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IMPACT OF SESQUITERPENE LACTONES ON THE CUTANEOUS INFLAMMATORY RESPONSE. A SYSTEMATIC REVIEW, *IN SILICO, IN VITRO* AND *IN VIVO* INTEGRATED APPROACH

Alfenas/MG

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Tese apresentada como parte dos requisitos para obtenção do título de Doutora em Biociências Aplicadas à Saúde pela Universidade Federal de Alfenas. Área de concentração: Fisiopatologia.

Orientador: Prof. Dr. Rômulo Dias Novaes

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Dedico este trabalho aos meus pais Dercílio e Cleusa
por nunca me deixarem desistir.

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“Consagre ao Senhor tudo o que você faz, e os seus planos serão bem-sucedidos”.

(Provérbios, 16:3)

ABSTRACT

Sesquiterpene lactones (SL) are indicated as potential scaffolds for anti-inflammatory drug design. However, cutaneous hypersensitivity reactions are proposed as limitations for SL therapeutic use. In addition, the anti-inflammatory applicability of SL remains underestimated since the impact of SL on inflammatory nociception and tissue repair are overlooked. Therefore, we initially developed a systematic review investigated the impact of SL on the skin and skin-related cells. Then, we investigated the impact of tagitinin F (TAG-F) on LPS-challenge macrophages, carrageenan-induced paw edema and mechanical hyperalgesia, and excisional skin wounds in mice. Thirty studies and forty-nine SL were analyzed in our systematic review. *In vivo* studies indicated that most SL induced cutaneous contact dermatitis associated with edema, erythema, and inflammatory infiltrate. Conversely, *in vitro* evidence indicated a dose-dependent anti-inflammatory effect of SL, which were mainly associated to NF-κB, cytokines, 5-lipoxygenase (5-LOX) and cyclooxygenase (COX-2) downregulation in keratinocytes, fibroblasts and cells involved in cutaneous immunological responses. From molecular docking, different affinity between SL and the enzymes 5-LOX, COX-2, MMP-1, 2 and 9 was identified. The current evidence supports the cutaneous immunomodulatory effects of SL. Although *in vitro* and *in vivo* studies indicate opposite anti- or pro-inflammatory effects, this contradiction exhibits a dose-dependent component. In our experimental study, RAW 264.7 macrophages in culture were challenge with LPS and treated with TAG-F (10, 50, 100, 500 μM). The paw of BALB/c mice was injected with carrageenan, treated with 0.5% and 1% TAG-F and evaluated during 6h post-treatment. Excisional wounds were also produced in BALB/c mice and treated with 0.5% and 1% TAG-F during 7 days. Our results indicated a consistent dose-dependent downregulation in 5-LOX, COX-1 and COX-2, matrix metalloproteinase (MMP-1 and MMP-2) activity; as well as attenuation in prostaglandin E2 (PGE2), leukotriene B4 (LTB4), and tumor necrosis factor-α (TNF-α) production in both models *in vitro* and *in vivo*. *In vivo*, TAG-F also attenuated carrageenan-induced paw oedema and mechanical hyperalgesia in mice. From the excisional skin wound, TAG-F was also effective in reducing neutrophils and macrophages infiltration and stimulating collagen deposition in the scar tissue, accelerating tissue maturation. Together, our findings indicate that the anti-inflammatory effect of TAG-F is more comprehensive than previously suggested, exerting a significant impact on the control of inflammatory pain and modulating central metabolic processes linked to skin wounds healing.

Keywords: Dermatitis, experimental pathology, sesquiterpene lactones.

RESUMO

As lactonas sesquiterpênicas (LS) são moléculas potencialmente úteis para o desenho de medicamentos anti-inflamatórios. No entanto, reações de hipersensibilidade cutânea são indicadas como limitações para o uso terapêutico de LS. Além disso, a aplicabilidade anti-inflamatória dessas moléculas permanece subestimada, uma vez que o seu impacto na nocicepção inflamatória e no reparo tecidual é negligenciado. Portanto, inicialmente desenvolvemos uma revisão sistemática que investigou o impacto de SL na pele e em células relacionadas à pele. Em seguida, investigamos o impacto da tagitinina F (TAG-F) sobre macrófagos estimulados por LPS, edema de pata induzido por carragenina e hiperalgesia mecânica, bem como em feridas excisionais de pele em camundongos. Trinta estudos e quarenta e nove LS foram revisados. Estudos *in vivo* indicaram que a maioria das LS causam dermatite associadas a edema, eritema e infiltrado inflamatório. Por outro lado, evidências *in vitro* indicaram um efeito anti-inflamatório dose-dependente de LS, o qual foi principalmente associado a inibição de NF-κB, citocinas, 5-lipoxigenase (5-LOX) e cicloxigenase-2 (COX-2) em queratinócitos, fibroblastos e células envolvidas na resposta imunológica cutânea. Na docagem molecular, foram identificados diferentes graus de afinidade entre LS e as enzimas 5-LOX, COX-2, MMP-1, 2 e 9. A evidência atual apoia os efeitos imunomoduladores cutâneos das LS. Embora estudos *in vitro* e *in vivo* indiquem efeitos anti- ou pró-inflamatórios opostos, essa contradição exibe um componente dose-dependente. Em nosso estudo experimental, macrófagos RAW 264.7 em cultura foram desafiados com LPS e tratados com TAG-F (10, 50, 100, 500 μM). A pata de camundongos BALB/c foi injetada com carragenina e tratada com TAG-F a 0,5% e 1%. Feridas excisionais também foram produzidas em camundongos BALB/c e tratadas com 0,5% e 1% de TAG-F durante 7 dias. Nossos resultados indicaram que TAG-F provocou inibição dose-dependente da atividade da 5-LOX, COX 1 e 2, metaloproteinases da matriz 1 e 2, além de atenuar a produção de prostaglandina E2, leucotrieno B4 e fator de necrose tumoral-α em ambos os modelos *in vitro* e *in vivo*. *In vivo*, TAG-F atenuou o edema de pata induzido por carragenina e a hiperalgesia mecânica em camundongos. Em ferida excisional de pele, TAG-F reduziu a infiltração de neutrófilos e macrófagos e estimulou a deposição de colágeno no tecido cicatricial, acelerando a maturação do tecido. Nossos achados indicaram que o efeito anti-inflamatório do TAG-F é mais abrangente do que previamente sugerido, exercendo um impacto significativo no controle da dor inflamatória e na modulação de processos metabólicos centrais ligados à cicatrização de feridas na pele.

Palavras-chave: Dermatite, patologia experimental, lactonas sesquiterpênicas.

LISTA DE ABREVIATURAS E SIGLAS

GM-CSF - fator estimulador de colônia de macrófagos-granulócitos

MAPK - proteínas quinases ativadas por mitógenos

IP-10 – interferon gama induzido por proteína 10

MIP-2 - proteína inflamatória de macrófagos 2

NF- κB - fator de transcrição nuclear-κB

TNF-α – fator de necrose tumoral alfa

MMP – metaloproteinase de matriz

DNCB – 2,4-dinitroclorobenzeno

LS – lactona sesquiterpênica

AP-1 - proteína ativadora-1

IFN-γ – interferon gama

COX – cicloxigenase

iNOS – óxido nítrico

TAG-F – tagitinina F

LOX – lipoxigenase

IL - interleucina

SUMÁRIO

1 INTRODUÇÃO GERAL

Lactonas sesquiterpênicas (LS) são um grupo de metabólitos secundários da classe dos sesquiterpenóides identificados principalmente em plantas da família Compositae e Asteraceae, que possuem ampla distribuição geográfica em todo o mundo (CHAGAS-PAULA et al., 2012, ABE et al., 2015; TAGNE; MARINO; COSENTINO, 2018). Embora estudos etnobotânicos e etnomédicos tenham documentado o uso sistemático de plantas ricas em LS na medicina popular nas culturas oriental e ocidental, seus efeitos, aplicabilidade, mecanismos de ação e segurança biológica permanecem pouco explorados (DUPUIS et al., 1980; MÁÑEZ et al., 1999; SECA et al., 2014).

O mecanismo de ação do SL não é totalmente compreendido. No entanto, há evidências consistentes indicando que os efeitos biológicos induzidos por essas moléculas são dependentes do grupo funcional α-metileno-γ-lactona, que é tipicamente encontrado em LS (ARANTES et al., 2011; AMORIM et al., 2013).

Embora algumas LS exerçam efeitos antioxidantes e antiinflamatórios (LEE et al., 2018; WANG et al., 2018; ZHANG et al., 2018), um grupo dessas moléculas é capaz de induzir citotoxicidade cutânea, cujo potencial pró-inflamatório está associado ao desenvolvimento de dermatite grave (FRAGINALS et al., 1991; SCHMIDT E CHUNG, 1993; HOFMANN et al., 2014). Reações cutâneas clássicas induzidas por LS, como edema, congestão vascular, eritema, coceira, hiperalgesia, recrutamento de leucócitos, bolhas na pele e descamação revelam que essas moléculas desencadeiam eventos imunomediados cutâneos potentes (BARBIER E BENEZRA, 1982; CHEMINAT; STAMPF; BENEZRA, 1984; FRAGINALS et al., 1991). Normalmente, a hipersensibilidade da pele é mediada por células, citocinas (GRABBE E SCHWARZ, 1998; LEVIN E MAIBACH, 2002) e enzimas da pele, como ciclooxigenase (COX) (KIM E CHOI, 2019), lipoxigenase (LOX) (OLIVEIRA et al., 2013) e metaloproteinases de matriz (MMP) (LOHBERGER et al., 2013).

No entanto, estudos anteriores sugerem que as reações cutâneas a diferentes tipos de LS podem estar associadas à ativação diferencial de vias metabólicas anti-inflamatórias (LIN et al., 2016; WANG et al., 2018) e pró-inflamatórias (BARBIER E BENEZRA, 1982; CHEMINAT; STAMPF; BENEZRA, 1984). Como as evidências atuais são fragmentadas, ainda não está claro quais vias de sinalização celular envolvidas no controle da resposta imune da pele são moduladas pelo LS. Na hipersensibilidade cutânea, os produtos metabólicos COX, LOX e MMP têm estado diretamente envolvidos na ativação de vias de sinalização imunomoduladoras centrais, como NF-κB (NAM et al., 2015; LEE et al., 2018) e MAPK / ERK (KIM et al., 2015).

Dentro do grupo LS, diferentes tipos de tagitininas (A, C e F) têm se destacado por compartilhar propriedades antimicrobianas, antiparasitárias e antiinflamatórias (CHAGAS-PAULA et al., 2015a, b; GONÇALVES-SANTOS et al., 2019). Em estudos desenvolvidos anteriormente, baixas concentrações de tagitinina F (TAG-F) foram eficazes no alívio da dermatite induzida por irritante de contato, um efeito mediado pela inibição dupla da atividade COX-2 e 5-LOX, bem como da prostaglandina, produção de leucotrieno e TNF-α; que induziu uma redução acentuada da inflamação cutânea (CHAGAS-PAULA et al., 2015a, b). Embora esses efeitos indiquem o potencial biotecnológico da tagitinina F para o desenvolvimento de terapias antiinflamatórias, a extensão desses efeitos e a aplicabilidade dessa molécula em diferentes condições inflamatórias permanecem incertas.

Neste sentido, a introdução de novas moléculas terapêuticas para aplicação comercial exige uma avaliação detalhada de seus riscos e benefícios a serem oferecidos, o que torna relevante o estudo das LSs ao longo dos anos, assim como sua aplicabilidade terapêutica no decorrer da evolução dos estudos de pesquisa biomédica. Afinal, as lactonas sesquiterpenicas seriam moléculas anti ou pró-inflamatórias para o sistema imune e para as alterações relacionadas à pele?

Para responder esta pergunta, esta tese tem como objetivo avaliar a resposta imunológica da pele frente à administração tópica das lactonas sesquiterpênicas a partir de uma revisão sistemática e uma abordagem integrada *in silico*, e a partir de uma experimentação *in vitro* com macrófagos e *in vivo*, em modelos animais de edema de pata induzido por carragenina e modelos de ferida excisional, a fim de evidenciar a aplicabilidade terapêutica das lactonas sesquiterpênicas, em especial da tagitinina F.

2 REVISÃO DE LITERATURA

2.1 Lactonas sesquiterpênicas

As lactonas sesquiterpênicas (LSs), ou sesquiterpenlactona, ou ainda sesquiterpenos lactonizados, como também podem ser chamados, são compostos disponíveis na natureza, originados de um grande grupo de metabólitos secundários da família *Asteraceae* (BARUAH et al., 1994). Com diferentes aplicabilidades biológicas, as lactonas sesquiterpênicas possuem um grande potencial para utilização na medicina, devido às suas atividades antitumoral e citotóxica (ARNASON et al., 1987), antibacteriana e antifúngica (PICMAN, 1986), anti-inflamatória (ABAD et al., 1994), antimalárica (FRANÇOIS et al., 1996), e esquistossomicida (VICHNEWISK et al., 1976).

Dentre os vários grupos constituintes dos produtos naturais, os terpenos, isoprenóides e terpenóides estão presentes em maior ambudância. Os terpenoides por sua vez, dão origem aos sesquiterpenos, que após um processo biossintético, passa a apresentar um grupo lactona em seu esqueleto carbônico, dando origem as lactonas sesquiterpênicas, classificadas com bases em seus esqueletos carbocíclicos e caracterizando sua atividade biológica (MANN et al., 1994; RODRIGUEZ; TOWERS; MITCHELL, 1976).

Principalmente, a ação das LSs se dá pelo mecanismo de alquilação, a partir do estabelecimento de ligações covalentes com as moléculas biológicas. Apesar de vários estudos sobre esses compostos, esse mecanismo de ação é muito inespecífico, apresentando baixa seletividade, e portanto, toxicidade inespecífica, de acordo com a via de administração e com a dose utilizada (SCHMIDT, 2006).

Desde muitos anos, humanos e animais são expostos às LSs de várias formas, até mesmo pelo fato de estarem presentes em espécies de plantas silvestres, principalmente, pelo contato direto com essas plantas ou pela administração de compostos medicinais ou medicamentos à base de LS. Como o uso empírico de plantas tem sido amplamente praticado por milhares de anos, as LSs vêm sido estudadas pelo seu valor terapêutico e pela sua capacidade de causar reações de hipersensibilidade e toxicidade (GURIB-FAKIM, 2006). Dentre as dermatites, ou reações de hipersensibilidade, vários estudos vêm mostrando o potencial irritativo e alergênico das plantas contendo LSs (STAMPF et al., 1978; PICMAN; PICMAN; TOWERS et al., 1982; FRAGINALS et al., 1991; SCHMIDT E CHUNG, 1993).

2.2 Dermatites e reações de hipersensibilidade

As dermatites e dermatoses são alterações referentes à pele, que podem ser agudas ou crônicas. As dermatites são inflamações mediadas por fatores imunológicos locais ou sistêmicos, de diversas causas, sendo que algumas permanecem desconhecidas. Normalmente, as alterações agudas se apresentam por dias a semanas, caracterizadas por inflamação, edema, e lesões epidérmica, vascular ou subcutânea. Enquanto as lesões do tipo crônicas podem persistir por meses a anos, e frequentemente, apresentam estruturas significativas de crescimento epidérmico alterado ou fibrose dérmica (MURPHY; MIHM; MARTIN, 2000). As dermatites podem ser divididas em: dermatite de contato ou hipersensibilidade de contato, dermatite seborreica, dermatite numular, dermatite atópica e dermatite herpetiforme (SAMPAIO; CASTRO; RIVITTI, 2000).

As dermatoses referem-se a um conjunto de doenças de pele caracterizadas por manifestações mais graves, como bolhas e escamações. Entre as dermatoses agudas, estão a urticária, dermatite eczematosa aguda e o eritema multiforme. As dermatoses crônicas são principalmente, a psoríase, o líquen plano e o lúpus eritematoso (MURPHY; MIHM; MARTIN, 2000).

A dermatite de contato é uma alteração definida por inflamação e prurido na pele (BÁNVÖLGYI et al., 2005) causada por contato direto com agentes externos que possuem substâncias irritantes, com potencial de ação tóxica (dermatite de contato irritativa), ou compostos alergênicos, os quais provocam uma reação de hipersensibilidade tardia (dermatite de contato alérgica) (ENGLISH, 2004). A dermatite de contato é a alteração de pele mais comum nos países industrializados, sendo uma das doenças ocupacionais mais frequentes (SAINT-MEZARD et al., 2004; BELSITO, 2000). A dermatite do tipo irritativa é marcada por três mudanças patofisiológicas principais, além do infiltrado neutrofílico: destruição da barreira da pele (estrato córneo), alterações nas células da epiderme e a liberação de mediadores, todos interligados (SMITH; BAKER; WILLIAMS-JR, 2002). Entre os mediadores destacam-se TNF-α, IFN-γ, GM-CSF, IL-1a, IL-2, IL-6, IL-8, IL-10, IP-10, MIP-2 com predominância das células CD4+ sobre as CD8+ (LEVIN E MAIBACH, 2002).

A dermatite de contato alérgica, ou também conhecida hipersensibilidade de contato, é marcada pelas alterações da pele provenientes de células T, com uma resposta do tipo tardia (GRABBE E SCHWARZ, 1998). O processo se dá pela ligação dos antígenos sensibilizantes, também conhecidos como haptenos, às proteínas da epiderme do hospedeiro, uma vez que por serem moléculas instáveis, de baixo peso molecular e não imunogênicas, não possuem capacidade por si, e necessitam de ser ligar às proteínas, iniciando um processo inflamatório pela ativação da imunidade inata (SAINT-MEZARD et al., 2004). Desta forma, mediadores

também são ativados, entre eles TNF-α, IFN-γ, GM-CSF, IL-1a, IL-6, IP-10, MIP-2, IL-1b, IL-4. Além disso, assim como na dermatite irritativa, há uma prevalência das células T CD4+, em comparação a TCD8+ (LEVIN E MAIBACH, 2002).

Alteração de pele comum, que também afeta grandemente o indivíduo acometido, é a psoríase. Caracterizada por uma alteração cutânea inflamatória com preferência pela pele e as articulações, a psoríase é uma doença multifatorial, envolvendo fatores genéticos, imunológicos e ambientais, que se interligam até a permanência das manifestações clínicas da pele e articulares (RODRIGO et al., 2010; RUIZ; AZEVEDO; SANTOS, 2012). Fisiopatologicamente, ocorre uma hiperproliferação e diferenciação anormal da epiderme, levando a uma queratinização incompleta, devido ao encurtamento do ciclo epidérmico. Além disso, observa-se infiltração de linfócitos T, especialmente CD4+, células dendríticas, mastócitos e neutrófilos. Como uma cascata, ao ser ativadas células do sistema imune, mediadores inflamatórios também são chamados, como citocinas do tipo Th1, particularmente INF-γ, TNF-α e IL-12, citocinas do tipo Th17, IL-23, IL-17, IL-22 (RODRIGO et al., 2010). Além disso, os queratinócitos, as células dendríticas e as células T CD4+ e CD8+ também são responsáveis pela ativação de uma série de citocinas pró-inflamatórias, vinculando o processo inflamatório da psoríase (SANCHEZ, 2010).

Porquanto, em uma inflamação cutânea, inicialmente, uma cascata de fatos é desencadeada após um estímulo na pele e, em suas respectivas células, incluindo aumento da microcirculação e da permeabilidade vascular, aumento do recrutamento de leucócitos, aumento da interação entre os tipos celulares do tecido e consequente secreção de diversos mediadores pró-inflamatórios (NICKLOFF E NESTLÉ, 2004; SHERWOOD E TOLIVER-KINSKY, 2004). Em um primeiro momento, os queratinócitos são os principais envolvidos na defesa do sistema imune da pele, produzindo mediadores pró-inflamatórios, como citocinas, que aumentam após a ativação dos queratinócitos (WILLIAMS E KUPPER, 1996), bem como outras células da epiderme e da derme, incluindo fibroblastos, melanócitos, macrófagos e células endoteliais, que também estão na linha de produção de citocinas (BURBACH; ANSEL; ARMSTRONG, 2000; KUPPER, 1990). Entre as citocinas produzidas pelos queratinócitos estão as interleucinas (IL-1α, IL-1β), fator de necrose tumoral (TNF-α), fatores de crescimento, fator estimulador de colônia de macrófagos-granulócitos (GM-CSF) e algumas quimiocinas, constituindo as citocinas primárias, que vão desempenhar um papel relevante na homeostasia e na modulação da resposta imune, iniciando a cascata do processo inflamatório (UCHI et al., 2000).

Por conseguinte, as citocinas primarias ativam outras vias de sinalização, como das proteínas quinases (PKC, PKA), proteínas quinases ativadas por mitógenos (MAPK), ativando fatores de transcrição, como fator de transcrição nuclear-κB (NF- κB) e a proteína ativadora-1 (AP-1). Quando ativados, esses fatores de transcrição induzem a transcrição de citocinas (TNF-α, IL-1, IL-2, IL-6, IL-8, GM-CSF, TGFB1- fator de crescimento tumoral), de quimiocinas, moléculas responsáveis por adesão e enzimas produtoras de mediadores inflamatórios secundários (óxido nítrico (iNOS) e cicloxigenase-2 (COX-2)) (PASCUAL E GLASS, 2006; DELHASE; WINYARD; WILLOUGHBY, 2003; BARNES E KARIN, 1997). Desta forma, a IL-1 e o TNF-α são os principais responsáveis por desencadear a resposta inflamatória inata e ainda atuar na resposta imunológica (UCHI et al., 2000).

2.3 Efeitos das lactonas sesquiterpênicas na pele

Wang et al. (2018), em um estudo com células HaCat, investigaram os efeitos das lactonas sesquiterpenicas alantolactona e isoalantolactona nestas células, e comprovaram o efeito em camundongos sensibilizados com DNCB. Os autores observaram que as LSs podem inibir a ativação de NF-κB após indução de inflamação com TNF-α, além de reduzir a expressão de TNF-α, IL-1 e IL-4 em células HaCat. Ao comprovar os efeitos em animais, a aplicação tópica da LS atenuou a severidade da dermatite induzida por DNCB, o que mostra o potencial anti-inflamatório das LSs (WANG et al., 2018). Assim como outros diversos estudos, os efeitos anti-inflamatórios das LSs foram identificados em animais (RECIO et al., 2000; SOSA et al., 2001; SUR et al., 2009; LIN et al., 2016) e células da pele (KIM et al., 2015; NAM et al., 2015; LEE et al., 2018; ZHANG et al., 2018).

