell, 2006.

4. Smith A. Select committee report into social care in the community. Available at: http://www.dhss.gov.uk/reports/report015285.html (last accessed 7 November 2003).

We recommend the use of a tool such as EndNote for reference management and formatting. EndNote reference styles can be found here.

Reporting standards and statistics

- All manuscripts should follow the SAMPL guidelines for general reporting of the methods and results. Aforementioned study-specific guidelines (e.g. CONSORT, STROBE) should be used for details of the statistical analysis.
  Methods should at least:
  - be described with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results
  - Indicate the aim of the statistical analysis (primary objective, secondary objective, exploratory or ancillary analysis)
  - Report the minimal clinical important difference (if possible)
  - Describe how missing data was handled (if data is missing)
  - Report the alpha-level (one or two-sided) and the statistical package

  Results should at least:

  - Be presented in the same order of importance as described in the methods
  - Include effect sizes and their 95 % confidence intervals with the appropriate degree of precision, in addition to p-values
  - Include both absolute and relative measures
  - Provide enough detail that the results can be incorporated into other analysis

  In addition to these recommendations, all applicable general and study-specific SAMPL guidelines should be followed. Further explanation can be found in the editorial 'Guidelines for statistical reporting in the British Journal of Dermatology'

Supporting Information

The *BJD* encourages the submission of underlying datasets, appendices, movie files etc. as online-only Supporting Information. Supporting Information should be uploaded during manuscript submission (see Section 3) using the file designation 'Supplementary file for review'.

Supporting Information should be important ancillary information that is relevant to the parent article but that does not or cannot appear in the printed edition of the Journal. Supporting Information will be published as submitted, and will not be corrected or checked for scientific content, typographical errors or functionality.

5. DECLARATIONS

Original publication

Submission of a manuscript will be held to imply that it contains original unpublished work and is not being submitted for publication elsewhere at the same time. The author must supply a full statement to the Editor about all submissions and previous reports that might be regarded as redundant or duplicate publication of the same or very similar work. The *BJD* employs a plagiarism detection system. By submitting your manuscript, you accept that your manuscript may be screened for plagiarism against previously published works.

Conflicts of interest

Authors are responsible for disclosing all financial and personal relationships between themselves and others that might be perceived by others as biasing their work. To prevent ambiguity, authors must state explicitly whether potential conflicts do or do not exist.

Ethics

When reporting experiments on human subjects, indicate whether the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional or regional) and with the Declaration of Helsinki of 1975, as revised in 1983. Do not use patients' names, initials or hospital

numbers, especially in illustrative material. When reporting experiments on animals, indicate whether the institution's or a national research council's guide for, or any national law on, the care and use of laboratory animals was followed. A statement describing explicitly the ethical background to the studies being reported should be included in all manuscripts in the Materials and methods section. Ethics committee or institutional review board approval should be stated. Exceptions are observational studies using routinely collected anonymous data such as regional/national cancer registries, claims data from insurance companies, or other national registries. If studies using these types of data do have approval from specific data protection agencies, please state accordingly.

Patients have a right to privacy that should not be infringed without informed consent. Identifying information should not be published in written descriptions, photographs and pedigrees unless the information is essential for scientific purposes and the patient (or parent or guardian) gives written informed consent for publication. Identifying details should be omitted if they are not essential, but patient data should never be altered or falsified in an attempt to attain anonymity. Complete anonymity is difficult to achieve and informed consent should be obtained if there is any doubt. For example, masking the eye region in photographs of patients is an inadequate protection of anonymity.

Authorship

All persons designated as authors should qualify for authorship, and all those who qualify should be listed. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content. One or more authors should take responsibility for the integrity of the work as a whole, from inception to published article. Authorship credit should be based only on (i) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; (ii) drafting the article or revising it critically for important intellectual content and (iii) final approval of the version to be published. Conditions (i), (ii) and (iii) must all be met. Acquisition of funding, the collection of data or general supervision of the research group, by themselves, do not justify authorship. All others who contributed to the work who are not authors should be named in the Acknowledgments section.

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