Contudo, da mesma forma que as LSs podem ser anti-inflamatórias, alguns estudos investigaram seus efeitos contrários, ou seja, seu potencial de induzir um processo inflamatório a partir de uma reação de hipersensibilidade de contato. Um estudo com camundongos investigou a sensibilização induzida com alantolactona em diferentes concentrações, a fim de elucidar em qual concentração a LS poderia ser irritativa. A sensibilização, assim como o teste foi realizado na orelha e o grau de irritação foi medido, assim como análise histológica foi realizada. Os autores observaram uma reação imunológica, confirmada na histologia, com infiltrado linfocitária significante, mostrando reação positiva à sensibilização com alantolactona nos animais (FRAGINALS et al., 1991). Esses achados corroboram com os resultados encontrados por outros autores, que também investigaram os efeitos sensibilizantes das LSs em animais (DUPUIS et al., 1980; BARBIER E BENEZRA, 1982; CHEMINAT;

STAMPF; BENEZRA, 1984; SCHMIDT E CHUNG, 1993) e em células (ALONSO BLASI et al., 1992; HOFMANN et al., 2014).

2.4 EFFECT OF SESQUITERPENE LACTONES ON THE SKIN AND SKIN-RELATED CELLS. A SYSTEMATIC REVIEW AND *IN SILICO* INTEGRATED APPROACH

Abstract

Although anti-inflammatory properties are attributed to sesquiterpene lactones (SL), cutaneous hypersensitivity reactions are proposed as limitations for SL therapeutic use. Therefore, this systematic review investigated the impact of SL on the skin and skin-related cells. Studies indexed in electronic databases were screened from the PRISMA strategy. Data on experimental models, SL investigated, treatment outcomes, and interactions between SL and target skin enzymes were analyzed. The risk of bias in animal studies was verified from the SYRCLE's tool. Thirty studies (15 *in vivo* and 10 *in vitro*, 5 *in vitro* and *in vivo*) and forty-nine SL were analyzed. Mice, guinea pig, keratinocytes and fibroblasts were predominantly investigated from *in vivo* and *in vitro* studies. *In vivo* studies indicated that most SL induced cutaneous contact dermatitis associated with edema, erythema, and inflammatory infiltrate. Conversely, *in vitro* evidence was consistent with a dose-dependent anti-inflammatory effect of SL, which were mainly associated to NF-κB, cytokines, 5-LOX, and COX-2 downregulation in keratinocytes, fibroblasts and cells involved in cutaneous immunological responses. From molecular docking, different affinity between SL and the enzymes 5-LOX, COX-2, MMP-1, 2 and 9 was identified, showing better inuviscolide, budlein A and α-methylene-γ-butyrolactone affinity profiles. *In vivo* studies presented uncertain to high-risk of bias mainly associated with underreporting of randomization and experimental blinding. The current evidence supports the cutaneous immunomodulatory effects of SL. Although *in vitro* and *in vivo* studies indicate opposite anti- or pro-inflammatory effects, this contradiction exhibits a dose-dependent component. In addition, the anti-inflammatory pathways activated by SL are better understood from in vitro evidence. However, additional studies are required to elucidating specific anti-inflammatory and proinflammatory mechanisms triggered by SL *in vivo*. Thus, controlling the sources of bias described in this review can contribute to improving the quality of the evidence in further investigations.

Keywords: Dermatitis, experimental pathology, natural products, sesquiterpene lactones.

1. Introduction

Sesquiterpene lactones (SL) are naturally available molecules originating from a large group of secondary metabolites from plants of the Asteraceae family (Baruah et al., 1994), examples of plants in this family are guaco (*Mikania laevigata*), gorse (*Baccharis trimera*),

fish-bake (*Vernonia polianthes*), marigold (*Calendula officinalis*), marcela (*Achyrocline satureioides*) and sunflower Mexican (*Tithonia diversifolia*) (Ayeni et al., 1997). Humans and animals have extensive exposure to SL due to the wide geographical distribution of wild plant species that produce these molecules, as well as direct contact with medicinal SL-based products (Gurib-Fakim, 2006). Ethnobotanical and ethnomedical studies has documented a systematic use of different Asteraceae species in traditional medicine (Souza, 2009; Silva and Moura, 2011), which is currently supported by modern pharmacological evidences. In this sense, SL were associated with potent antioxidant (Shoaib et al., 2017, Onoja et al., 2020), immunomodulatory (Abad et al., 1994), antitumor (Arnason et al., 1987), antibacterial, antifungal (Picman, 1986), and antiparasitic (François et al., 1996; Vichnewski et al., 1976) activities; which indicates a marked biotechnological and therapeutic potential for these molecules (Silva and Moura, 2011).

As biological effects of SL are mainly mediated by unspecific alkylation mechanism, some molecules may exhibit low selectivity and some degree of rout- and dose-dependent toxicity (Schmidt, 2006; Ivanescu et al., 2015), which brings relevant limitations to the therapeutic applicability of SL. Hypersensitivity and toxicity reactions are the most frequent side effects associated with SL-rich plants products (Gurib-Fakim, 2006). Although the allergenic potential of plants containing SL is well documented (Stampf et al., 1978; Picman et al., 1982; Fraginals et al., 1991; Schmidt and Chung, 1993), the chemical characteristics of allergenic SL and their toxic mechanisms of action remains poorly understood. However, the primary mechanism of action of SL that underlies their cytotoxicity is associated to the presence of an α-methylene-γ-lactone chemical group (αMγL), which reacts with nucleophiles (sulfhydryl or amino groups) in enzymes and transcription factors, alkylating them irreversibly (Schmidt, 2006; Arantes et al., 2011). Yet, the number of alkylating groups, lipophilicity, molecular geometry and size, chemical environment, functional groups neighboring the reactive αMγL, and the target sulfhydryl groups can also influence the effect of SL (Chaturvedi, 2011; Chadwick et al., 2013).

Although some SL exert antioxidant and anti-inflammatory effects (Lee et al., 2018; Wang et al., 2018; Zhang et al., 2018), a group of these molecules is able to inducing cutaneous cytotoxicity, whose pro-inflammatory potential is associated with the development of severe dermatitis (Fraginals et al., 1991; Schmidt and Chung, 1993; Hofmann et al., 2014). Classical cutaneous reactions induced by SL, such as edema, vascular congestion, erythema, itchy, hyperalgesia, leucocytes recruitment, skin blisters and flaking reveals that these molecules trigger potent cutaneous immunomediated events (Barbier and Benezra 1982; Cheminat et al.

1984; Fraginals et al. 1991). Typically, skin hypersensitivity is mediated by cells (i.e., keratinocytes, dendritic cells, macrophages, T lymphocytes, mast cells and fibroblasts), cytokines (i.e., Th1, Th2, Th17 and Treg) (Grabbe and Schwarz, 1998; Levin and Maibach, 2002) and skin enzymes such as cyclooxygenase (COX) (Kim and Choi, 2019), lipoxygenase (LOX) (Oliveira et al., 2013) and matrix metalloproteinases (MMP) (Lohberger et al., 2013). However, it is still poorly understood how SL interacts with these molecules to modulate the cutaneous immune response.

Previous studies suggest that skin reactions to different types of SL may be associated with differential activation of anti-inflammatory (Lin et al., 2016; Wang et al., 2018) and pro-inflammatory (Barbier and Benezra 1982; Cheminat et al., 1984) metabolic pathways. As current evidence is fragmented, it is still unclear which cell signaling pathways involved in controlling the skin's immune response are modulated by SL. In cutaneous hypersensitivity, COX, LOX and MMP metabolic products have been directly involved in the activation of central immunomodulatory signaling pathways such as NF-κB (Nam et al., 2015; Lee et al., 2018) and MAPK/ERK (Kim et al., 2015). Therefore, mapping the types of SL that induce cutaneous hypersensitivity, analyzing their chemical characteristics, the signaling pathways influenced by these molecules, as well as potential interactions with skin enzymes can contribute to broaden the understanding of the skin's immune mechanisms modulated by these molecules. In this sense, we used a systematic review framework to explore the main characteristics of the experimental models, treatments and anti- and proinflammatory effects and an integrated in silico approach to explore the potential interactions between SL and skin enzymes in order to investigate cutaneous reactions to SL. The risk of bias associated with the current evidence *in vivo* was evaluated, and the main sources of bias were also pointed out.

2. Methodology

2.1. Search strategy

The PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) statement was adopted for conducting this systematic review (Moher et al. 2009). A two-level search strategy was developed to maximize the retrieval of relevant research registers. The first level was based on a direct advanced search in the comprehensive electronic databases PubMed/Medline (www.ncbi.nlm.nih.gov/pubmed), Scopus (www.scopus.com) and Web of Science (www.webofknowledge.com). The second level (indirect search) intended to find additional studies from a detailed screening of the reference list of all relevant studies identified

in all databases (Felizardo et al., 2018). In both direct and indirect strategies, the researchers LPS and RDN independently searched original articles that investigated the cutaneous response to topical and epicutaneous administration of sesquiterpene lactones published up to January 14, 2020.

The advanced search applied to electronic databases was based on specific filters developed from two components: (i) Skin condition: normal skin or skin disease (Dermatitis, Psoriasis, Skin Disease) and (ii) Bioactive molecules (Sesquiterpenlactone, Sesquiterpene lactone, Lactonized sesquiterpene). A search filter was initially developed for PubMed/Medline according standardized descriptors (MeSH terms) organized in the hierarchical tree of the MeSH database (www.ncbi.nlm.nih.gov/mesh). To expand the recovery of relevant indexed studies and those in the indexing process, the commands [MeSH Terms] and [TIAB] were combined. The same search matrix used in the Pubmed/Medline database was adapted for Scopus and Web of Science by using the search algorithms TITLE-ABS-KEY or TS=, respectively (Pereira et al., 2017). To reduce search noise, standardized limit algorithms were applied to exclude review articles and book chapters in all databases. The full search strategy is described in table S1. No language or chronological restriction were applied in the search strategy.

2.2. Eligibility criteria

The researchers LPS and RDN performed the selection of potentially relevant studies. Initially, the researchers screened specific data of publication (authors, journal, volume, number, edition, and year) and the abstract of all papers recovered in electronic databases (Felizardo et al., 2018). Duplicate studies were excluded and only *in vivo* pre-clinical and clinical studies in animals investigating the cutaneous response to topical administration or epicutaneous of isolated sesquiterpene lactones and *in vitro* studies with skin cells or cells related to the immune response that incubated the cells with sesquiterpene lactones were submitted to the analysis of eligibility and considered for potential inclusion in the systematic review.

After initial screening, all potentially relevant studies were recovered in full-text and evaluated for eligibility. Study exclusion was based on well-defined criteria as follows: (i) studies testing synthetic substances, (ii) no full-text available, (iii) secondary studies (i.e., editorials, commentaries, letters to the editor, literature reviews without original data), (iv) grey literature (i.e., studies published in journals that are not indexed or submitted to peer review). The researchers independently analyzed the eligibility criteria and all doubts were resolved by

consensus, reached through discussion. In order to extend the recovery of relevant studies, the reference lists of the selected relevant papers were manually screened for potentially relevant papers (Felizardo et al., 2018).

2.3. Data extraction and synthesis from in vivo and in vitro studies

Considering a detailed characterization of all studies included in the systematic review, qualitative and quantitative data were extracted by using structured tables. Each table was constructed from basic methodological requirements used to characterize the studies according to different descriptive levels as follows: (i) publication characteristics: authors, years and countries; (ii) Characteristics of the experimental model: species, sex, age, weight; (iii) Characteristics of the target organ: normal skin or model of contact dermatitis; (iv) characteristics of the administered treatment: origin of sesquiterpene lactones (i.e., plant family and species, part of the plant used), bioactive formulation (i.e., extract fractions, and isolated substances), dosimetry (i.e. dose, route, frequency and duration of the treatment), type and chemical structure of sesquiterpene lactones; (v) histopathological outcomes measures: cutaneous reactions (i.e., edema, erythema, and inflammatory infiltrate); and (vi) cellular and molecular outcomes: cell death, immunological effectors (i.e., cytokines and antibodies), prooxidant (reactive oxygen species) and antioxidant (i.e., antioxidant enzymes) molecules.

The same characteristics described in the descriptive levels (i), (iv), and (vi) were also extracted and analyzed from *in vitro* studies. Parameters such as (vii) cell lineages used, and (viii) model of cell challenge, stimulation or treatment, were additionally collected and summarized in extraction tables. Research findings were respectively grouped in negative or positive outcomes when SL induced cytotoxic/proinflammatory or anti-inflammatory responses.

2.4. Risk of bias assessment from in vivo studies

The risk of reporting bias in animal studies was analyzed from the SYRCLE's (Systematic Review Center for Laboratory Animal Experimentation) tool (Hooijmans et al., 2014). This instrument is based on the Cochrane Collaboration's tool for assessing risk of bias in randomized trials and is adjusted for aspects of bias that play a specific role in animal intervention studies (Higgins et al., 2011). To increase transparency and applicability, standardized signaling questions guide the researcher judgment based on the following domains: (i) sequence generation, (ii) baseline characteristics, (iii) allocation concealment, (iv) random housing, (v) blinding, (vi) random outcome assessment, (vii) incomplete outcome data,

(viii) selective outcome, and (ix) other sources of bias (risks related to contamination and drug use, errors in analysis and risks of specific bias in each study). Two reviewers (LPS and RDN) assessed the risk of bias for each study, and disagreements were resolved by discussion and consensus. The adherence to individual quality criteria obtained in SYRCLE's toll was graphically expressed (Nogueira et al., 2018).

2.5. Molecular docking

Eicosanoids are biologically active lipid mediators derived from arachidonic acid and have an important role in injury and inflammatory responses. Cyclooxygenase-1 and cyclooxygenase-2 mediate the production of prostaglandins, while 5-lipoxygenase mediates the production of leukotrienes. These mediators can recruit immune cells to the site of injury and inflammation and interact with various wound cells, including modulation of keratinocyte activity (Sivamani, 2014). In addition to COX and LOX, matrix metalloproteinases also play an important role in the migration of keratinocytes and in wound healing (Pilcher et al, 1997), which makes these enzymes interesting in the study of the skin's immunological biology.

Molecular docking analysis was performed in Schrödinger software suite Maestro version 10.2.010 (Schrödinger, New York, USA, 2015a), using the crystal structure of 5-lipoxygenase (ALOX5, PDB code: 3V98), cyclooxygenase-2 (COX-2, PDB code: 5KIR), matrix metalloproteinase-1 (MMP-1, PDB code: 4AUO), matrix metalloproteinase-2 (MMP-2, PDB code: 1CK7) and matrix metalloproteinase-9 (MMP-9, PDB code: 5CUH). For ligand preparation, the LigPrep program was used with OPLS_3 force field (Schrödinger, New York, USA, 2015b) and ionization state for pH 7.0 ± 2.0 (using Epik) (Schrödinger, New York, USA, 2015c). The protein structures preparation was realized by the Protein Preparation Wizard program, with hydrogen bonding network optimization in pH 7.0 and minimization performed using the OPLS-3 force field in the Macromodel module (Schrödinger, New York, USA, 2015d). For the docking analysis, the Induced Fit Docking (IFD) protocol was used, which performed the prediction of the protein structure and the refinement of the compounds by the Prime program, as well as the docking and provides the score by the Glide program, considering the protein and the ligand flexible (Schrödinger, New York, USA, 2015d). The grid box area was defined as 20×20×20 Å in the active site region. The force field used was OPLS_3. The final ligand protein complexes were visualized using the Maestro interface and figures were generated using its graphical interface (Schrödinger, New York, USA, 2015a). To compare the score of the interaction between sesquiterpene lactones and enzymes, drugs commercially

known as inhibitors of 5-LOX, COX-2 and MMP-1, 2 and 9 were used, being, respectively, zileutron, celecoxib and batimastat.

3. Results

3.1. Studies identified from databases search

From our search strategy (Table S1), 580 research records were recovered, and 30 relevant studies published from 1978 to 2018 were included in the systematic review (Figure 1 - PRISMA flowchart). From all 30 papers submitted to data extraction, 10 studies (33.3%) used *in vitro* systems, 15 studies (50%) investigated animal models and 5 studies (16,6%) used experiments *in vivo* and *in vitro*. The geographic origin of all studies was France (30%, n= 9), Republic of Korea (13.3%, n= 4), China and Germany (10%, n= 3, each), Canada, Italy and Spain (6.6%, n= 2, each), followed by Japan, South Korea, Sweden, United States of America and United Kingdom (3.3%, n= 1, each) (Table S2 and Figure 2).

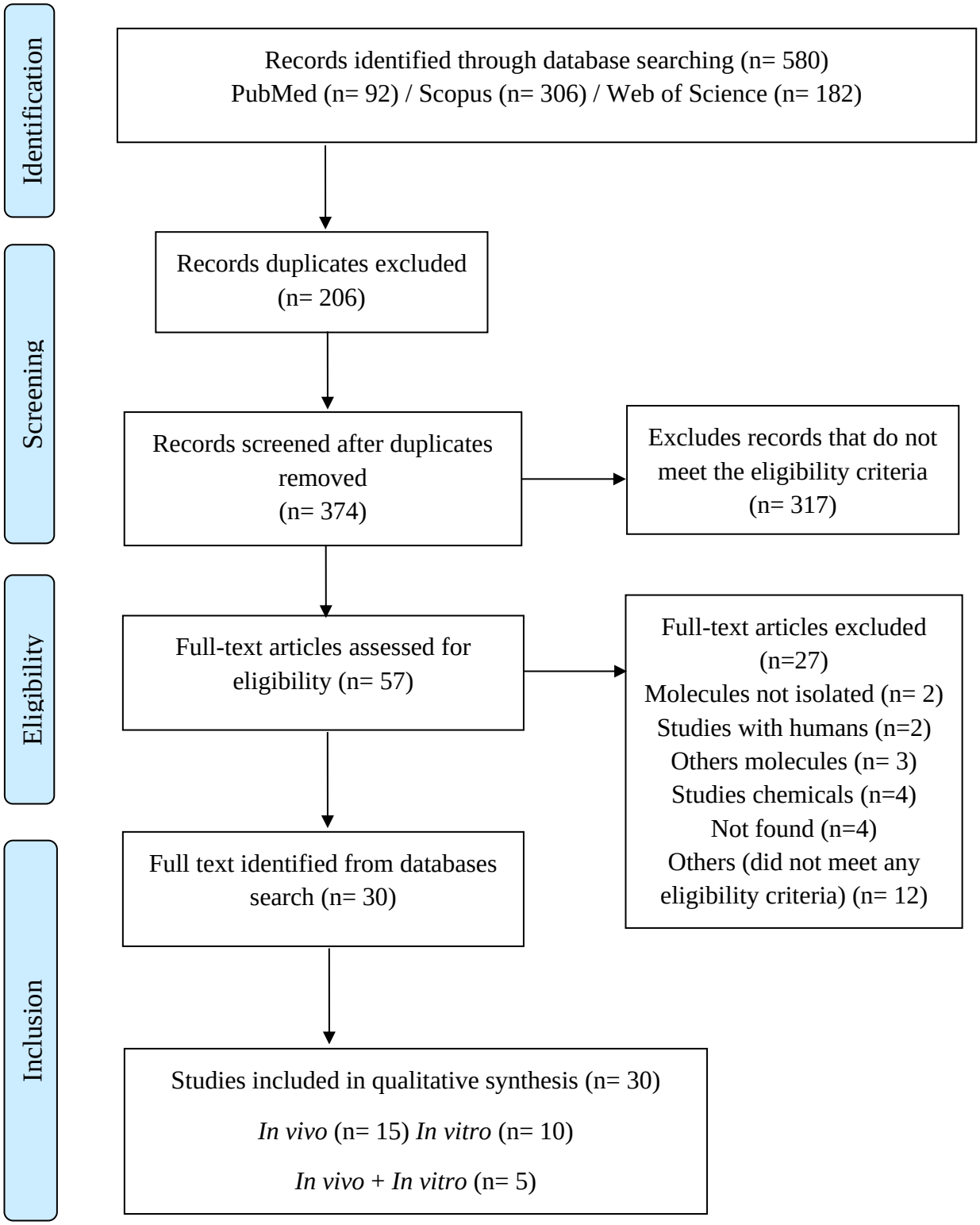

Figure 1. Flowchart detailing selection of studies included in systematic review. Based on PRISMA statement "Preferred Reporting Items for Systematic Reviews and Meta-Analyses". www.prisma-statement.org

Figure 2. Countries of study authors.

3.2. Sesquiterpene lactones sources

In vivo and *in vitro* studies investigated 49 SL (Tables S2-S5). Alantholactone and isoalantholactone were most frequently used in both *in vivo* and *in vitro* studies. All molecules analyzed in each study can be found in Tables S2 to S5, Figures 2 and 3. In most studies (30%, n= 9), SL were extracted from plants of specie *Inula helenium*, followed from *Arnica montana* (10%, n= 3), *Laurus nobilis*, *Parthenium hysterophorus* and *Tanacetum parthenium* (6.6%, n= 2, each), *Achillea pannonica, Ambrosia arborescens, Aucklandia lappa, Cynara scolymus, Inula japonica, Inula viscosa, Ixeris dentata* and *Saussurea costus* (3.3%, n= 1, each) were also reported, observed in Table S3 and Figure 3.

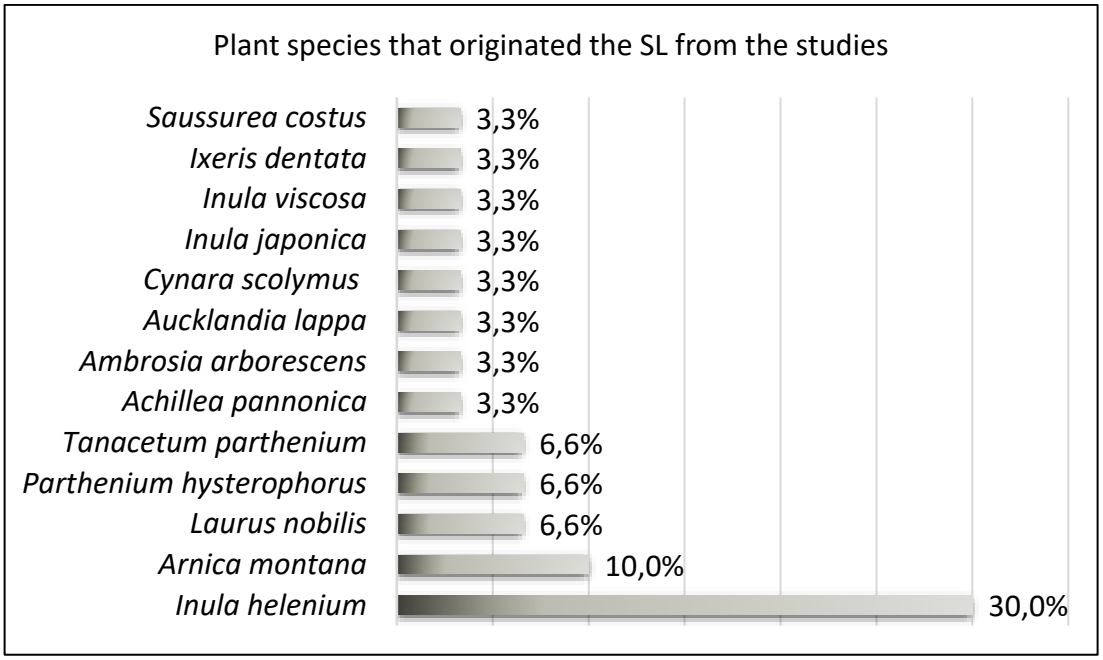

Figure 3. Plant species that originated the SL from the studies.

3.3. Preclinical studies with animal models

3.3.1. Characteristics of the animal models

In vivo studies used mice (60%, n= 12) or guinea pigs (40%, n= 8). Balb/c lineage was mainly reported (41.66%, n= 5), and 5 studies (41.66%) compared more than one strain (Balb/c, DBA/2, C3H/He, and C57BL/6). Other studies used *Swiss* (16.66%, n= 2) or ICR, WSP and CD-1 (8.33%, n= 1, each) mice. In studies with guinea pigs, the albino Himalayan (50%, n= 4) and albino Hartley (25%, n= 2) lineages were more frequent. Only one study reported the lineage Pirbright White (12.5%). This parameter was not specified in 1 study (12.5%, n= 1). Most studies (n= 15, 75%) used female animals, and this information was neglected in 2 studies (10%). The age of the animals ranged from 5 to 16 weeks in mice, in guinea pigs this information was not reported, and 12 (60%) studies did not report animal's age. The weight of the animals ranged from 250 to 600 g in guinea pigs and 18 to 30 g in mice. This parameter was not reported in 13 studies (65%). All these data are detailed in (Table S2).

3.3.2. Characteristics of the skin sensitization and dermatitis model

As seen in Table S3, 11 studies (55%) used sesquiterpene lactones to sensitize the animal's skin. Among these studies, 4 (36%) used an emulsified SL in Freund's complete adjuvant (FCA), 2 (18%) diluted the molecules in acetone and olive oil, 2 (18%) in ethanol, 1 (9%) study carried out two sensitizations (acetone plus olive oil, and FCA), and 2 (18%) studies did not inform the vehicle used. Two animal studies (10%) carried out sensitization only with FCA. Two studies (10%) induced sensitization with 2,4-dinitrochlorobenzene (DNCB) or 2,4,6-trinitro-1-chlorobenzene (TNCB). One study (5%) performed sensitization with croton oil and 4 studies (20%) used others sensitization agents, including 12-O-tetradecanoylphorbol-13-acetate (TPA), arachidonic acid, oxazolone, carrageenan, ethylphenylpropiolate, and serotonin.

3.3.3. Characteristics of the cutaneous challenge/treatment

As seen in Table S4, cutaneous administration of SL was mainly based on solutions containing ethanol (40%, n= 8), acetone:olive oil (20%, n= 4) and acetone (10%, n = 2); followed by dichloromethane, emollient cream, croton oil and dimethyl ketone (Me_2CO) (5%, n= 1, each). This information was not reported in two studies (10%). The administered dose ranged from 0.1 to 10%. The main routes of administration were topical (55%, n= 11) and epicutaneous (40%, n= 8). Most studies used a single SL administration (65%, n=13). In animal

models exposed to some skin sensitizing agent, SL were administered before, during or after dermatitis induction. The treatment with SL varied from 1 h to 17 days.

3.3.4. Main preclinical evidence in vivo

As reported in Table S5 and Figure 4, 35.7% (n= 15) of all SL tested *in vivo* exhibited only proinflammatory activity, which was consistently associated with induction of dermatitis associated with mild to severe skin erythema, edema, epidermal and dermal thickening, and inflammatory infiltrate. Six SL (14.3%) presented exclusive anti-inflammatory effects, attenuating the development of contact dermatitis (erythema, edema and skin thickening) by attenuating gene expression and/or production of proinflammatory cytokines (i.e., TNF, IL-1, IL-4, IL-5, IL-6, IL13), reducing inflammatory infiltrate and immunoglobulin E (IgE) titers, and increasing the production of anti-inflammatory cytokines (i.e., IL-10). Three SL (7.1%) were associated with anti-inflammatory or proinflammatory effects in different studies. Fifteen (35.7%) SL were unable to inducing anti-inflammatory or proinflammatory cutaneous effects. Three SL (7.1%) were associated with anti-inflammatory activity in some studies and indifferent in other studies. The lactones categorized according to their cutaneous effects *in vivo* can be seen in Figure 4.

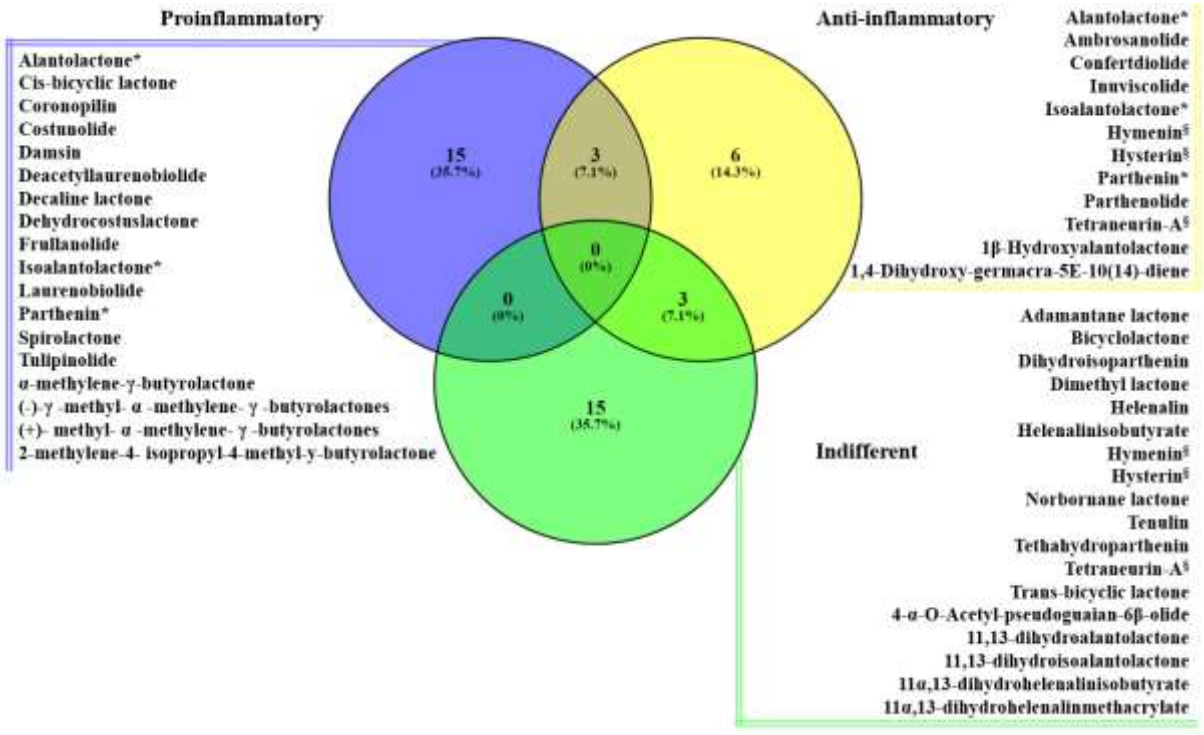

Figure 4. Sesquiterpene lactones (SL) categorized from their cutaneous effects *in vivo*. Molecules with indifferent effects were unable to induce detectable proinflammatory or anti-

inflammatory effects (similar results compared to control treatments). The effects were based on direct topical treatments with SL or considering the therapeutic response of these molecules in animal models of contact irritants-induced dermatitis. *Alantolactone, isoalantolactone, and parthenin exhibited proinflammatory and anti-inflammatory effects in at least two different studies. §Hymenin, hysterin, and tetraneurin-A exhibited anti-inflammatory effects or was ineffective in inducing cutaneous responses.

In Figure 5, the main evidence found in animal studies is observed, observing the anti and pro inflammatory findings, leading or not to inflammation.

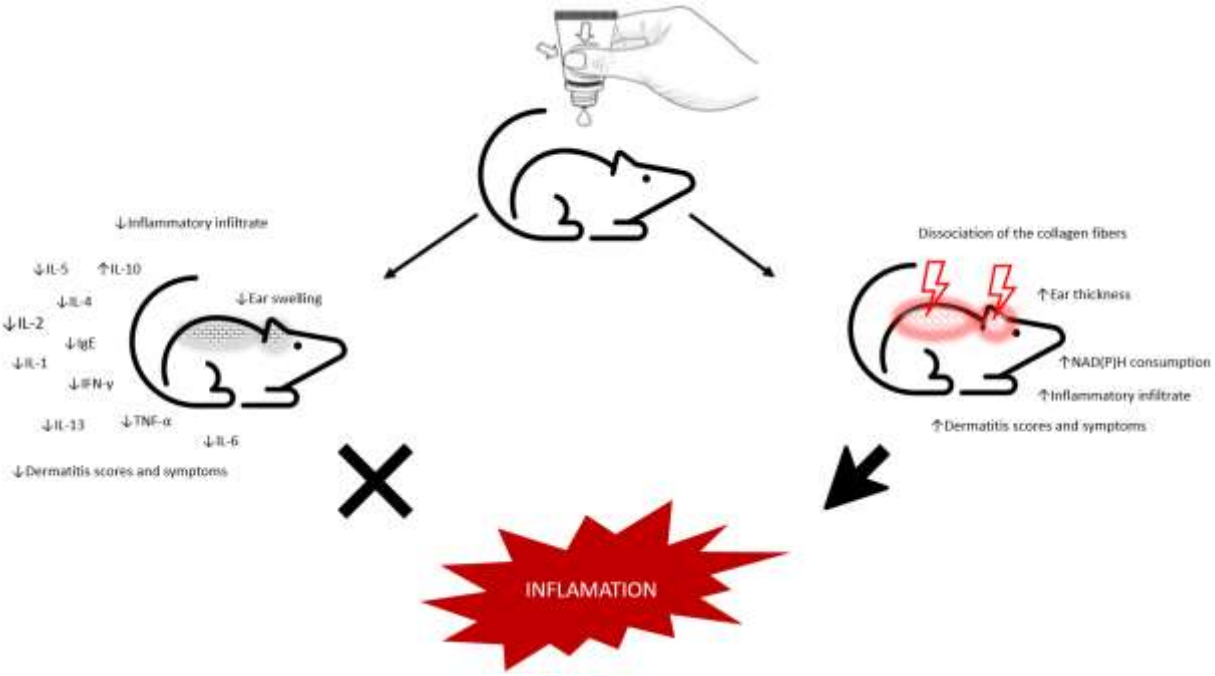

Figure 5. Evidence from *in vivo* studies.

3.4. Preclinical studies with in vitro models

3.4.1. Characteristics of cell cultures

As reported in Table S2, most *in vitro* studies investigated the effect of SL on human keratinocytes (53.33%, n= 8), especially HaCaT lineage predominating (75%, n= 6). Lymphocytes, mast cells, dendritic cells, macrophages, epidermal keratinocyte, and basophilic cells were also used (6.66%, n= 1, each). Most cells were cultured in DMEM (40%, n= 6) or RPMI (33.33%, n= 5) medium. Bovine pituitary extract, serum-free medium KGM and keratinocyte basal medium (KBM-GOLD), serum-free Epilife, serum-free keratinocyte growth

were also reported in 1 study each (6.66%). Only one study that did not report the culture medium used (6.66%).

3.4.2. Characteristics of in vitro cell stimulation

Cell induction/stimulation *in vitro* can be seen in Table S3. Fifteen studies (50%) investigated the effects of sesquiterpene lactones from *in vitro* models. Of these, 4 studies (26.6%) stimulated cells with the SL alone (n= 2, 50%), SL plus tumor necrosis factor (TNF-α) and interferon gamma (IFN-γ) (n= 1, 25%), and SL with TNFα and UVB exposure (n= 1, 25%). From all *in vitro* studies, 4 (26.6%) performed a pre-incubation with lipopolysaccharide (LPS) and 1 (25%) study also performed TNF-α stimulation. Three studies (20%) used incubation with tumor necrosis factor (TNFα). Two studies (13.3%) incubated cells with anti-DNP IgE antibody, including IL-5 in one of these studies. One study (6.6%) performed the stimulation with ultraviolet light, and 1 study (6.6%) did not inform the mode of cell stimulation. In 5 studies (33.3%), SL were diluted in the culture media and administered at 0.5 to 100μM. The period of cell incubation with SL ranged from 20 min to 24 h, with a duration of the assays ranging from 24 to 48 h.

3.4.3. Characteristics of the challenge/treatment with SL in vitro

As reported in Table S4, of the 15 *in vitro* studies, 6 (40%) reported that SL was directly dissolved in culture medium and add to cell culture, 1 (6.6%) used tinctures rich in SL, and 8 (53.3%) studies did not report the specific formulation administered. The doses of SL administered ranged from 0.625 μM to 40 μM, and 0.6 μg/ml to 300 μg/mL. Regarding cell challenge/treatment, 1 study (6.6%) used lymphocytes from animals pre-treated with sesquiterpene lactones, in 6 studies (40%) cells were pre-treated with SL, in 5 studies (33.3%) the cells were incubated the presence or absence of SL, and 3 studies (20%) incubated cell with SL after antigen stimulation. The incubation period with SL ranged from 20 min to 48h. Only 1 study (6.6%) did not reported this information.

3.4.4. Main preclinical evidence in vitro

From SL tested *in vitro,* only four molecules share proinflammatory and anti-inflammatory activities (Table S5 and Figure 6). Proinflammatory effects were associated with increased lymphocytes proliferation, reduced total glutathione, and cell cytotoxicity in higher doses (≥100 μM). Most SL (n= 12) exhibited exclusive anti-inflammatory activity, which was consistently associated with attenuation of gene expression and/or production of

proinflammatory cytokines (i.e., TNF, IL-1, IL-4, IL-5, IL-6, IL-8, IL-22, MCP-1, and CXCL10); downregulation of gene expression (i.e., SOCS3, HBD-2, ICAM- 1, TARC, MDC, RANTES, GROα, p65, p105, Bcl-2, TARC, MDC), activation (i.e., Tyr705, STAT3, ERK1/2, EGFR, Ser727 STAT1, ERK, NF-κB, IkB, JNK, and p38 MAPK), and production (i.e., cyclin D1, PCNA, p-RB, phospho-Akt, mTOR, Toll-like 4 receptor, prostaglandin E2) of molecules involved in inflammatory pathways. Anti-inflammatory activity of SL was also associated with increased Nrf2 activation, CYP1A1, Nrf2, Nqo1, HSP70B' gene expression and IκB levels, inhibition of 5-lipoxygenase, phosphodiesterase-3, phosphodiesterase-4, β-hexosaminidase, cyclooxygenase-2 activity; as well as inhibition of reactive oxygen species production, cell activation (i.e., dendritic cells, basophil cells) and proliferation (i.e., keratinocytes, B lymphocytes). The lactones categorized according to their effects *in vitro* can be seen in Figure 5.

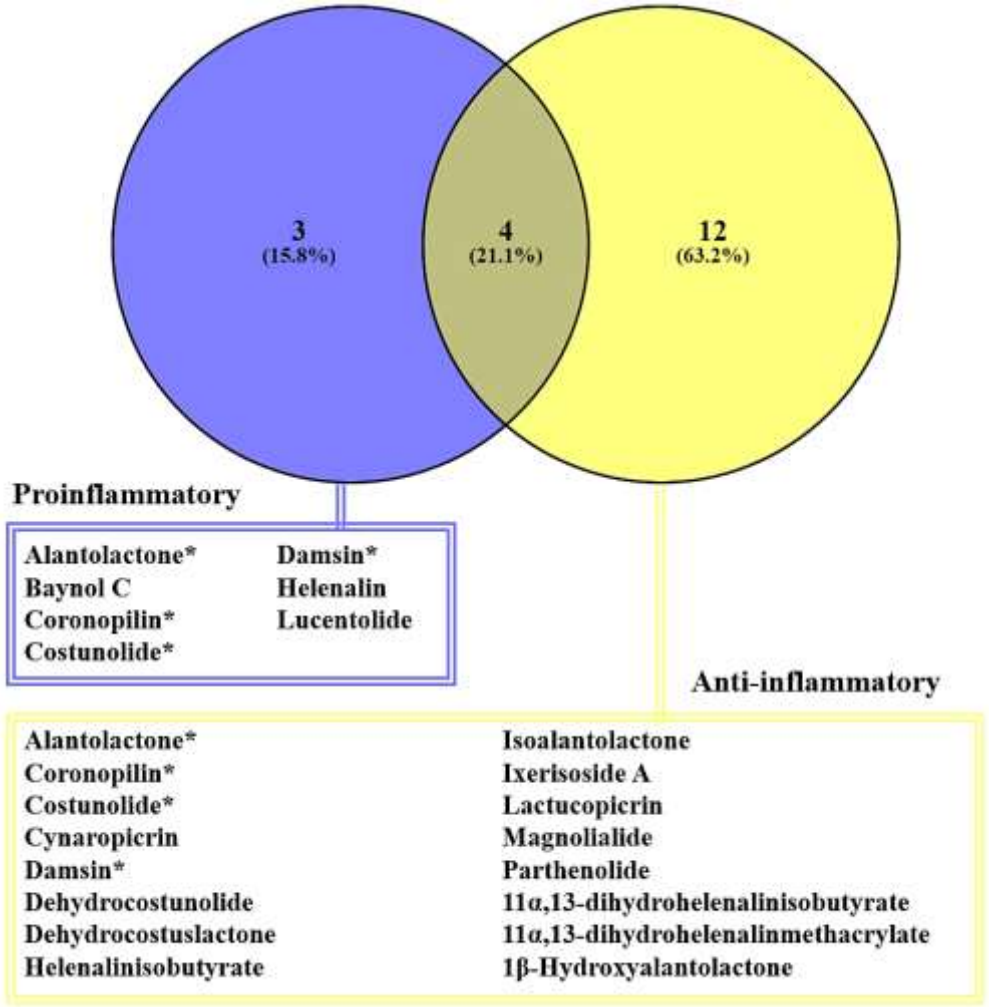

Figure 6. Sesquiterpene lactones (SL) categorized from their cellular effects *in vitro*. *Alantolactone, costunolide, coronopilin, and damsim exhibited proinflammatory and anti-inflammatory effects in at least two different studies.

Considering that the current evidence of anti-inflammatory mechanisms triggered by SL are more consistent, the main molecules and metabolic pathways modulated by SL in skin-related cells are summarized in Figure 7. Although SL act by different mechanisms in different cell lines, the anti-inflammatory effects are especially triggered by the inhibition in the production of cytokines and enzymes involved in the metabolism of arachidonic acid. Inhibition of the NF-κB pathway was most often associated with attenuation of cytokine production.

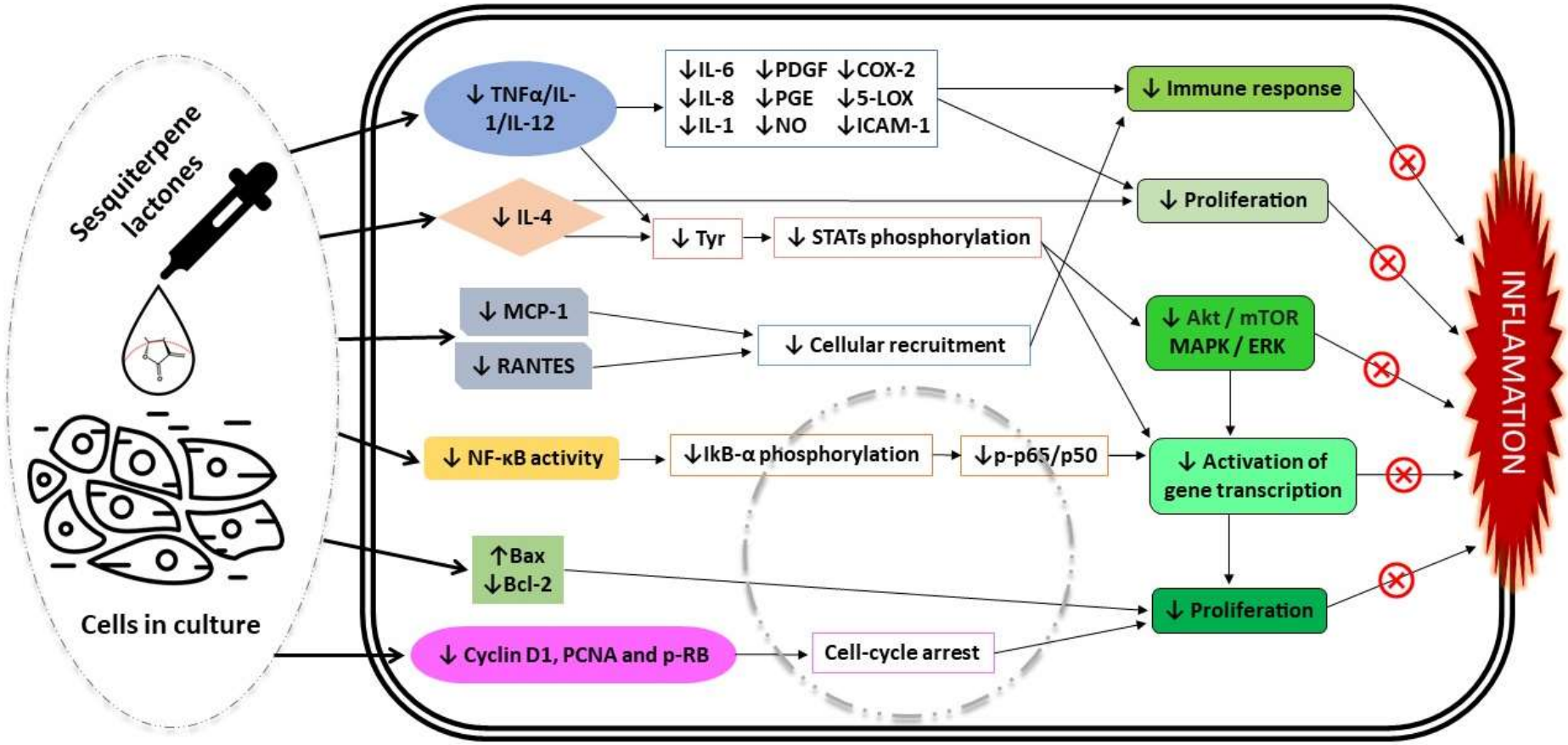

Figure 7. General anti-inflammatory mechanisms triggered by sesquiterpene lactones (SL) identified from *in vitro* studies with skin-related cells. *In vitro* evidence is based on keratinocytes, fibroblasts, dendritic cells, macrophages, mast cells, basophilic cells, and pro-B cells. Anti-inflammatory effects were obtained from lactone doses < 100µM. Cell responses to specific SL are described in the supplementary files (Table S5).

3.5. In silico SL-enzyme interaction

From computational modeling *in silico*, molecular superimposition between SL with the active site of all target enzymes (5-LOX, COX-2, MMP-1, MMP-2, and MMP-9) were obtained. The superimpositions of SL presenting the strongest molecular interactions with each enzymatic target were presented in Figures 8 and 9. Interestingly, the SL inuviscolide presented a better interaction with 5-LOX and MMP-9, and the LS budlein A with COX-2 and MMP-1 (Figures 8 and 9 and Tables 1, 2, 3 and 5). In addition, α-methylene-γ-butyrolactone and MMP-2 exhibited a more strong interaction (Figure 10 and Table 4).

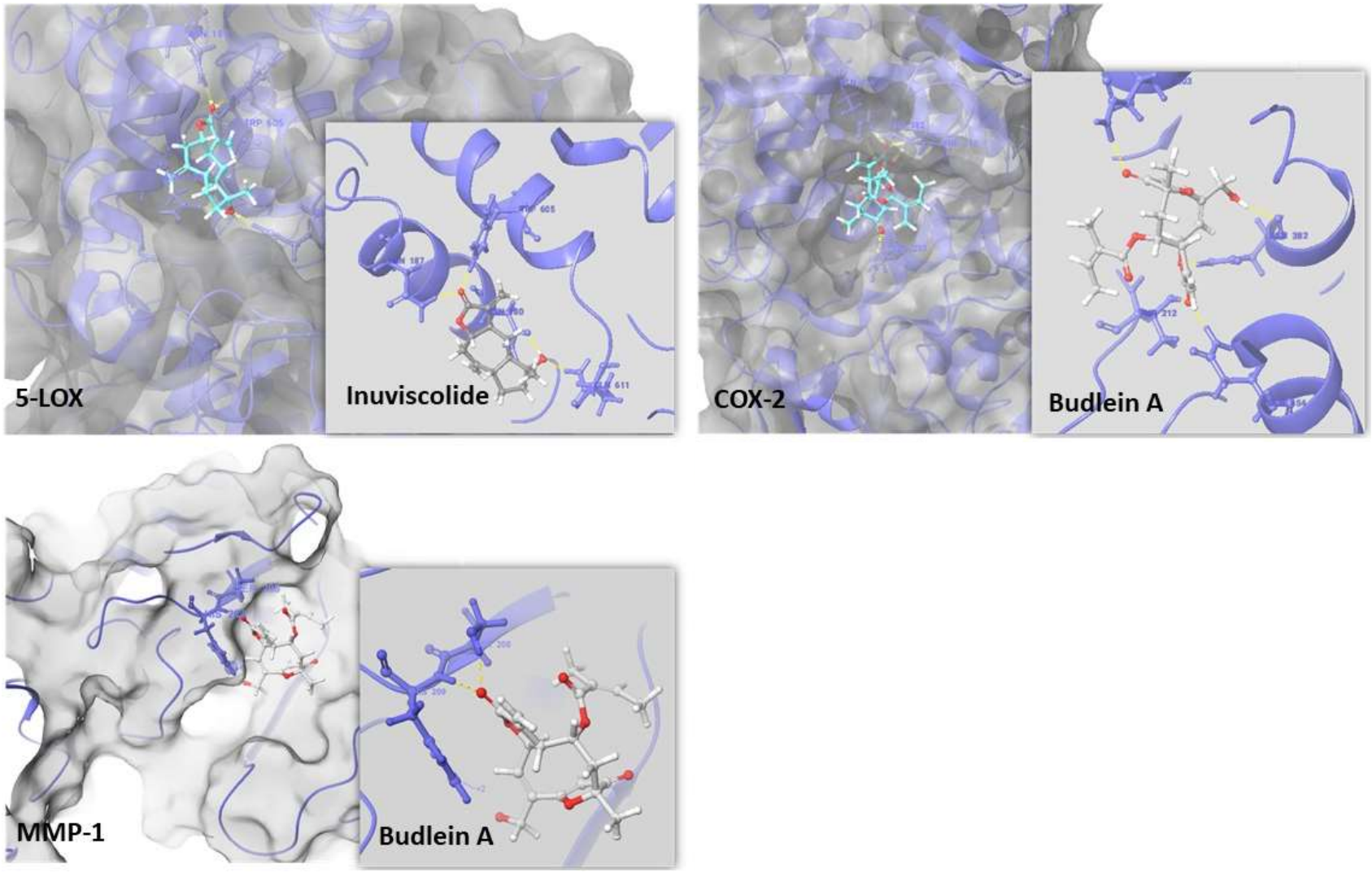

Figure 8. Representation of molecular docking results of sesquiterpene lactones with the best Glide Score with the active site of the target enzymes 5-lipoxygenase (5-LOX), cyclooxygenase 2 (COX-2) and matrix metalloproteinase 1 (MMP-1).

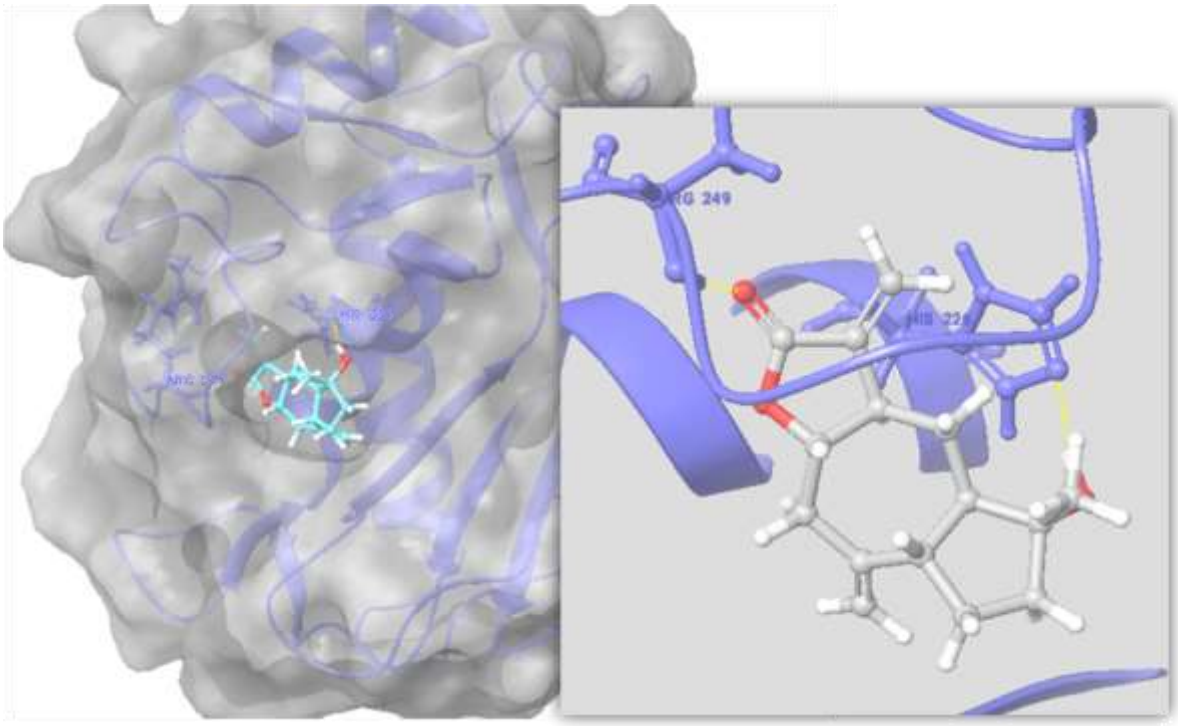

Figure 9. Representation of molecular docking results of Inuviscolide with matrix metalloproteinase 9 (MMP-9) active site.

In molecular docking analysis, all chemical interactions between SL and specific amino acids of the active site of all target enzymes was determined. The better interactions and chemical links established were represented in Figure 10 and Tables 1 to 5. Hydrophobic amino acids were predominant in the active site of MMP-1, MMP-2, and MMP-9; while a similar distribution of hydrophobic and polar amino acids was identified in 5-LOX and COX-2 active site.

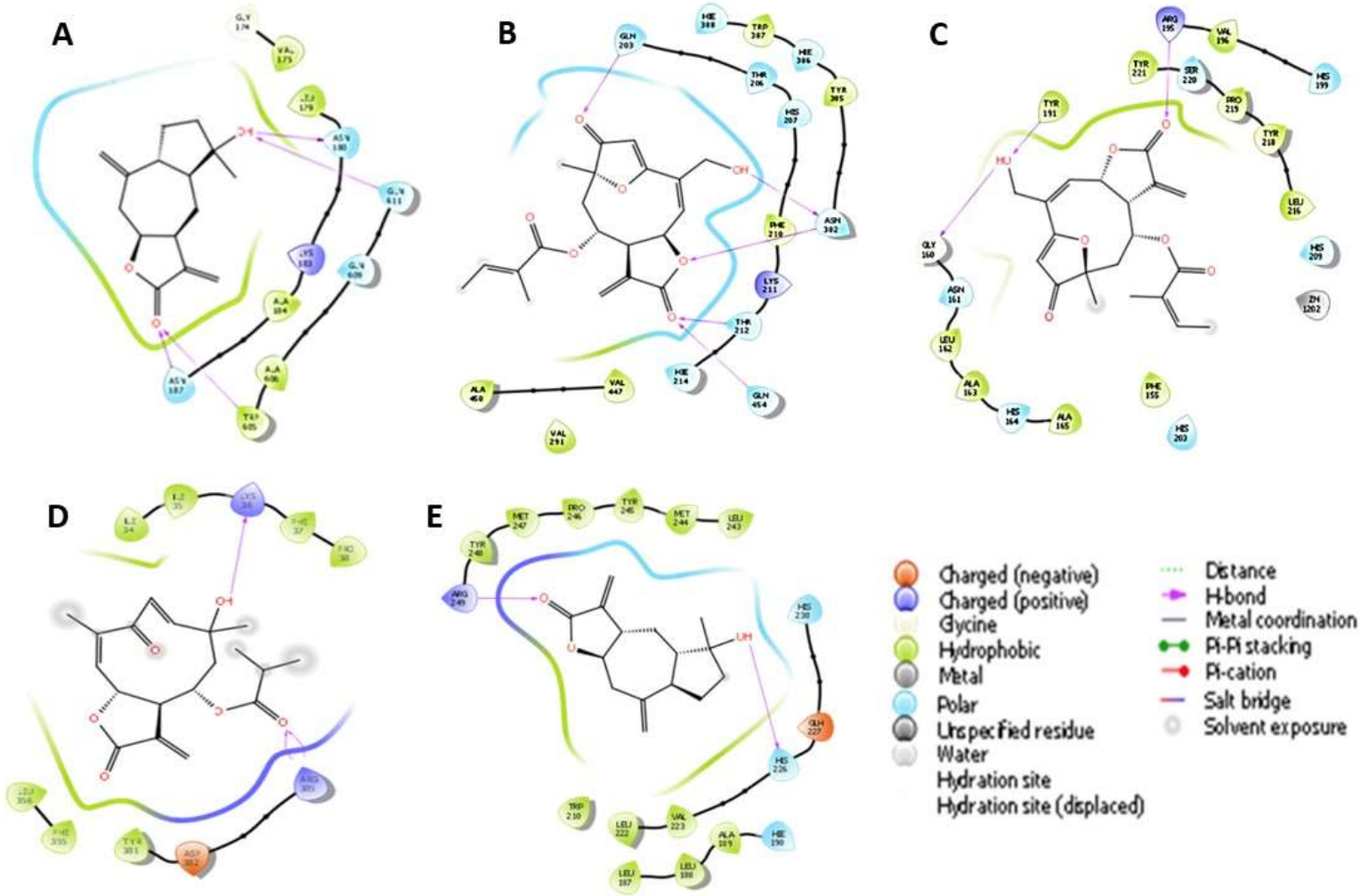

Figure 10. Interactions between amino acids of 5-lipoxygenase and Inuviscolide **(A)**, cyclooxygenase 2 and Budlein A **(B)**, metalloproteinase 1 and Budlein A **(C)**, matrix metalloproteinase 2 and α-methylene-y-butyrolactone C **(D)**, matrix metalloproteinase 9 and Inuviscolide **(E)**. All interactions are based on the active site of all target enzymes.

In silico analysis indicated different profiles and chemical strengths of ligand-enzyme interaction of SL with 5-LOX, COX-2, MMP-1, MMP-2, and MMP-9 (Tables 1 to 5). From SL and 5-LOX modeling, only inuviscolide presented a better *GScore* compared to zileutron. In addition, the SL coronopilin, tenulin, tetraneurin A, and parthenin presented a similar *GScore* among them and compared to zileutron (Table 1).

Table 1. Values of Glide Score (*GScore*), number of interactions by Hydrogen bonds (*Hbond*) and van der Waals (*good vdW*) between the lactones and 5-lipoxygenase (5-LOX, PDB code: 3V98).

5-LOX - 3V98				
Ligand	**GScore (kcal.mol^{-1})**	**H bond**	**Amino acids that perform H bond**	**Good vdW**
Zileutron*	-7.692	2	Asn180, Gln611	215
Inuviscolide	-7.802	4	Asn187, Trp605, Gln611, Asn180	159
Coronopilin	-7.381	2	Asn180, Gln611	200
Tenulin	-7.357	4	Trp605, Phe610, Gln611, Asn180	216
Tetraneurin A	-7.334	5	Asn187,Trp605, Asn180 (2), Gln611	208
Parthenin	-7.034	4	Trp605, Asn180 (2), Gln611	154
Costunolide	-6.878	2	Asn187, Trp605	216
Helenalin	-6.819	3	Asn187, Trp605, Asn180	181
Budlein A	-6.693	4	Asn180, Asn613, Glu612, Gln611	230
10-acetoxy-8,9-epoxythymolisobutyrate	-6.641	3	Trp605, Asn187, Asn180	234
Confertdiolide	-6.554	4	Trp605, Asn187, Asn180, Gln611	142
α-methylene-y-butyrolactone	-6.465	1	Asp176	142
11α,13-dihydrohelenalin methacrylate	-6.347	4	Asn180, Gln611, Trp605, Asn187	149
Hymenin	-6.344	5	Glu612, Gln609, Ala606, Ile673 (2)	199
1β-Hydroxyalantolactone (IJ-5)	-6.330	3	Asn187, Asn180, Gln611	203
Alantolactone	-6.299	1	Asn187	210
Isoalantolactone	-6.261	1	Asn187	217
Parthenolide	-6.249	2	Asn187, Trp605	181
Dehydrocostuslactone	-6.172	1	Asn187	176
11α,13-dihydrohelenalin isobutyrate	-6.159	2	Lys183;Gln611	202
Artesunate	-6.147	3	Gln611, Asn613, Asp170	245
Damsin	-6.032	2	Gln611, Asn180	151

*Zileutron, was used as specific control of the molecular docking since is a potent 5-LOX inhibitor.

Molecular docking between SL and COX-2 indicated that only budlein A exhibited a better *GScore* than celecoxib. In addition, SL such as artesunate, 11α,13-dihydrohelenalin isobutyrate, inuviscolide and coronopilin presented similar interactions with COX-2, which were the closest to the results obtained to celecoxib (Table 2).

Table 2. Values of Glide Score (*GScore*), number of interactions by Hydrogen bonds (*Hbond*) and van der Waals (*good vdW*) between the lactones and Cyclooxygenase 2 (PDB code: 5KIR).

COX-2 - 5KIR				
Ligand	**GScore (kcal.mol^{-1})**	**H bond**	**Amino acids that perform H bond**	**Good vdW**
Celecoxib*	-8.406	1	Phe210	272
Budlein A	-8.484	5	Gln454, Thr212, Asn382 (2), Gln203	235
Artesunate	-8.366	5	Thr212, Hie214, Asn283, Gln454, Hie386	292
11α,13-dihydrohelenalin isobutyrate	-8.264	5	Gln454, Asn382, Thr212, Hie388, Gln203	226
Inuviscolide	-8.068	1	Hie388	239
Coronopilin	-8.007	3	Asn382, Gln454 (2)	179
Helenalin	-7.979	3	Thr212, Asn382, Gln203	150
Dehydrocostuslactone	-7.951	3	Thr212 (2), Asn382	187
Tetraneurin A	-7.775	5	Asn382, Hie386, Gln454, Gln289, His207	162
Damsin	-7.622	3	Thr212 (2), Asn382	134
Confertdiolide	-7.550	1	Gln203	303
Isoalantolactone	-7.503	2	Asn382, Thr212	170
Parthenin	-7.385	3	Gln289, Thr212, Hie214	149
11α,13-dihydrohelenalin methacrylate	-7.335	3	Thr212, His207, Gln203	234
10-acetoxy-8,9-epoxythymol isobutyrate	-7.263	4	Asn382, Thr212, Gln203, Hie388	181
1β-Hydroxyalantolactone (IJ-5)	-7.213	2	Phe210, Asn382	214
Tenulin	-7.033	3	Phe210, Hie214, Gln289	168
Costunolide	-6.904	3	Gln454, Hie214, Thr212	220
Parthenolide	-6.854	3	Gln454,Thr212 (2)	199
Alantolactone	-6.794	2	Asn382, Thr212	183
α-methylene-y-butyrolactone	-6.259	2	Hie386, Thr383	107
Hymenin	-6.171	4	Gln454, Val447, Hie388, Gln203	122

*Celecoxib was used as specific control of the molecular docking since is a potent COX-2 inhibitor.

The interaction between SL and MMP-1 indicated that only budlein A and artesunate presented a better *GScore* than batimastat. In addition, tetraneurin A and parthenin presented similar interaction, which were the closest to batimastat (Table 3).

Table 3. Values of Glide Score (*GScore*), number of interactions by Hydrogen bonds (*Hbond*) and van der Waals (*good vdW*) between the lactones and Matrix metalloproteinase 1 (MMP-1, PDB code: 4AUO).

MMP-1 - 4AUO				
Ligand	**GScore (kcal.mol^{-1})**	**H bond**	**Amino acids that perform H bond**	**Good vdW**
Batimastat*	-7.780	6	Asn161, Leu162, Ala163(2), Ser220, Tyr221	279
Budlein A	-8.000	2	His209, Ser208	206
Artesunate	-7.924	1	Leu162	270
Tetraneurin A	-7.060	2	Gly160, Arg195	321
Parthenin	-7.013	2	Gly160, Leu162	212
Coronopilin	-6.998	1	Gly160	229
Damsin	-6.983	0	-	262
Dehydrocostuslactone	-6.827	0	-	226
Alantolactone	-6.698	0	-	250
Tenulin	-6.654	1	Asn161	240
Isoalantolactone	-6.502	0	-	244
1β-Hydroxyalantolactone (IJ-5)	-6,343	1	Ser220	200
Inuviscolide	-6.127	0	-	220
Confertdiolide	-5.852	0	-	242
10-acetoxy-8,9-epoxythymol isobutyrate	-5.736	2	Ala163, Leu162	223
11α,13-dihydrohelenalin isobutyrate	-5.692	2	Leu162, Ala163	203
Parthenolide	-5.282	0	-	215
11α,13-dihydrohelenalin methacrylate	-5.253	1	Arg195	275
Costunolide	-4.880	0	-	183
α-methylene-y-butyrolactone	-4.727	0	-	92
Hymenin	-4.400	1	Gly160	136

*Batimastat was used as specific control of the molecular docking since is a potent MMP inhibitor.

From SL and MMP-2 modeling, α-methylene-y-butyrolactone, dehydrocostuslactone, alantolactone, helenalin, coronopilin, confertdiolide, 1β-hydroxyalantolactone, costunolide, damsin, parthenolide, and hymenin presented a better *GScore* than batimastat (Table 4).

Table 4. Values of Glide Score (*GScore*), number of interactions by Hydrogen bonds (*Hbond*) and van der Waals (*good vdW*) between the lactones and Matrix metalloproteinase 2 (MMP-2, PDB code: 1CK7).

MMP-2 - 1CK7				
Ligand	**GScore (kcal.mol^{-1})**	**H bond**	**Amino acids that perform H bond**	**Good vdW**
Batimastat*	-4.263	4	Lys36, Tyr381, Arg385(2)	179
α-methylene-y-butyrolactone	-4.757	1	Arg385	127
Dehydrocostuslactone	-4.750	1	Tyr381	167
Alantolactone	-4.520	1	Lys36	106
Helenalin	-4.500	2	Tyr381, Arg385	127
Coronopilin	-4.475	4	Lys36; Arg385 (2), Tyr381	77
Confertdiolide	-4.412	3	Arg385, Tyr381, Lys36	100
1β-Hydroxyalantolactone (IJ-5)	-4.388	2	Arg385, Lys36	142
Costunolide	-4.354	2	Tyr381, Arg385	139
Damsin	-4.340	0	-	196
Parthenolide	-4.323	0	-	197
Hymenin	-4.318	3	Arg385, Asp382, Lys36	204
Tetraneurin A	-4.246	3	Arg385 (2), Lys36	131
10-acetoxy-8,9-epoxythymol isobutyrate	-4.214	2	Arg385, Lys36	251
Tenulin	-4.138	2	Tyr381, Lys36	109
Artesunate	-4.111	0	-	179
11α,13-dihydrohelenalinmethacrylate	-4.082	3	Arg385 (2), Tyr381	161
Isoalantolactone	-4.067	2	Tyr381, Arg385	82
Parthenin	-3.990	2	Arg385 (2)	156
Budlein A	-3.966	2	Arg385, Lys36	181
Inuviscolide	-3.909	1	Tyr381	98
11α,13-dihydrohelenalin isobutyrate	-3.538	1	Tyr381	185

*Batimastat was used as specific control of the molecular docking since is a potent MMP inhibitor.

The interaction between SL and MMP-9 indicated that most molecules investigated presented a better *GScore* compared to batimastat. The best result was obtained to inuviscolide (Table 5).

Table 5: Values of Glide Score (*GScore*), number of interactions by Hydrogen bonds (*Hbond*) and van der Waals (*good vdW*) between the lactones and Matrix metalloproteinase 9 (MMP-9, PDB code: 5CUH).

MMP-9 - 5CUH				
Ligand	**GScore (kcal.mol^{-1})**	**H bond**	**Amino acids that perform H bond**	**Good vdW**
Batimastat*	-6.882	4	Gly186, Leu188, Ala189, Glu227	294
Inuviscolide	-9.702	2	Arg249, His226	284
Isoalantolactone	-8.940	1	Arg249	241
Artesunate	-8.463	3	Arg249 (3)	312
Coronopilin	-8.430	2	Glu227, Ala189	219
Confertdiolide	-8.400	1	Arg249	277
Alantolactone	-8.335	1	Arg249	240
Dehydrocostuslactone	-8.219	1	Glu227	218
Tetraneurin A	-8.125	2	His226, Glu227	228
Helenalin	-7.894	1	Glu227	240
1β-Hydroxyalantolactone (IJ-5)	-7.742	0		257
11α,13-dihydrohelenalin methacrylate	-7.691	1	Glu227	279
Damsin	-7.623	1	Ala189	269
11α,13-dihydrohelenalin isobutyrate	-7.395	1	Ala189	279
Budlein A	-7.336	1	Hie236	303
Costunolide	-7.233	2	Glu227, Ala191	222
Parthenolide	-7.167	0	-	280
Hymenin	-6.863	3	Leu243, Ala189, Glu227	241
Parthenin	-6.654	1	Glu227	231
10-acetoxy-8,9-epoxythymol isobutyrate	-6.469	3	Ala189, Leu188, Tyr248	347
Tenulin	-6.253	1	Glu227	233
α-methylene-y-butyrolactone	-5.741	1	Glu227	96

*Batimastat was used as specific control of the molecular docking since is a potent MMP inhibitor.

3.6. Bias report from in vivo studies

The bias analysis based on the SYRCLE's animal studies tool is detailed in Table S6. Of the 15 animal studies (50%), none of the studies fully met all established criteria. From a comprehensive analysis, 12 studies (60%) had a low risk of bias, while 7 (35%) had a high risk of bias and 1 (5%) did not report all necessary information. As for the performance of the studies, 55% (n = 11) had a low risk of bias, 25% (n = 5) had a high risk of bias, and 20% (n = 4) an uncertain high risk of bias. When evaluating the incomplete data in the research reports, or if all animals had been included in the results, the studies presented a low risk of bias (35%, n = 7), followed by high risk (20%, n = 4) and uncertain high risk of bias (45%, n= 9). When evaluated in relation to study reports, most published all expected results, presenting a low risk of bias (70%, n = 14), while 30% (n = 6) had a high risk of bias. In relation to other criteria, such as the use of medicines or whether new animals had been added to the groups to replace the lost ones, 45% (n = 9) had a low risk of bias, and 55% (n = 11) had a high risk of bias. As for the allocation of animals, randomized housing, random selection for evaluation of results and blinding of the evaluators, most studies (95%, n= 19) did not bring all the information clearly.

4. Discussion

Our findings indicated that the effect of SL on the skin were investigated since 1978 in 12 different countries. Most studies were originated from France, Republic of Korea, Canada, China, Germany, Spain, Italy, United States of America and Sweden. The greater concentration of studies in developed countries is not completely understood, since the research motivation was not always explicit. However, it may be linked to the high frequency of skin diseases in these countries (Lewis and Finlay, 2004; Hay et al., 2014; Svensson et al., 2018). This proposition is reinforced considering that most studies on SL-induced cutaneous hypersensitivity admits that dermatitis arising from direct contact with plants rich in SL widely distributed in their countries (Dupuis et al., 1980; Paulsen, 2017). Conversely, some studies also admit a pharmacological potential of SL to treat skin diseases, indicating an important concern with the discover of new molecules with biotechnological applicability on dermatological conditions, especially inflammatory skin diseases such as contact dermatitis (Máñez et al., 1999; Sosa et al., 2001).

The geographic distribution of studies and plant species used to obtain SL also exhibits an ethnobotanical and ethnomedical basis closed correlated with traditional health care

practices widespread in ancient and geographically well-delimited populations, whose transgenerational records of plants with medicinal properties provide clues to modern scientific research (Kubelka et al., 1999; Seca et al., 2014). In fact, the plant species identified in this systematic review, especially *Inula helenium*, *Inula japonica*, *Inula viscosa*, *Arnica montana*, *Tanacetum parthenium* and *Laurus nobilis* exhibits a consistent use in popular medicine (Máñez et al., 1999; Lass et al., 2008; Sur et al., 2009; Lee et al., 2013). In this sense, reports of skin diseases from the contact with the species *Laurus nobilis*, a SL-rich plant commonly encountered in France, motivated the research of allergenic molecules by Cheminat et al. (1984). From reports of cutaneous reactions after exposition to plants of the *Compositae* family, Dupuis et al. (1980) established a direct link between the content of SL in this species and contact dermatitis. In this study, an important action mechanism associated to SL toxicity was proposed, which is based on processes of nucleophilic addition through the α-methylene-γ-butyrolactone conjugate system. This process is responsible for covalent binding to the carrier molecule, especially proteins, which facilitates its irritating action (Dupuis et al., 1980). In a similar perspective, by associating the high occurrence of cutaneous allergic reactions after the introduction of plant species of the *Compositae* family in India, Picman et al. (1982) also established a causal link between SL and the development of contact dermatitis.

Until 1993, according to the studies selected for this systematic review, the focus was on activity on the toxic and skin irritating action of SL (Schmidt and Chung, 1993). From that date to the present moment, we have identified a gradual increase in studies with the aim of prospecting and analyzing SL with anti-inflammatory potential. Thus, Máñez et al. (1999) examined the anti-inflammatory activity of *Inula viscosa* extract, a SL-rich plant widespread in Mediterranean countries. Máñez et al. (1999) based their studies in ethonomedical evidence, since *Inula viscosa* is topically used in folk medicine of Mediterranean countries as an anti-inflammatory, anti-scab and healing agent. Sosa et al. (2001) also reported a wide applicability of the species *Achillea* (*Asteraceae*) in folk medicine for its anti-inflammatory properties, which is currently attributed to SL. Similarly, the high content of SL in *Inula helenium* species provides a rational basis to explain the wide applicability of this plant species in traditional Chinese medicine for the treatment of inflammatory diseases (Seca et al., 2014).

From plant species selection and SL extraction, skin and cellular responses to these molecules were tested in studies with animal models or cells in culture. Mice and guinea pig were consistently used in preclinical studies *in vivo*. Following the timeline, we identified that guinea pig (*Cavia porcellus)* was predominantly used until 1990. Since then, mice (*Mus muscullus*) were more frequently used is studies on the cutaneous effects of SL (Alonso Blasi

et al., 1992a). In fact, mice and guinea pig are useful animal models in preclinical investigations on skin biology. They present low cost of acquisition, easy handling, and exhibits a similar skin structure and metabolism compared to human skin (Harkness and Wagner, 1993; Todo, 2017). In this sense, the skin reaction to SL can be better compared, especially considering a high degree of overlapping in cutaneous manifestations of toxicity activated by these molecules in humans and animal models (Schmidt and Chung, 1993; Gurib-Fakim, 2006). In addition, the anti-inflammatory mechanism activated by SL in different mammal species is also similarly mediated by COX-2, 5-LOX, and NFk-B inhibition; which are molecules with expression, structures and function highly conserved comparing mice, guinea pig and humans (Kim et al., 2015; Sur et al., 2009; Lass et al., 2008). In studies with mice, younger isogenic Balb/c mice were most used, a characteristic potentially related to the high sensitivity of this model to contact allergens, making Balb/c mice a relevant model in preclinical dermatological research (Bailey, 1978; Fraginals, 1991). The use of younger animals was also aligned with the models of contact dermatitis, since the immune response can change with aging, leading to nonspecific inductions (Kim, 2013).

In studies *in vitro*, cells lineages relevant to skin biology were investigated. Most studies were based on keratinocytes, fibroblasts, dendritic cells, lymphocytes, and mast cells. HaCat cells are a lineage of human keratinocytes transformed into adult cells, which are widely used in scientific research on skin biology and *in vitro* models of dermatological diseases (Boukamp et al., 1988; Seo et al., 2012). While HaCat cells are often used in modes of skin barrier, fibroblasts are typical dermal cells involved with extracellular matrix biosynthesis, maintenance and remodeling (Lovell et al., 1987; Cole et al., 2018). To regulates skin structure and function, keratinocytes and fibroblasts acts synergistically with defense cells, especially dendritic cells, mast cells and dermal lymphocytes; regulating the cutaneous immune response to biological, physical and chemical challenges (Kupper, 1990; Burbach et al., 2000; Rodrigo et al., 2010; Sanchez, 2010). As these cells are directly involved in skin biology and easily cultivable *in vitro*, studies directed to the effect of SL on specific skin cells are relevant and necessary; especially considering that each cell type exerts a distinct but complementary role in skin metabolism, structure and function (Lass et al., 2008; Svensson et al., 2018).

From *in vivo* studies, 42 SL were investigated, and most studies used alantolactone and isoalantolactone originated from helenin, a phytochemical mixture of the two isomeric lactones found in many plant species, mainly in *Inula helenium* (Xu R. et al., 2014). Interestingly, we identified that 15 SL summarized in Figure 2 exhibited only proinflammatory properties. Conversely, 6 SL presented an exclusive anti-inflammatory potential, while 15 molecules were

ineffective in inducing any cutaneous response. In addition, 3 SL showed pro and anti-inflammatory potential. From this systematic review, we identified that the current evidence indicates that SL induces a broad spectrum of cutaneous effects, which can be opposed. However, a group of these molecules were topically inert, a characteristic that does not exclude biological effects of these SL on different organs or tissues. In general, most studies investigated the anti-inflammatory activity of SL. It is interesting to note that until the 1993, only the proinflammatory effects of the molecules were investigated (Schmidt and Chung, 1993), a finding potentially motivated by the frequent reports of contact dermatitis associated to SL-rich plant species reported by the authors. From the 1990, the anti-inflammatory activity of SLs began to be more objectively investigated considering a potential biotechnological and therapeutic applicability (Máñez et al., 1999).

From our findings, it becomes clear that despite the recognition of SL as pro or anti-inflammatory agents, the toxicity and immunomodulatory mechanisms activated in the skin by these molecules remains poorly understood (Schmidt, 2006; Amorim et al., 2013). Thus, the allergenic effects of proinflammatory SL are basically attributed to a broad spectrum of skin manifestations such erythema, edema, epidermal and dermal thickening, and inflammatory infiltrate; which are classical signals of cutaneous hypersensitivity induced by contact allergens. In their studies with mice, Alonso Blasi et al. (1992b) indicated that intradermal administration of alantolactone combined to Freund's adjuvant is able to sensitize the skin, inducing edema and infiltration of inflammatory cells. However, isoalantholactone was unable to induce a similar effect in Balb/c and DBA/2 mice. These findings corroborate the study by Dupuis et al. (1980) with guinea pigs, which concluded that allantolactone can induce cutaneous hypersensitivity reactions and also trigger cross-reactions to other lactones carrying the α-methylene-γ-butyrolactone system. However, no cross-reaction with dimethyl lactone and spironolactone was detected by Dupuis et al., (1980), proving that not all lactones induce hypersensitivity. Thus, these findings exhibit a marked relevance, since corroborate the evidence that the α-methylene group attached to the γ-lactone ring is a relevant prerequisite for allergenic activity of SL (Bleumink et al., 1976). These studies also highlight that some SL act as haptens and do not have intrinsic immunogenic characteristics. However, these molecules are able to trigger immune reactions when combined with carrier macromolecules, including skin proteins (Cheminat et al., 1984; Alonso-Blasi et al., 1992a). Thus, adjuvants are relevant tolls in studies with SL, since they can also act as haptens. In this sense, vehicles such as FCA (Freund's Complete Adjuvant) are useful since help antigens, including potentially

immunogenic SL, to trigger a rapid and effective immune response even in the presence of low antigenic load (Gupta and Sibaer, 1995; He et al., 2000).

Although the anti-inflammatory effects *in vivo* are slightly better understood, it is now evident that further mechanistic studies are required. By using models of dermatitis induced by cutaneous irritants (i.e., FCA, 2,4-dinitro-1- chlorobenzene [DNCB], 12-O-tetradecanoylphorbol-13-acetate [TPA], arachidonic acid, oxazolone, carrageenan, ethylphenylpropiolate, and serotonin); anti-inflammatory properties of SL were attributed to an inhibitory effect on the infiltration of inflammatory cells (Máñez et al., 1999; Lass et al., 2008), immunoglobulin E (IgE) production (Lin et al., 2016; Wang et al., 2018), gene expression and biosynthesis of cytokines such as TNF, IL-1, IL-4, IL-5, IL-6, IL13; as well as upregulation of the anti-inflammatory cytokine IL-10 (Sur et al., 2009; Lass et al., 2010; Lin et al., 2016; Wang et al., 2018). Considering the anti-inflammatory effects of SL *in vivo*, Lin et al. (2016) demonstrated that 1β-Hydroxyalantolactone inhibited skin inflammation by reducing IgE and IL-4 production in mice exposed to DNCB. Studies using DNCB confirmed the activation of the skin's immune response by increasing the migration of dendritic cells and expression of chemokine receptors Cys-Cys and CCR7, as well as IL-1β and TNF-α; the latter involved with the activation of MAPK signaling pathway, suggesting that DNCB needs MAPK activation to trigger its irritant effects (Boislève et al., 2004). In addition, by stimulating IL-1β, IL- 4, IL-6 and IL-18 production, inflammatory infiltrate, skin increased edema, dermal and epidermal thickening; TNCB is also a useful sensitization agent in dermatitis models (Harada et al., 2005). From this model, Lass et al. (2008) identified that while high concentrations of the SL 11α,13-dihydrohelenalinisobutyrate; 11α,13-dihydrohelenalinmethacrylate, and helenalinisobutyrate inhibited NF-κB DNA binding, low concentrations induced opposite effects. Another study demonstrated the attenuation of dermatitis in mice after topical application of alantolactone and isoalantolactone, which reduced by 31 to 38% IL-4, IL-5 and IL-13 gene expression, and attenuated at least 80% IgE, IFN-γ and TNF-α production in DNCB dermatitis model (Wang et al., 2018). Potent anti-inflammatory effects were also reported by Sur et al. (2009), which observed that topical treatment with parthenolide inhibited contact hypersensitivity by reducing TNF-α, IL-2 and IFN-γ production, and ear edema in oxazolone-induced dermatitis.

From *in vitro* studies, 19 SL were investigated in all studies reviewed. Interestingly, most studies were focused and reported objective anti-inflammatory properties of all SL tested. However, alantolactone, coronopilin, costunolide and damsin also induced some negative effects on culture cells, which indicated potential proinflammatory and cytotoxic properties with a dose-dependent profile. In this sense, baynol C, coronopilin, damsim, helenalin and

lucentolide reduced the viability of HDFa fibroblasts, HaCaT keratinocytes, and RBL-2H3 basophilic cells only when administered in high concentrations (≥100 μM) (Svensson et al., 2018; Takei et al., 2015; Lee et al., 2013). In addition, alantolactone, isoalantolactone, dehydrocostuslactone and costunolide reduced glutathione levels in HaCaT keratinocytes (Hofmann et al., 2014; Scarponi et al., 2014), while alantolactone also triggered a proinflammatory effect by stimulating lymphocytes proliferation (Alonso Blasi et al., 1992a).

Although the mechanisms linked to the cytotoxic and proinflammatory effects of SL are poorly understood, the evidence on the anti-inflammatory potential of these molecules is more objective. In general, while cytotoxicity was reported in high doses of SL, low concentrations ranging from 1 to 10 μM were associated with marked anti-inflammatory effects *in vitro*. In this sense, Kim et al. (2015) reported that ixerisoside A pretreatment reduced COX-2, IL-6 and IL-8 gene expression induced by ultraviolet light irritation on HaCaT keratinocytes; an effect associated to dose-dependent downregulation of ERK, JNK and p38 MAPK activation. In LPS-stimulated RAW267.4 macrophages, parthenolide also inhibited 5-LOX and phosphodiesterase (isoforms 3 and 4) activities, as well as nitrite and prostaglandin E2 (PGE_2) production in a dose-dependent manner; which are molecular effectors directly involved in proinflammatory pathways (Sur et al., 2009). A similar effect was reported by Nam et al. (2015), which identified COX-2, IL-1β, and PGE_2 downregulation in LPS-stimulated HaCaT keratinocytes treated with parthenolide, an effect associated to marked reduction in IkB phosphorylation and NF-κB activation, as well as in phospho-Akt and mTOR levels. Lass et al. (2008) also reported a potent anti-inflammatory effect of SL-rich *Arnica* tinctures, which was evidenced by reduced NF-κB activation and IL-12 production by LPS-stimulated dendritic cells. Alantolactone and isoalantolactone were also effective in attenuating IL-1, IL-4 and TNF-α gene expression in TNF-α-stimulated HaCat keratinocytes, an anti-inflammatory response mediated by inhibition of IκB, p65, and NF-κB activation. In addition, costunolide, dehydrocostuslactone, alantolactone (Seo et al., 2015), 1β-Hydroxyalantolactone (Lin et al., 2016), coronopilin, damsin (Svensson et al., 2018), cynaropicrin (Takei et al. 2015), and magnolialide (Lee et al., 2013) presented inhibitory effects on gene expression an production of several cytokines (i.e., TNF-α, IL-1, IL-4 IL-6, IL-8, IL-12, and MCP-1) in HaCaT keratinocytes, HDFa fibroblasts, Y16 pro-B-cells and RBL-2H3 basophilic cells stimulated with proinflammatory agents; including LPS, TNF-α, IFNγ, and ultraviolet light. In addition to reducing IL-6 and TNF-α production, cynaropicrin also exhibits antioxidant properties by upregulating antioxidant signaling pathways (i.e., CYP1A1, Nrf2 and Nqo1) and reducing ROS production in TNF-α- or ultraviolet light-stimulated keratinocytes (Takei et al. 2015).

From *in vitro* evidence, we identified important mechanisms of action of specific SL on skin cells. However, as most of the SL tested *in vivo* have not yet been investigated *in vitro*, the mechanistic basis of many potentially relevant lactones is still unknown. Thus, to deepen the discussion on potential mechanisms associated with the biological effects of specific SL, we developed an *in silico* model to solve potential interactions of these molecules with target enzymes involved in skin biology; such as COX-2, 5-LOX and MMP-1, MMP-2 and MMP-9 (Sur et al., 2009; Lohberger et al., 2013; Kim et al. 2015). According to the molecular docking, all SL tested exhibited some degree of interaction with target enzymes from hydrogen and saline bridges, Van Der Waals, cation-π, and π-π stacking interactions. From a detailed analysis of the nature of molecular interaction, we identified that inuviscolide (-7.802 kcal.mol^{-1}), coronopilin (-7.381 kcal.mol^{-1}), tenulin (-7.357 kcal.mol^{-1}), tetraneurin A (-7.334 kcal.mol^{-1}), parthenin (-7.034 kcal.mol^{-1}) and costunolide (-6.878 kcal.mol^{-1}) showed the best values of affinity with 5-LOX. It was possible to observe that the structural similarity of the first five lactones is potentially linked to the best standard of affinity front 5-LOX, which was also influenced by the pattern of Hydrogen bonds with the same amino acids residues Asn180, Trp605, Gln611 in 5-LOX active site. Currently, the effect of these SL on 5-LOX is poorly understood. However, some degree of inhibition on these enzymes deserve to be investigated, since a better result with inuviscolide was obtained, which is a SL with recognized inhibitory effect on 5-LOX (Sur et al., 2009; Nam et al., 2015). As 5-LOX is directly involved in cutaneous inflammatory responses from biosynthesis of pro-inflammatory leukotriene lipid mediators (Gilbert et al., 2012), this enzyme plays an important role in contact dermatitis, representing a relevant target for the discovery and development of anti-inflammatory molecules and drugs (Fiorucci et al., 2001; Lohberger et al., 2013).

In addition to 5-LOX, the best values of affinity with COX-2 were obtained to budlein A (-8.484 kcal.mol^{-1}), artesunate (-8.366 kcal.mol^{-1}), 11α,13-dihydrohelenalin isobutyrate (-8.264 kcal.mol^{-1}), inuviscolide (-8.068 kcal.mol^{-1}) and coronopilin (-8.007 kcal.mol^{-1}). In this case, we identified that these lactones presented larger chemical structures and macrorings, whose conformational freedom degree was associated to better values of affinity with the COX-2 due to the greater spatial volume of the active site in this enzyme. Interestingly, budlein A exhibited a better molecular affinity to COX-2 than celecoxib, which is a classical and specific COX-2 inhibitor in clinical use (Inagaki et al., 2000). COX-2 catalyzes the committed step in the biosynthesis of prostaglandins, prostacyclins and thromboxanes, and its expression is tissue-specific and induced by cytokines and growth hormones (Orlando and Malkowski, 2016). COX-2 is highly expressed in the skin, especially in inflammatory processes whose regulatory

cytokines such as TNF-α and INF-γ induces COX-2 gene expression (Desai et al., 2018). Currently, there is no evidence on the effect of budlein A on inflammatory dermatological diseases; however, inhibitory effect of this lactone on COX-2 expression was proved in a murine model of antigen-induced arthritis (Zarpelon et al., 2017).

Although often underestimated and overlooked, MMP are directly involved in immunological responses and tissue repair in skin diseases (Birkedal-Hansen et al., 1993; Page-McCaw et al., 2007). Matrix metalloproteinase 1 (MMP-1) is a typical vertebrate collagenase. It consists of an N-terminal catalytic domain containing an active-site zinc ion and a C-terminal hemopexin domain comprised of a four-bladed β-propeller, which are connected by a linker region (Mankaa et al., 2012). In addition, matrix metalloproteinases MMP-2 and MMP-9 cleaves type IV collagen. MMP-2 is primarily expressed during development of mesenchymal cells and in process of tissue repair (Morgunova et al., 1999). Unfortunately, the effect of SL on these enzymes is poorly unknown. Thus, we presented theoretical evidences that several SL, especially budlein A (-8.000 kcal.mol^{-1}), artesunate (-7.924 kcal.mol^{-1}), helenalin (-7.628 kcal.mol^{-1}), tetraneurin A (-7.060 kcal.mol^{-1}), parthenin (-7.013 kcal.mol^{-1}) and coronopilin (-6.998 kcal.mol^{-1}) presented the best value of affinity with MMP-1. Interestingly, these results were similar to the *GScore* obtained to batimastat, which is a recognized MMP inhibitor. In addition, most SL investigated presented a better affinity with MMP-2 and MMP-9 than batimastat, indicating promising inhibitory effects on MMP that requires further investigation from *in vitro* and *in vivo* studies. Despite scarce evidence, previous studies indicated that specific SL such as artesunate is effective in attenuating MMP-2 and MMP-9 gene expression in animal models of liver (Xu et al., 2014) and lung (Wang et al., 2016) inflammation, and in fibroblast-like synoviocytes isolated from patients with rheumatoid arthritis (Ma et al., 2017). Similarly, SL from *Arnica montana* suppress MMP-1 and MMP-13 mRNA levels in bovine and human articular chondrocytes when administered in low doses, an effect mainly attributed to helenalin, a major SL of this plant species (Jäger et al., 2009). Therefore, although the inhibitory potential of SL on MMP cannot be neglected, its contribution to the mechanisms of cutaneous hypersensitivity need to be better clarified.

Considering a more comprehensive analysis of the scientific evidence *in vivo*, the risk of bias was assessed as a quality criterion complementary to the information reported in the studies reviewed. In general, the *in vivo* evidence was based on relevant animal models to study the cutaneous response to SL. However, the SYRCLE's toll revealed specific limitations in the research reports, which were mainly associated to underreporting of important information such as procedure of animal's allocation in experimental groups, the way they were housed,

procedures of experimental randomization, methods applied in data collection, and blinding of the evaluators related to the experimental groups and results. Objectively, the specific limitations identified in each study reviewed do not indicates that the researchers did not evaluate these parameters. However, it is a clear indicator that several relevant information to understand the experimental protocol were not included in the research reports. Thus, limited research reports can indicate potential risk bias (reporting bias), which exerts variable influence on the reproducibility, internal and external validity of the evidence. In this sense, it is essential that the results of the research are interpreted considering the narrow limits of the experimental designs on which the evidence is based. As all elements associated with the risk of bias hinder the progression of preclinical to clinical studies, it is essential to understand the limitations of research in order to define more accurate, reliable, reproducible and applicable preclinical experiments, whose external validity may support clinical trials.

5. Conclusion

Based on this systematic review, we identified that SL can modulates several immunological effectors in the skin and in skin-related cells, inducing proinflammatory and/or anti-inflammatory effects *in vitro* and *in vivo*. Although most SL exhibit distinct allergenic profiles *in vivo*, these effects are potentially influenced by the dose administered, since even highly allergenic and cytotoxic SL can induce anti-inflammatory effects when used in low doses *in vitro*. There is consistent evidence that both cytotoxic and anti-inflammatory activities are associated to the presence α-methylene-γ-butyrolactone system, which is directly involved in alkylation reactions induced by SL. Although the mechanistic basis that explain the proinflammatory and anti-inflammatory effects of SL *in vivo* remains poorly understood, the anti-inflammatory mechanisms *in vitro* are clearer. Thus, the current evidence indicates consistently that low doses of SL is able in downregulating gene expression, synthesis or activity of several immunological effector such as TNF-α, IL-1, IL-4 IL-6, IL-8, IL-12, MCP-1, 5-LOX, and COX-2; which are effects partially associated to a potent inhibitory effect on NF-κB pathway. Reinforcing the current evidence, the affinity of SL with target enzymes involved in cutaneous immunological mechanisms, such as 5-LOX, COX-2 and MMP-1, MMP-2 and MMP-9, was in fact identified from molecular docking; suggesting a potential relevance of these enzymes in studies on the therapeutic applicability of specific SL as anti-inflammatory agents. Although studies have used relevant animal models to investigate skin biology, methodological limitations were identified due to underreported aspects. Thus, the

suggestive risk of bias from uncertain to high reduced the generalizability of the current evidence, which must be interpreted within the narrow limits in which it was built. By indicating these limitations, this systematic review can assist the development of further studies with a more controlled experimental design, in order to minimize risk of bias associated to the evidence and to elucidate the specific mechanisms by which SL act in the skin immune system.

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SUPPLEMENTARY FILES

Table S1. Detailed search strategy with search filters and number of studies recovered in all electronic databases.

PubMed-MEDLINE – Search filters	***Records***
#1 Disease model: (Dermatitis[MeSH Terms] OR Dermatitis[TIAB] OR Psoriasis[MeSH Terms] OR Psoriasis[TIAB] OR Skin Disease*[TIAB])	**178,706**
#2 Bioactive molecules: (Sesquiterpenlactone*[TIAB] OR "Sesquiterpene lactone*"[TIAB] OR "Lactonized sesquiterpene*"[TIAB])	**1,303**
#3 Combined search: (#1 AND #2)	**102**
#4 Research limit: (#1) AND #2 NOT Review[PT]	**92**
SCOPUS – *Search filters*	***Records***
#1 Disease model: (TITLE-ABS-KEY("Dermatitis") OR TITLE-ABS-KEY("Psoriasis") OR TITLE-ABS-KEY("Skin disease"))	**300,619**
#2 Bioactive molecules: (TITLE-ABS-KEY("Sesquiterpenlactone") OR TITLE-ABS-KEY("Sesquiterpene lactone") OR TITLE-ABS-KEY("Lactonized sesquiterpene"))	**5,738**
#3 Combined search: #1 AND #2	**388**
#4 Research limit (Document type – Exclude): Review and Book Chapter	**306**
WEB OF SCIENCE – *Search filters*	***Records***
#1 Disease model: TS=Dermatitis OR TS=Psoriasis OR TS=Skin disease	**214,090**
#2 Bioactive molecules: TS=Sesquiterpenlactone OR TS=Sesquiterpene lactone OR TS=Lactonized sesquiterpene	**6,648**
#3 Combined search: #1 AND #2	**227**
#4 Research limit (Document type – Exclude): Review and Book Chapter	**182**

Table S2. Characteristics of the experimental model of all studies *in vivo* included in the systematic review.

Authors	Year	Country	Animal species	Animal lineage/specie	Sex	Age	Weight	Sesquiterpene lactone
In vivo studies								
Alonso Blasi et al.	1992a	France	Mice	Balb/c, Balb/d and DBA/2	Male	6 – 8 W	N	Helenin (alantolactone, isoalantolactone)
Alonso Blasi et al.	1992b	France	Mice	C3H/He, DBA/2, Balb/c and Balb/b	Male	5 – 6 W	N	Alantolactone, isoalantolactone
Barbier and Benezra	1982	France	Guinea pigs	Hartley albino	Female	N	250 – 300g	(+)- and (-)-γ -methyl- α -methylene- γ -butyrolactones
Cheminat et al.	1984	France	Guinea pigs	Himalayan spotted albino	Female	N	250 – 300g	Costunolide, Dehydrocostuslactone, Tulipinolide, Deacetyllaurenobiolide
Dupuis et al.	1980	Canada	Guinea pigs	Himalayan spotted albino	Female	N	300 – 500g	Alantolactone, isoalantolactone, α-methylene-γ-butyrolactone, decaline lactone, lactone, dimethyl lactone, bicyclolactone, adamantane lactone, spirolactone.
Fraginals et al.	1991	France	Mice	C3H/He, DBA/2, Balb/c and Balb/b	Male	5 – 6 W	N	Helenin (alantolactone, isoalantolactone)
Gabriel-Robez et al.	1982	France	Guinea pigs	Himalayan spotted	N	N	N	Alantolactone and isoalantolactone
Lass et al.	2008	Germany	Mice	C57BL/6 (B6), Balb/c, T cell receptor (TCR) transgenic P14, C57BL/6 (MHC class II knockout-KO)	Female	6 – 8 W	N	11α,13-dihydrohelenalinisobutyrate; 11α,13-dihydrohelenalinmethacrylate, helenalinisobutyrate, *Arnica* tinctures.
Lass et al.	2010	Germany	Mice	C57BL⁄6 (B6) and TCR transgenic P14	Female	6 – 8 W	N	11α,13-dihydrohelenalinmethacrylate, helenalinisobutyrate

N, not reported. W, weeks.

Table S2. Characteristics of the experimental model of all studies *in vivo* included in the systematic review.

Authors	Year	Country	Animal species	Animal lineage/specie	Sex	Age	Weight	Sesquiterpene lactone
In vivo studies								
Lin et al.	2016	China	Mice	Balb/c	Female	6 – 8 W	N	1β-Hydroxyalantolactone (IJ-5)
Máñez et al.	1999	Spain	Mice	Swiss	Female	N	25 – 30g	Inuviscolide
Picman et al.	1982	Canada	Guinea pigs	Albino	Female	N	N	Parthenin, Hymenin, Coronopilin, Damsin, Dihydroisoparthenin, Tethahydroparthenin, Tetraneurin-A, Hysterin, Helenalin, Tenulin
Recio et al.	2000	Spain	Mice	Swiss	Female	N	25 – 30g	4-α-O-Acetyl-pseudoguaian-6β-olide, hymenin, ambrosanolide, tetraneurin A, patthenin, hysterin, confertdiolide
Schaeffer et al.	1990	France	Guinea pigs	Himalayan spotted albino	Female	N	N	Helenin (Alantolactone, Isoalantolactone), *cis*-bicyclic lactone, *trans*-bicyclic lactone.
Schmidt and Chung	1993	United Kingdom	Mice	WSP	Female	12 – 16 W	N	Helenin (alantolactone, isoalantolactone, 11,13-dihydroalantolactone and 11,13-dihydroisoalantolactone)
Sosa et al.	2001	Italy	Mice	N	N	N	N	1,4-Dihydroxy-germacra-5E-10(14)-diene (DHGD)
Stampf et al.	1978	France	Guinea pigs	Hartley albino	Female	N	400 – 600g	Norbornane lactone, Helenin (Alantolactone, Isoalantolactone), Costunolide, Laurenobiolide, Frullanolide, Spirolactone, α-methylene-γ-butyrolactone.
Stampf et al.	1982	France	Guinea pigs	Himalayan spotted albino, Hartley albino, Pirbright white.	Female	N	N	Helenin (alantolactone, isoalantolactone)

N, not reported. W, weeks.

Table S2. Characteristics of the experimental model of all studies *in vivo* included in the systematic review.

Authors	Year	Country	Animal species	Animal lineage/specie	Sex	Age	Weight	Sesquiterpene lactone
In vivo studies								
Sur et al.	2009	USA	Mice	CD-1	Female	N	N	Parthenolide, Feverfew tincture
Wang et al.	2018	China	Mice	ICR	Female	5 – 8 W	18 – 22g	Alantolactone, Isoalantolactone, Total sesquiterpene lactones (TSL-IHL)

N, not reported. W, weeks.

Table S2 Characteristics of the experimental model of all studies *in vitro* included in the systematic review.

Authors	Year	Country	Cells type	Cells lineage	Source	Culture medium	Sesquiterpene lactone
In vitro studies							
Alonso Blasi et al.	1992a	France	Lymphocytes	N	Balb/c, Balb/b and DBA/2 after induction with alantolactone and isoalantolactone	RPMI-1640 medium supplemented with 1 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 5 x 10^{-5} M final concentration of mercaptoethanol, 100μg/ml gentamycin and 10% heat-inactivated fetal calf serum.	Alantolactone and isoalantolactone
Hofmann et al.	2014	Germany	Human keratinocyte	HaCaT	N	N	Helenalin
Kim et al.	2015	Republic of Korea	Human keratinocyte	HaCaT	N	RPMI-1640 medium containing 5% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin sulfate.	Ixerisoside A
Lass et al.	2008	Germany	Dendritic cells	N	N	RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM of l-glutamine, 25 mM of HEPES buffer, 50 μg/ml of penicillin-streptomycin and 10 μM of 2-mercaptoethanol	11α,13-dihydrohelenalinisobutyrate; 11α,13-dihydrohelenalinmethacrylate, helenalinisobutyrate, Arnica tinctures.

N, not reported.

Table S2 Characteristics of the experimental model of all studies *in vitro* included in the systematic review.

Authors	Year	Country	Cells type	Cells lineage	Source	Culture medium	Sesquiterpene lactone
In vitro studies							
Lee et al.	2013	Republic of Korea	Rat basophilic leucemia; B cell	RBL-2H3; Y16	Both ATCC (American Type Culture Collection)	DMEM supplemented with 10% (v/v) fetal bovine serum, 100U/mL penicillin, and 100μg/mL streptomycin; RPMI-1640 supplemented with 24mM $NaHCO_3$, 100U/mL penicillin, 100μg/mL streptomycin, 50μM 2-mercaptoethanol, and 10U/mL of IL-5.	Magnolialide, Santamarine, Reynosin, Baynol C, 11,13-dehydrosantonin, (3aS,5aR,6R,9S,9aS, 9bS)-6,9-dihydroxy-5a,9-dimethyl-3-methylidene-3a,4,5,6,7,8,9a,9b-octahydrobenzo [g][1]benzofuran-2-one, and Lucentolide
Lee et al.	2018	Republic of Korea	Mast cells	RBL-2H3	ATCC (American Type Culture Collection)	High-glucose Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/mL penicillin, and 50 μg/mL streptomycin.	Alantolactone, dehydrocostuslactone, costunolide
Lin et al.	2016	China	Human keratinocyte	HaCaT	ATCC (American Type Culture Collection)	DMEM medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin.	1β-Hydroxyalantolactone (IJ-5)
Nam et al.	2015	South Korea	Human keratinocytes (human papillomavirus 16 E6/E7 transformed)	HEK001	ATCC (American Type Culture Collection)	Bovine pituitary extract, recombinant epidermal growth factor, 100 U/ml penicillin and 100 μg/ml streptomycin.	Parthenolide

N, not reported. DMEM, Dulbecco's modified Eagle's medium.

Table S2. Characteristics of the experimental model of all studies *in vitro* included in the systematic review.

Authors	Year	Country	Cells type	Cells lineage	Source	Culture medium	Sesquiterpene lactone
In vitro studies							
Scarponi et al.	2014	Italy	Human keratinocytes	N	Skin biopsies of healthy donors	Serum-free medium KGM and keratinocyte basal medium (KBM-GOLD)	Dehydrocostuslactone, Costunolide and Dehydrocostunolide
Seo et al.	2015	Republic of Korea	Human keratinocytes	HaCaT	CLS Cell Lines Service GmbH	Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 μg/ml) and streptomycin (100 μg/ml).	Costunolide, Dehydrocostuslactone, and Alantolactone
Sur et al.	2009	USA	Murine macrophage; Stable transfected NF-κB reporter cell line designed for monitoring the activity of NFκB transcription factor in cellbased assays	RAW267.4; 293/NFκB-luc	ATCC; Panomics	Serum-free Epilife medium supplemented with human keratinocyte growth supplement containing 0.2 % (v/v) bovine pituitary extract (BPE), 5 μg/ml bovine insulin, 0.18 μg/ml hydrocortisone, 5 μg/ml bovine transferrin and 0.2 ng/ml human epidermal growth factor; It was not informed how the cells 293/NFκB-luc were maintained.	Parthenolide, Feverfew tincture

N, not reported.

Table S2. Characteristics of the experimental model of all studies *in vitro* included in the systematic review.

Authors	Year	Country	Cells type	Cells lineage	Source	Culture medium	Sesquiterpene lactone
In vitro studies							
Svensson et al.	2018	Sweden	Primary human dermal fibroblasts, Human keratinocytes and Human monocytes	HDFa, HaCaT and THP-1, respectively.	Cascade Biologics, CLS Cell Line Service GmbH and ATCC, respectively.	HDFa and HaCaT cells were cultured in DMEM/Ham´s F12 (1:1) and THP-1 cells in RPMI-1640 medium, both supplemented with antibiotics (penicillin 50 U/ml, streptomycin 50 µg/ml) and 10% fetal bovine serum.	Coronopilin and damsin
Takei et al.	2015	Japan	Normal human epidermal keratinocyte	NHEKs	Clonetics-BioWhittaker	Serum-free keratinocyte growth medium supplemented with bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrine.	Cynaropicrin
Wang et al.	2018	China	Human keratinocyte	HaCaT	Shanghai Institute of Cell Biology, Chinese Academy of Sciences	Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin.	Alantolactone, Isoalantolactone, Total sesquiterpene lactones (TSL-IHL)
Zhang et al.	2018	China	Human skin cancer	SKMEL-5	Type Culture Collection of Chinese Academy of Sciences	RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100U/mL penicillin and 100µg/mL streptomycin	Lactucopicrin

N, not reported.

Table S3. Characteristics of sesquiterpene lactones and model of skin and cell sensitization used in all studies *in vivo* included in the systematic review

Authors	Year	Sesquiterpene lactones	Plant species / Sources	Dermatitis induction / Sensitization
In vivo studies				
Alonso Blasi et al.	1992a	Helenin (alantolactone, isoalantolactone)	Commercial source	• Sensitization: 100 µl 10% lactones in acetone:olive oil (4:1), shaved abdomen by epicutaneous route. • Sensitization: 100 µl 10-20% isoalantolactone in Freund's complete adjuvant (FCA), shaved dorsal area by epicutaneous and intradermal route.
Alonso Blasi et al.	1992b	Alantolactone, isoalantolactone	Roots of *Inula helenium* L.	• Sensitization: 100 µl of 3, 6, 10, 12 and 15% solutions of alantolactone in acetone:olive oil (4:1), by epicutaneous route on the shaved abdomen.
Barbier and Benezra	1982	(+)- and (-)-γ -methyl- α -methylene- γ -butyrolactones	(R)- and (S)-glutamic acid	• Sensitization: 100 µl intradermal injection 5% lactones emulsified in FCA (Freund's complete adjuvant) in the shaved nuchal region (3 alternate days).
Cheminat et al.	1984	Costunolide, Dehydrocostuslactone, Tulipinolide, Deacetyllaurenobiolide	*Laurus nobilis* L.	• Sensitization: 100 µl intradermal injection 1% extract emulsion in FCA/saline (1:1), in the nuchal region in alternate days).
Dupuis et al.	1980	Alantolactone, isoalantolactone, α-methylene-γ-butyrolactone, decaline lactone, lactone, dimethyl lactone, bicyclolactone, adamantane lactone, spirolactone.	Commercial source	• Sensitization: Two intradermal injections of emulsion - isotonic saline + complete Freund's adjuvant (1:1) (0.1 ml each, containing 50 µg of conjugate) and two intradermal injections (0.1 ml each) of a FCA-saline emulsion (1:1) without conjugate, in the post nuchal region.
Fraginals et al.	1991	Helenin (alantolactone, isoalantolactone)	Commercial source	• Sensitization: 100 µl of alantolactone in acetone:olive oil (4:1) solutions of 3, 6, 10, 12 and 15% concentration on the shaved abdominal area by epicutaneous route.

Table S3. Characteristics of sesquiterpene lactones and model of skin and cell sensitization used in all studies *in vivo* included in the systematic review

Authors	Year	Sesquiterpene lactones	Plant species / Sources	Dermatitis induction / Sensitization
In vivo studies				
Gabriel-Robez et al.	1982	Alantolactone and isoalantolactone	*Inula helenium*	• Sensitization: 0.1 ml intradermal injection of a 5% emulsion of alantolactone in a 1:1 saline-FCA (Freund's complete adjuvant) mixture in the nuchal area, on alternate days. Five injections were used to induce hypersensitivity.
Lass et al.	2008	11α,13-dihydrohelenalinisobutyrate; 11α,13-dihydrohelenalinmethacrylate, helenalinisobutyrate and Arnica tinctures.	Flowers of *Arnica montana*	• Sensitization in C57BL/6 and Balb/c mice: epicutaneously application on day 0 with 100µl of TNCB (2,4,6-trinitrochlorobenzene) (7%) in acetone, CE and SP tincture (undiluted), helenalinisobutyrate, dihydrohelenalinmethacrylate and dihydrohelenalinisobutyrat, all in EtOH (ethanol), on shaved abdominal skin. • Sensitization in C57BL/6 mice or MHC class II KO mice treated with anti-CD4: anti-CD4 monoclonal antibody was given intraperitoneally on days –3, –2, –1 before and on day +4 after the last sensitization (day 0).
Lass et al.	2010	11α,13-dihydrohelenalinmethacrylate, helenalinisobutyrate and Arnica tinctures.	Flowers of *Arnica montana*.	• Three days pretreatment with Arnica tincture on the shaved abdominal skin. Dermatitis: epicutaneous application of 100 µl 7% TNCB (2,4,6-trinitrochlorobenzene) in acetone on the shaved abdominal skin.
Lin et al.	2016	1β-Hydroxyalantolactone (IJ-5)	Aerial part of *Inula japonica*	• Sensitization: once on day 1 by painting 100 mL of 5% DNCB (2,4-Dinitrochlorobenzene) solution (dissolved in a 3:1 mixture of acetone and olive oil) on their shaved dorsal skin.

CE, Central European. SP, Spanish.

Table S3. Characteristics of sesquiterpene lactones and model of skin and cell sensitization used in all studies *in vivo* included in the systematic review

Authors	Year	Sesquiterpene lactones	Plant species / Sources	Dermatitis induction / Sensitization
In vivo studies				
Máñez et al.	1999	Inuviscolide	*Inula viscosa*	• *TPA-Induced:* topical application of 2.5 µg of TPA (12-O-tetradecanoylphorbol-13-acetate) dissolved in 20 µl of Me_2CO (dimethyl ketone) on the right ear. • *Arachidonic acid (AA)-Induced:* topical application of 2 mg/ear of AA in 20 µl of Me_2CO (dimethyl ketone). • *Mouse Ear Inflammation Induced by Multiple Topical Applications of TPA:* topical application of 10 µl of TPA (2.5 µg/ear) on alternate days on both the inner and outer surface of both ears.
Picman et al.	1982	Parthenin, Hymenin, Coronopilin, Damsin, Dihydroisoparthenin, Tethahydroparthenin, Tetraneurin-A, Hysterin, Helenalin, Tenulin	Hysterin, helenalin, damsin, tetraneurin-A and tenulin: donation. Parthenin, hymenin and coronopilin were isolated from *Parthenium hysterophorus.* Dihydroisoparthenin and Tethahydroparthenin: obtained of parthenin	• Sensitization: 50µl of a 10% parthenin solution in 80% ethanol was applied daily for 15 days (except on the 6^{th} and 12^{th} days) to the ears of animals. 18 days later, a 10% solution of parthenin was applied on their shaved flanks.

Table S3. Characteristics of sesquiterpene lactones and model of skin and cell sensitization used in all studies *in vivo* included in the systematic review

Authors	Year	Sesquiterpene lactones	Plant species / Sources	Dermatitis induction / Sensitization
In vivo studies				
Recio et al.	2000	4-α-O-Acetyl-pseudoguaian-6β-olide, hymenin, ambrosanolide, tetraneurin A, patthenin, hysterin, confertdiolide	Parthenium hysterophorus L. and Parthenium glomeratum	• Carrageenan-induced: a subplantar injection of 50µl of a 3% carrageenan solution into the right hind paw of the mouse. • (TPA)-induced: topical application of 10 µl of TPA (12-O-tetradecanoylphorbol-13-acetate) in acetone (2.5 µg/ear) on the right ear. • Ethyl-phenylpropiolate (EPP)-induced: EPP in acetone (1 mg/ear) was applied topically in ear. • Arachidonic acid (AA)-induced: AA was applied topically on the right ear (2 mg/20 µl). • Serotonin-induced in mice: serotonin (50 µl, 1% solution saline) was injected into right hind paw. • Mouse ear edema: 20 µl of TPA (2 µg/ear x 5 times) applied topically to both the inner and outer surface of both ears of each mouse on alternate days. • Oxazolone-induced: topical application on the ventral abdomen of 50µl of a 2% (w/v) of oxazolone in acetone on two consecutive days (days 1 and 2).
Schaeffer et al.	1990	Helenin (Alantolactone, Isoalantolactone), cis-bicyclic lactone, trans-bicyclic lactone.	Helenin: Commercial source. cis-bicyclic lactone and trans-bicyclic lactone: chemical synthesis	• Sensitization: Days 2 and 4, the animals received an intradermal injection in the shaved post-nuchal region of emulsion from Freund's Complete Adjuvant (FCA) of saline with 0.3% Helenin and a second emulsion with 0.3% of cis-lactone.

Table S3. Characteristics of sesquiterpene lactones and model of skin and cell sensitization used in all studies *in vivo* included in the systematic review

Authors	Year	Sesquiterpene lactones	Plant species / Sources	Dermatitis induction / Sensitization
In vivo studies				
Schmidt and Chung	1993	Helenin (alantolactone, isoalantolactone, 11,13-dihydroalantolactone and 11,13-dihydroisoalantolactone)	Helenin: Commercial source. 11,13-dihydroalantolactone and 11,13-dihydroisoalantolactone: obtained from alantolactone and isoalantolactone.	• Sensitization: two applications of xenobiotics in acetone spaced 24h. Sesquiterpene lactones were applied as 0.22M solutions (0.2 mL) to the shaved dorsal skin on days 1 and 2; isothiocyanates were applied as 0.37M solutions (25µl).
Sosa et al.	2001	1,4-Dihydroxy-germacra-5E-10(14)-diene (DHGD)	Flower of Achillea pannonica	• Cutaneous inflammation: 75 µg of Croton oil/ear in 15 µl acetone on the right ear.
Stampf et al.	1978	Norbornane lactone, Helenin (Alantolactone, Isoalantolactone), Costunolide, Laurenobiolide, Frullanolide, Spirolactone, α-methylene-γ-butyrolactone.	Helenin: Commercial source. Norbornane lactone, Spirolactone, α-methylene-γ-butyrolactone: chemical synthesis. Costunolide, Laurenobiolide, Frullanolide: donation.	• Sensitization: solutions with alantolactone, isoalantolactone and laurel essential oil residue in olive oil-acetone (4:1) with added Freund's complete adjuvant.

Table S3. Characteristics of sesquiterpene lactones and model of skin and cell sensitization used in all studies *in vivo* included in the systematic review

Authors	Year	Sesquiterpene lactones	Plant species / Sources	Dermatitis induction / Sensitization
In vivo studies				
Stampf et al.	1982	Helenin (alantolactone, isoalantolactone)	Commercial source	• *OET (open epicutaneos test):* 0.1ml of a 5% ethanol solution of alantolactone or 0.1mL of a 10% acetone solution of isoalantolactone or 15% ethanol solution of helenin. • *FCAT (Freund's Complete Adjuvant Technique):* 0,1ml of the emulsion with Freund's Complete Adjuvant with a final 0.1% concentration in alantolactone, another with a 0.2% concentration and a third one with a 0.3% concentration in helenin injected intradermally in the shaved post-nuchal region. • *DT (Draize test):* 0.05ml of the suspension of helenin in saline was injected intradermally into the clipped flank. 9 injections were repeated on alternate days. • *GPMT (Guinea pig maximization test):* three series of intradermal injections on shaved post-nuchal: 2 injections of the substance (0.2%) in saline, 2 injections of the substance (0.2%) in FCA-saline (1:1) emulsion (0.1ml) and 2 injections of a 1:1 FCA-saline emulsion (0.1ml). • *OT (Optimization test):* on day 0 animals received 2 intradermal injections (0.05ml and 0.1ml) of the substance (0.1%), in saline on shaved flank. On days 2 and 4, injections (0,1ml) of a 0.1% suspension of the substance in saline into the same flank, On alternate days (starting on day 6), intradermal injections (0.1ml) of the FCA emulsions (0.3%) to day 16.

Table S3. Characteristics of sesquiterpene lactones and model of skin and cell sensitization used in all studies *in vivo* included in the systematic review.

Authors	Year	Sesquiterpene lactones	Plant species / Sources	Dermatitis induction / Sensitization
In vivo studies				
Sur et al.	2009	Parthenolide	*Tanacetum parthenium*	• *TPA-induced ear oedema:* 1µg/ear of TPA (12-O-tetradecanoylphorbol-13-acetate) applied to the left ear. • *Oxazolone-induced:* oxazolone applied to the right ear.
Wang et al.	2018	Alantolactone, Isoalantolactone, Total sesquiterpene lactones (TSL-IHL)	Roots of *Inula helenium*	• Sensitization: 100 µl of 7% DNCB (2,4-dinitrochlorobenzene) dissolved acetone:olive oil (4:1) once on day 1 by topically applying on their shaved back skin.

Table S3 (*continuation*). Characteristics of sesquiterpene lactones and protocol of cell challenge used in all studies *in vitro* included in the systematic review.

Authors	Year	Sesquiterpene lactone	Plant species / Source	Induction mode
In vitro studies				
Alonso Blasi et al.	1992a	Alantolactone and isoalantolactone	Commercial source	• Stimulation: lymphocytes were stimulated by adding 20 µl per well of a $2x10^{-5}$ M, $1x10^{-5}$ M, $2x10^{-6}$ M, $1x10^{-6}$ M, $5x10^{-7}$ M or $2,5x10^{-7}$ M solution of alantolactone or isoalantolactone in complete RPMI medium.
Hofmann et al.	2014	Helenalin, A.montana extract	Flowers of Arnica montana	• Stimulation: HaCaT cells were exposed with 125 µg/mL A. montana or 5 µM helenalin.
Kim et al.	2015	Ixerisoside A	Whole plant: Ixeris dentata	• Stimulation: the medium was replaced with fresh RPMI-1640 medium and exposed to UVB (100mJ/cm2).
Lass et al.	2008	11α,13-dihydrohelenalinisobutyrate; 11α,13-dihydrohelenalinmethacrylate, helenalinisobutyrate, Arnica tinctures	Arnica montana	• Stimulation: Dendritic cells were incubated for 48 h with 1 µg/ml of LPS (lipopolysaccharide) from *Escherichia coli*. LPS was added to the cells 1 h before tincture treatment.
Lee et al.	2013	Magnolialide, Santamarine, Reynosin, Baynol C, 11,13-dehydrosantonin, (3aS,5aR,6R,9S,9aS, 9bS)-6,9-dihydroxy-5a,9-dimethyl-3-methylidene-3a,4,5,6,7,8,9a,9b-octahydrobenzo [g][1]benzofuran-2-one, and Lucentolide	Laurus nobilis	• Stimulation RBL-2H3 cells: cells were incubated with monoclonal mouse anti-dinitrophenyl IgE anti-body (anti-DNP IgE) (1 µg/mL) for 24h at 37ºC. • Stimulation Y16 cells: cells were incubated with 50 µM 2-mercaptoethanol, and 10 U/mL of IL-5 and other compounds as antibiotic, for 48h.

Table S3 (*continuation*). Characteristics of sesquiterpene lactones and protocol of cell challenge used in all studies *in vitro* included in the systematic review.

Authors	Year	Sesquiterpene lactone	Plant species / Source	Induction mode
In vitro studies				
Lee et al.	2018	Alantolactone, dehydrocostuslactone, costunolide	Commercial source	• Stimulation: incubation with 0.2 µg/mL monoclonal anti-dinitrophenyl mouse immunoglobulin E overnight at 37°C in a 5% CO2 incubator.
Lin et al.	2016	1β-Hydroxyalantolactone (IJ-5)	Aerial part: Inula japonica	• Stimulation: 20 ng/ml TNF-α for 10 min (for Western blotting assay) or 6 h (for qRT-PCR).
Nam et al.	2015	Parthenolide	EMD-Calbiochem	• Stimulation: Keratinocytes were treated with 1 µg/ml lipopolysaccharide (LPS) for 30min, 4h, 24h, varying according to the analysis.
Scarponi et al.	2014	Dehydrocostuslactone (DCE), Costunolide (CS) and Dehydrocostunolide (HCS)	Commercial source Dehydrocostunolide : obtained from Costunolide.	• Stimulation: 50 ng/ml human recombinant IL-22, 200 U/ml human recombinant IFN-γ or 50 ng/ml human recombinant TNF-α in keratinocyte basal medium (KBM-GOLD) for different time periods.
Seo et al.	2015	Costunolide, Dehydrocostuslactone, and Alantolactone	Roots of *Aucklandia lappa*.	• Stimulation: cells were treated with sesquiterpene lactones in 1ml of serum-free medium supplemented with tumor necrosis factor (TNF-α) and interferon gamma (IFN-γ) (each 10 ng/ml) for 24h.
Sur et al.	2009	Parthenolide	*Tanacetum parthenium*	• Stimulation: Macrophages were stimulated with 100 ng/mL LPS. Cells 293/NFκB-luc were treated with 100 µg/ml TNF-an alone or in the presence of the NF-κB inhibitor BAY11-7082.
Svensson D. et al.	2018	Coronopilin and damsin	Aerial parts of *Ambrosia arborescens*	• Stimulation: incubation with lipopolysaccharide (LPS, 0.5 µg/ml, *Escherichia coli*) for 24h.

Table S3 (*continuation*). Characteristics of sesquiterpene lactones and protocol of cell challenge used in all studies *in vitro* included in the systematic review.

Authors	Year	Sesquiterpene lactone	Plant species / Source	Induction mode
In vitro studies				
Takei et al.	2015	Cynaropicrin (Cyn)	Extrasynthese	• Stimulation 1: NHEKs were treated with Cyn BaP, TNF-α, or DMSO (Dimethyl sulfoxide, control). • Stimulation 2: UVB treatment of cells was performed using a Philips TL20W/12RS lamp with an emission peak at 310 nm. Cells were irradiated at 50 mJ/cm^2 in phosphate-buffered saline (PBS) with Mg_2^+/Ca_2^+.
Wang et al.	2018	Alantolactone, Isoalantolactone, Total sesquiterpene lactones (TSL-IHL)	Roots of Inula helenium	• Stimulation: 20 ng/ml of TNF-α for 20min to 6 h.
Zhang et al.	2018	Lactucopicrin	Commercial source	N

N, not reported.

Table S4. Characteristics of treatments administered in all studies *in vivo* identified in the systematic review.

Authors	Year	Formulation administered	Dose	Administration route	Treatment / Challenge	Duration of treatment
In vivo studies						
Alonso Blasi et al.	1992a	Acetone: olive oil (4:1)	1% helenin, 1% alantolactone, 1% isoalantotactone	Topical (ear)	• Challenge: Single dose 25 µl 1% helenin, alantolactone or isoalantolactone in acetone:olive oil (4:1).	1 d
Alonso Blasi et al.	1992b	Acetone: olive oil (4:1)	1% alantolactone, 1% isoalantotactone	Topical (ear)	• Challenge: Single dose 25 µl 1% of the lactones in acetone:olive oil (4:1).	1 d
Barbier and Benezra	1982	Dichloromethane (CH_2C1_2)	(-)-α-methylene-γ-methyl-γ -butyrolactone (5%, w/v, in a 1:1 FCA-saline emulsion), (+)-α -methylene- γ -methyl- γ -butyrolactone (5%, w/v, under the same conditions), and the racemate of α -methylene- γ -methyl- γ -butyrolactone (10%, w/v, under the same conditions).	Epicutaneous (shaved flanks)	• Challenge: Single dose 25 µl 5-10% lactones in dichloromethane by epicutaneous route on the shaved flank.	1 d
Cheminat et al.	1984	Ethanol solution	3-30% crude extract, 1% costunolide, dehydrocostuslactone, tulipinolide, deacetyllaurenobiolide.	Epicutaneous (shaved flanks)	• Challenge: Single dose 25 µl 1-30% lactones by epicutaneous route.	1 d

Table S4. Characteristics of treatments administered in all studies *in vivo* identified in the systematic review.

Authors	Year	Formulation administered	Dose	Administration route	Treatment / Challenge	Duration of treatment
In vivo studies						
Dupuis et al.	1980	Ethanol and methylene chloride	Conjugate alantolactone-skin: 50 mg (0.2 mmol). Cross-reaction: alantolactone (0.1%), isoalantolactone (0.1%), α-methylene-γ-butyrolactone, decaline lactone, lactone, dimethyl lactone, bicyclolactone, adamantane lactone, spirolactone with 1%.	Epicutaneous (shaved flanks)	• Challenge: Single dose 25 µl of the lactones by shaved flank over an area of $2cm^2$.	1 d
Fraginals et al.	1991	Acetone: olive oil (4:1)	1% alantolactone, 1% isoalantotactone	Topical (ear)	• Challenge: Single dose 25 µl of a 1% lactones solution in acetone:olive oil (4:1).	1 d
Gabriel-Robez et al.	1982	Ethanol solution	1% alantolactone or isoalantolactone	Epicutaneous (shaved flanks)	• Challenge: 1% alantolatone or isoalantolactone on a $2cm^2$ area on the shaved flank by open epicutaneous test.	1 d
Lass et al.	2008	Tincture and ethanol	Helenalinisobutyrate: 1.2 mM = 39.89 mg/100 ml tincture or ethanol (EtOH) and 12 mm. 11a.13-dihydrohelenalinmethacrylate: 2.12 mM = 69.93 mg/100 ml tincture or EtOH and 21.2 mM. 11a,13-dihydrohelenalinisobutyrate: 2.12 and 21.2 mM.	Topical (skin testing on both ears)	• Challenge C57BL/6 and Balb/c mice: on day 5 with 20 µl of 1% TNCB (2,4,6-trinitrochlorobenzene) or 20 µl of the Arnica tinctures or sesquiterpene lactones applied on both ears. • Challenge in C57BL/6 and MHC class II KO mice: on days 5 and 6 with 25 µl of tincture.	1 to 2 d

Table S4. Characteristics of treatments administered in all studies *in vivo* identified in the systematic review.

Authors	Year	Formulation administered	Dose	Administration route	Treatment / Challenge	Duration of treatment
In vivo studies						
Lass et al.	2010	Tincture and ethanol	N	Topical (ears)	• Challenge: Single dose of 20 µl of 1% TNCB (2,4,6-trinitrochlorobenzene) or 20 µl of the Arnica tinctures applied on both ears.	1 d
Lin et al.	2016	N	10 mg/kg	Topical / Intraperitoneal	• Challenge: From day 5, the mice received five times every 3 d by painting the inner and outer surfaces of the right ears with 20 mL of 0.2% DNCB (2,4-Dinitrochlorobenzene). In the IJ-5 treatment group, 10 mg/kg IJ-5 was administrated through i.p. injection 1 h before every DNCB challenge.	17 d
Máñez et al.	1999	Me2CO (dimethyl ketone)	0.5 mg/ear	Topical (ears)	• TPA-Induced challenge: single dose applied topically of 0.5mg/ear of pure compounds simultaneously with TPA dissolved in Me2CO (dimethyl ketone). • Arachidonic acid (AA)-Induced challenge: single dose applied topically of 0.5mg/ear of pure compounds 30 min before AA dissolved in Me2CO. • Mouse Ear Inflammation Induced by Multiple Topical Applications of TPA challenge: 2x daily for 4 days, in the morning immediately after TPA (12-O-tetradecanoylphorbol-13-acetate) application, and 6 h later of 0.5mg/ear of pure compounds dissolved in Me2CO, applied topically.	• TPA-Induced: 4h. • Arachidonic acid (AA)-Induced: 1h. • Mouse ear inflammation induced by multiple topical applications of TPA: 4 d
Picman et al.	1982	Ethanol solution	10% parthenin solution in 80% ethanol	Epicutaneous (shaved flanks)	• Challenge: 50µl of 10% solutions of individual sesquiterpene lactones applied on the shaved flanks.	1 d

N, not reported.

Table S4. Characteristics of treatments administered in all studies *in vivo* identified in the systematic review.

Authors	Year	Formulation administered	Dose	Administration route	Treatment / Challenge	Duration of treatment
In vivo studies						
Recio et al.	2000	*Carrageenan-induced:* EtOH/Tween 8O/H_2O (2:2:20, v/v). *(TPA)-induced, Ethyl-phenylpropiolate (EPP)-induced, Arachidonic acid (AA)-induced, Mouse ear edema:* acetone solution. *Serotonin-induced paw edema in mice, Oxazolone-induced:* N.	*Carrageenan-induced:* 100 mg/kg (0,5 ml). *(TPA)-induced, Ethyl-phenylpropiolate (EPP)-induced, Arachidonic acid (AA)-induced and Mouse ear edema:* 0,5 mg/ear. *Serotonin-induced paw edema in mice:* 50 mg/kg (0,1 mL). *Oxazolone-induced:* 20 µL to right ears.	*Carrageenan-induced:* orally. *(TPA)-induced, Ethyl-phenylpropiolate (EPP)-induced, Arachidonic acid (AA)-induced, Mouse ear edema, Oxazolone-induced:* topically. *Serotonin-induced paw edema in mice:* Subcutaneous.	• *Carrageenan-induced:* sesquiterpene lactones dissolved in EtOH/Tween 80/H_2O (2:2:20, v/v) were administered orally at 100mg/kg (0.5 ml) 1 h before carrageenan injection. • *(TPA)-induced:* compounds dissolved in acetone were applied topically (0.5 mg/ear) simultaneously with TPA (12-O-tetradecanoylphorbol-13-acetate). • *Ethyl-phenylpropiolate (EPP)-induced:* compounds (0.5 mg/ear) dissolved in acetone were applied topically 16 h before induction of ear edema. • *Arachidonic acid (AA)-induced:* compounds in acetone were applied topically (0.5 mg/ear) 30 min before the application of AA on the right ear. • *Serotonin-induced paw edema in mice:* compounds (50 mg/kg) were administered (0.1 ml, s.c.) 3 h before the subplantar injection of serotonin. • *Mouse ear edema:* compounds were dissolved in acetone and applied topically (0.5 mg/ear) twice daily for four days, in the morning immediately after TPA application and 6 h later. • *Oxazolone-induced:* application of 30 µl of 2% oxazolone to both ears. Rechallenge: Sesquiterpene lactones were applied (20 µl) to right ears 6 h after challenge (single application) and 24, 48, 72 and 96 h after challenge (repeated dosage).	1 to 4 d

N, not reported.

Table S4. Characteristics of treatments administered in all studies *in vivo* identified in the systematic review.

Authors	Year	Formulation administered	Dose	Administration route	Treatment / Challenge	Duration of treatment
In vivo studies						
Schaeffer et al.	1990	Ethanol solution	3, 1, 0.3 and 0.1%	Epicutaneous (shaved flanks)	• Challenge: 25µl solution of the sensitizer in ethanol was applied on a 2 cm^2 area of the shaved flank of the animals. Animals sensitizes to helenin on day 0 were challenged to helenin, cis-lactone and trans-lactone on day 29. Animals sensitized to cis-lactone on day 0 were challenged to cis-lactone, Helenin and trans-lactone on day 29.	1 d
Schmidt and Chung	1993	Acetone	0,022M	Topical (skin testing on inner surface of ear)	• Challenge and cross-challenge: 20 µl of 0.022 M solutions of sesquiterpene lactones and 20 µl of 0.03 M solutions of isothiocyanates.	1 d
Sosa et al.	2001	Croton oil solution	*DHGD:* 0.000µmol/cm^2, 0.250µmol/cm^2, 0.400µmol/cm^2, 0.630µmol/cm^2, 0.750µmol/cm^2, 1000µmol/cm^2. *Indomethacin:* 0.000µmol/cm^2, 0.125µmol/cm^2, 0.250µmol/cm^2, 0.500µmol/cm^2, 0.750µmol/cm^2. *Hydrocortisone:* 0.0000µmol/cm^2, 0.0062µmol/cm^2, 0.0125µmol/cm^2, 0.0250µmol/cm^2, 0.0500µmol/cm^2, 0.1000µmol/cm^2.	Topical (ear)	N	N

DHGD, 1,4-Dihydroxy-germacra-5E-10(14)-diene. N, not reported.

Table S4. Characteristics of treatments administered in all studies *in vivo* identified in the systematic review.

Authors	Year	Formulation administered	Dose	Administration route	Treatment / Challenge	Duration of treatment
In vivo studies						
Stampf et al.	1978	Olive oil-acetone	1% alantolactone, 1% isoalantolactone. The doses of the other compounds were not reported.	Epicutaneous (shaved flanks)	• Challenge: 20 µl of a solution of the sesquiterpene lactones in olive oil-acetone (1:9) was deposited on the animal's shaved flank.	1 d
Stampf et al.	1982	Ethanol solution	OET: helenin 0.1% and 0.03%, alantolactone 0.1%, isoalantolactone 0.1%. FCAT: helenin 0.1% and 0.03%, alantolactone 0.1%, isoalantolactone 0.1%. DT: helenin 0.3% and 0.1%. GPMT: alantolactone 0.1% and 0.03%, isoalantolactone 0.1% and 0.03%. OT: helenin 0.03% and 0.01%.	Epicutaneous (shaved flanks) and intradermal	• Challenge: 25 µl solution of the sesquiterpene lactones in ethanol was applied on a 2cm^2 area of the shaved flank.	1 d
Sur et al.	2009	N	TPA-induced: 1% Parthenolide. Feverfew tincture: 0.01%, 0,1% and 1%. Oxazolone-induced: 0.01% and 0.1% of Parthenolide and Feverfew tincture.	Topical (ears)	• Challenge TPA-induced: immediately after TPA-induced, parthenolide or tincture was applied to the TPA-treated ear. • Challenge oxazolone-induced: oxazolone applied to the right ear and one hour after application of oxazolone, parthenolide or Feverfew was applied to the oxazolone-treated ear.	1 d

N, not reported.

Table S4. Characteristics of treatments administered in all studies *in vivo* identified in the systematic review.

Authors	Year	Formulation administered	Dose	Administration route	Treatment / Challenge	Duration of treatment
In vivo studies						
Wang et al.	2018	Emollient cream	1% Total sesquiterpene lactones (TSL-IHL)	Topical (dorsal skin and ears)	• Challenge: 5 times every 3 days by painting 20 µl of 0.2% DNCB (2,4-Dinitrochlorobenzene) solution on the inner and outer surfaces of the right ears. • Rechallenge: 300 µl of emollient cream containing TSL-IHL (1%, W/W) applied topically on the dorsal skin and ears once per day for 17 days.	17 d

Table S4 (*continuation*). Characteristics of treatments administered in all studies *in vitro* identified in the systematic review.

Authors	Year	Formulation administered	Dose	Pre-treatment/ Treatment	Incubation period
In vitro studies					
Alonso Blasi et al.	1992a	Medium	10% alantolactone, 10% and 20% isoalantolactone in animals.	• Lymphocytes were taken from predefined animals. Balb/c, Balb/b and DBA/2 mice received epicutaneous induction with 10% alantolactone. Another group of Balb/c mice received epicutaneous induction with 20% isoalantolactone and another group received intradermal induction with 10% isoalantolactone.	Unclear
Hofmann et al.	2014	N	125 µg/mL *Arnica montana* extract, 5 µM helenalin	• HaCaT cells with 125 µg/mL *A. montana* or 5 µM helenalin.	8h
Kim et al.	2015	Medium	MTS assay: 2.5, 5, 10, and 20 µM. Other analysis: 5 and 10 µM.	• Exposed to UVB in the presence or absence of IXA.	24h
Lass et al.	2008	Tincture and ethanol	Different concentrations	• Cells were incubated for 48 h with LPS in the presence or absence of graded concentrations of the CE or SP Arnica tincture.	48h
Lee et al.	2013	Fresh medium	MTT assay: 0, 0.8, 4, 20, 50, 100 µM all compounds. Other analysis: Magnolialide 10, 20, 30 and 40 µM.	• RBL-2H3 cells were stimulated with anti-DNP IgE, washed or not, and incubated with sesquiterpene lactones for 20 min to 3 h, varying according to the analysis. • Y16 cells were stimulated and treated with isolated compounds for 48h.	20 min to 48h
Lee et al.	2018	PIPES buffer (piperazine-N,N′-bis-(2-ethanesulfonic acid)	Different concentrations of alantolactone, costunolide, or dehydrocostuslactone	• Cells were pre-treated with 400 µl of PIPES buffer containing different concentrations of sesquiterpene lactones at 37ºC for 30 min.	30 min
Lin et al.	2016	N	2.5 - 10 µM	• Cells were pre-treated with IJ-5 for 1 h.	1 h

N, not reported.

Table S4 (*continuation*). Characteristics of treatments administered in all studies *in vitro* identified in the systematic review.

Authors	Year	Formulation administered	Dose	Pre-treatment/ Treatment	Incubation period
In vitro studies					
Nam et al.	2015	N	0.5 - 10 μM	• HEK001 were pre-treated with parthenolide for 20 min to 24h and exposed to LPS in combination with parthenolide for 24h.	20 min to 24 h
Scarponi et al.	2014	N	DCE, CS and HCS (all at 12.5 mM)	• Cells were pretreated with DCE, CS and HCS (all at 12.5 mM) for 1 h.	1 h
Seo et al.	2015	RT-qPCR: serum-free medium supplemented with tumor necrosis factor (TNF-α) and interferon gamma (IFN-γ)	CCK-8 assay: costunolide or dehydrocostus lactone at 0, 1.25, 2.5, 5 or 10 μM and alantolactone at 0, 0.625, 1.25, 2.5 or 5 μM	• HaCaT cells were incubated with costunolide or dehydrocostus lactone or with alantolactone in 1ml of serum-free medium supplemented with tumor necrosis factor and interferon for 24h with different concentrations.	24 h
Sur et al.	2009	N	1, 5, 10, 50, 100 and 200 μg/ml	• Macrophages were stimulated in the presence or absence of various concentrations of parthenolide and Feverfew for 18h. Cells 293/NFκB-luc were treated in the presence of the NF-κB inhibitor BAY11-7082 or Parthenolide-Feverfew for 24 hours.	18 to 24h
Svensson et al.	2018	N	75, 150, 225 and 300 μg/ml all compounds	• Cells were pre-treated with sesquiterpene lactonas, dexamethasone or vehicle for 30 min and incubated for 24 h in the presence or absence of sesquiterpene lactones, dexamethasone and LPS.	30 min to 24h

N, not reported.

Table S4 (*continuation*). Characteristics of treatments administered in all studies *in vitro* identified in the systematic review.

Authors	Year	Formulation administered	Dose	Pre-treatment/ Treatment	Incubation period
In vitro studies					
Takei et al.	2015	Culture medium	0.5 and 1 μM	• NHEKs were treated with Cyn BaP, TNFa, or DMSO (Dimethyl sulfoxide) (control) for 6 to 18h. • Cells irradiated with UVB: After irradiation, PBS was immediately removed, and cells received fresh medium containing the Cyn or DMSO for 6 to 18h, varying according to the analysis.	6 to 18 h
Wang et al.	2018	N	0.6, 1.2 and 2.4 μg/ml	• HaCat cells pretreated with sesquiterpene lactones for 2 h.	2h
Zhang et al.	2018	N	0, 7.5, 15, and 30 μM	• Cells were treated with different concentrations of lactucopicrin for 24 h and then, incubated with respective controls used in each analysis.	24 h

N, not reported.

Table S5. Summary of the main results *in vivo* obtained from in all sesquiterpene lactones investigated in the studies included in the systematic review.

Studies in vivo				
Authors	**Sesquiterpene lactone**	**Challenge/Treatment**	**Positive results**	**Negative results**
Alonso Blasi et al. 1992a	Helenin (alantolactone, isoalantolactone)	Alone lactones and Freund's incomplete adjuvant + lactones	Not observed	• 10% isoalantolactone: increased ear thickness in mice. • 10% alantolactone: severe toxicity and mice mortality.
Alonso Blasi et al. 1992b	Alantolactone, isoalantolactone	Alone lactones	Not observed	• Alantolactone: dermal edema with inflammatory infiltrate in mice.
Barbier and Benezra 1982	(+)- and (-)-γ-methyl-α-methylene-γ-butyrolactones	Freund's complete adjuvant + lactones	Not observed	• Induction of allergic contact dermatitis with skin erythema and edema in guinea pigs. • Enantiometer (+): More intense allergenic effect in guinea pigs.
Cheminat et al. 1984	Costunolide, Dehydrocostuslactone, Tulipinolide, Deacetyllaurenobiolide	Freund's complete adjuvant + lactones	Not observed	• Costunolide: Dermatitis with slight to intense erythema and edema in guinea pigs. • Deacetyllaurenobiolide, tulipinolide, and Dehydrocostuslactone: Dermatitis with intense erythema and edema in guinea pigs.
Dupuis et al. 1980	Alantolactone, isoalantolactone, α-methylene-γ-butyrolactone, decaline lactone, 2-methylene-4-isopropyl-4-methyl-y-butyrolactone, dimethyl lactone, bicyclolactone, adamantane lactone, spirolactone.	Freund's complete adjuvant + lactones	Not observed	• Alantolactone, isoalantolactone, α-methylene-γ-butyrolactone, decaline lactone, and 2-methylene-4- isopropyl-4-methyl-γ-butyrolactone: Delayed cutaneous hypersensitivity in dorsal skin of guinea pigs.

Table S5. Summary of the main results *in vivo* obtained from in all sesquiterpene lactones investigated in the studies included in the systematic review.

Studies in vivo				
Authors	**Sesquiterpene lactone**	**Challenge /Treatment**	**Positive results**	**Negative results**
Fraginals et al. 1991	Helenin (alantolactone, isoalantolactone)	Lactones in acetone	Not observed.	• Dermatitis with dose-dependent increase of ear thickness, dermal edema and dissociation collagen fibers, and lympho-histiocytary inflammatory infiltrate in mice.
Gabriel-Robez et al. 1982	Alantolactone and isoalantolactone	Freund's complete adjuvant + lactones	Not observed.	• Dermatitis: Intense skin reaction to open epicutaneous test in guinea pigs.
Lass et al. 2008	Arnica tincture, 11α,13-dihydrohelenalinisobutyrate; 11α,13-dihydrohelenalinmethacrylate, helenalinisobutyrate	TNCB + treatment with lactones	• Arnica tinctures: Attenuated 2,4,6-Trinitrochlorobenzene (TNCB)- induced ear swelling.	• Arnica tincture: Slight dermatitis with skin inflammation by T cell infiltration in mice.
Lass et al. 2010	Arnica tinctures, 11α,13-dihydrohelenalinmethacrylate, helenalinisobutyrate.	TNCB + treatment with lactones	• Arnica tinctures increased IL-10 levels in mice.	• Arnica tinctures: Slight skin inflammation with T cell infiltration in mice.
Lin et al. 2016	1β-Hydroxyalantolactone	DNCB + treatment with lactone	• Attenuated the severity of dinitrochlorobenzene (DNCB)-induced dermatitis in mice. • Reduced ear swelling, epidermis and dermis thickening, inflammatory infiltrate, IgE, IL-4, and IL-6 serum levels, and TNF, IL-1, IL-4, and IL-6 mRNA expression in mice.	Not observed.

TPA: 12-O-tetradecanoylphorbol-13-acetate. AA: arachidonic acid. TNCB, 2,4,6-trinitrochlorobenzene. DNCB, 2,4-Dinitrochlorobenzene

Table S5. Summary of the main results *in vivo* obtained from in all sesquiterpene lactones investigated in the studies included in the systematic review.

Studies in vivo				
Authors	**Sesquiterpene lactone**	**Challenge /Treatment**	**Positive results**	**Negative results**
Máñez et al. 1999	Inuviscolide	TPA or AA + treatment with lactone	• Reduced arachidonic acid-induced edema in mice.	Not observed.
Picman et al. 1982	Parthenin, Hymenin, Coronopilin, Damsin, Dihydroisoparthenin, Tethahydroparthenin, Tetraneurin-A, Hysterin, Helenalin, Tenulin	Parthenin + lactones	Not observed.	• Parthenin: Strong dermatitis with confluent erythema in guinea pigs. • Coronopilin: Slight dermatitis with confluent erythema in guinea pigs. • Damsin: Slight dermatitis with spotted erythema in guinea pigs.
Recio et al. 2000	4-α-O-Acetyl-pseudoguaian-6β-olide, hymenin, ambrosanolide, tetraneurin A, parthenin, hysterin, confertdiolide	Carrageenan or TPA or EPP or AA or Serotonin or Oxazolone + treatment with lactones	• Confertdiolide: reduced carrageenan- and TPA-induced edema in mice. • All the compounds inhibited TPA-induced dermatitis in mice. • Ambrosanolide, parthenin, confertdiolide inhibited EPP-induced edema and exhibited anti-inflammatory effects on plantar subcutaneous injection of serotonin-induced edema in mice. • Hysterin and confertdiolide: decreased epithelium thickness and mastocytes number.	Not observed.
Schaeffer et al. 1990	Helenin (Alantolactone, Isoalantolactone), cis-bicyclic lactone, trans-bicyclic lactone.	Freund's complete adjuvant + lactones	Not observed.	• Cis-bicyclic lactone and Helenin: Dermatitis with erythema to edema covering the whole test area.

TPA: 12-O-tetradecanoylphorbol-13-acetate. EPP: Ethyl-phenylpropiolate. AA: arachidonic acid.

Table S5. Summary of the main results *in vivo* obtained from in all sesquiterpene lactones investigated in the studies included in the systematic review.

Studies in vivo				
Authors	**Sesquiterpene lactone**	**Challenge /Treatment**	**Positive results**	**Negative results**
Schmidt and Chung, 1993	Helenin (alantolactone, isoalantolactone), 11,13-dihydroalantolactone and 11,13-dihydroisoalantolactone)	Xenobiotic + lactones	• Alantolactone, isoalantolactone: Increased glutathione and glutathione disulphide skin levels in mice.	• Dermatitis with ear edema in open epicutaneous test in mice.
Sosa et al. 2001	1,4-Dihydroxy-germacra-5E-10(14)-diene (DHGD)	Croton oil + treatment with lactones	• Dose-dependent edema reduction in mice. • Attenuated vascular dilatation, inflammatory infiltrate, and dermis thickening in mice.	Not observed.
Stampf et al. 1978	Norbornane lactone, Helenin (Alantolactone, Isoalantolactone), Costunolide, Laurenobiolide, Frullanolide, Spirolactone, α-methylene-γ-butyrolactone.	Freund's complete adjuvant + lactones	Not observed.	• Alantolactone and isoalantolactone: dermatitis with intense erythema, leucocytes infiltration and exudation in guinea pigs. • Sensitized by alantolactone: cross-react to isoalantolactone, spirolactone, frullanolide, laurenobiolide, and costunolide inducing leucocytes infiltration and confluent erythema in guinea pigs. • Sensitized by isoalantolactone: cross-react to alantolactone, spirolactone, frullanolide, and costunolide inducing leucocytes infiltration and confluent erythema in guinea pigs

Table S5. Summary of the main results *in vivo* obtained from in all sesquiterpene lactones investigated in the studies included in the systematic review.

Studies in vivo				
Authors	**Sesquiterpene lactone**	**Challenge /Treatment**	**Positive results**	**Negative results**
Stampf et al. 1982	Helenin (alantolactone, isoalantolactone)	Alone lactones and Freund's complete adjuvant + lactones	Not observed.	• Isoalantolactone: slight erythema covering part of the test area. • Alantolactone: Strong erythema covering the whole test area.
Sur et al. 2009	Feverfew tincture reduced in parthenolide	TPA or Oxazolone + treatment with lactones	• Feverfew tincture and parthenolide reduced TPA-induced dermatitis in mice. • Parthenolide: decreased TPA-induced edema; and TNF, IL-2, and IFNγ levels in oxazolone-induced ear edema in mice. • Parthenolide: applied before challenge by methyl nicotinate reduced erythema.	Not observed.
Wang et al. *2018*	Alantolactone, Isoalantolactone, total sesquiterpene lactones (TSL-IHL)	DNCB + treatment with lactone	• TSL-IHL: Dermatitis scores, erythema, hemorrhage, excoriation, erosion, epidermal thickening, and inflammatory infiltrate were attenuated in mice. • TSL-IHL: decreased ear swelling, IgE, IFN-γ, TNF serum levels, and IL-4, IL-5, IL-13 mRNA expression in mice.	Not observed.

TSL-IHL: Total sesquiterpene lactones. DNCB: Dinitrofluorobenzene. TPA: 12-O-tetradecanoylphorbol-13-acetate.

Table S5 (*continuation).* Summary of the main results *in vitro* obtained from all sesquiterpene lactones investigated in the studies included in the systematic review.

Studies in vitro				
Authors	**Sesquiterpene lactone**	**Challenge /Treatment**	**Positive results**	**Negative results**
Alonso Blasi et al. 1992a	Helenin (alantolactone, isoalantolactone)	Lactones	Not observed.	• Alantolactone: stimulated lymphocytes proliferation
Hofmann et al. *2014*	Helenalin (alantolactone, isoalantolactone), A. montana extract	DNCB or Nickel sulfate or Cadmium chloride + treatment with lactone	• Helenalin and A. montana extract: dose-dependent increase in HSP70B' gene expression in HaCaT cells.	• Helenalin and A. montana extract: dose-dependent reduction in tGSH concentrations in HaCaT cells.
Kim et al. 2015	Ixerisoside A	Exposed to Ultraviolet B + treatment with lactone	• Dose-dependent inhibition of IL-6 and IL-8 production by HaCaT cells. • Inhibited IL-6 and IL-8 gene expression in HaCaT cells. • Suppressed COX-2 expression. • Dose-dependent inhibition of ERK, JNK, and p38 MAPK phosphorylation in HaCaT cells.	Not observed.
Lass et al. 2008	Arnica tincture, 11α,13-dihydrohelenalinisobutyrate; 11α,13-dihydrohelenalinmethacrylate, helenalinisobutyrate	Lipopolysaccharide + lactones	• Inhibition of NF-κB activation and DNA binding in dendritic cells. • Tinctures: prevented dendritic cells activation.	Not observed.

DNCB: Dinitrofluorobenzene.

Table S5 (*continuation).* Summary of the main results *in vitro* obtained from all sesquiterpene lactones investigated in the studies included in the systematic review.

Studies in vitro				
Authors	**Sesquiterpene lactone**	**Challenge /Treatment**	**Positive results**	**Negative results**
Lee et al. 2013	Magnolialide, Santamarine, Reynosin, Baynol C, 11,13-dehydrosantonin, (3aS,5aR,6R,9S,9aS, 9bS)-6,9-dihydroxy-5a,9-dimethyl-3-methylidene-3a,4,5,6,7,8,9a,9b-octahydrobenzo [g][1]benzofuran-2-one, and Lucentolide	Antigens + Lactones	• Magnolialide inhibited the release of β-hexosaminidase, reduced IL-4 gene expression in RBL-2H3 cells; and inhibited IL-5-induced proliferation in Y16 cell.	• Baynol C and lucentolide: Cytotoxic at high concentration (100 μM) on RBL-2H3 cells.
Lee et al. 2018	Alantolactone, dehydrocostuslactone, costunolide	Monoclonal anti-dinitrophenyl mouse immunoglobulin E + treatment with lactones	• Dose-dependent inhibition of antigen-induced release of β-hexosaminidase in RBL-2H3 cells. • Alantolactone and costunolide: inhibited RBL-2H3 cells degranulation at concentrations higher than 10μM.	Not observed.
Lin et al. 2016	1β-Hydroxyalantolactone	TNFα + treatment with lactone	• Dose-dependent inhibition of TNF, IL-1, and IL-6 gene expression in HaCaT cells. • Dose-dependent inhibition of TNF production, induced IkB phosphorylation and degradation in HaCaT cells.	Not observed.

Table S5 (*continuation).* Summary of the main results *in vitro* obtained from all sesquiterpene lactones investigated in the studies included in the systematic review.

Studies in vitro				
Authors	**Sesquiterpene lactone**	**Challenge /Treatment**	**Positive results**	**Negative results**
Nam et al. 2015	Parthenolide	LPS (Lipopolysaccharide) + treatment with lactones	• Dose-dependent reduction in IL-1β and PGE2 production by keratinocytes. • Inhibited inducible enzyme COX-2 activity and Toll-like 4 receptors levels. • Prevented IkB phosphorylation and NF-κB activation, and reduced NF-κB-DNA binding activity. Reduced phospho-Akt and mTOR levels.	Not observed.
Scarponi et al. 2014	Dehydrocostuslactone (DCE), Costunolide (CS) and Dehydrocostunolide (HCS)	Lactones + IL-22, IFNγ and TNFα	• DCE and CS: decreased IL-22 production, SOCS3, CCL2 and HBD-2 mRNA induced by IL-22; inhibited CCL2, CXCL10 and ICAM- 1 mRNA in keratinocytes. • DCE or CS: inhibited IFN-γ-induced Tyr701 and Ser727 STAT1 phosphorylation; reduced keratinocytes proliferation, and reducing the nuclear accumulation of cyclin D1, PCNA and p-RB. • DCE and CS: induced of keratinocytes arrest in G2/M phases and decreased the percentage of cells in S and G0/G1 phases.	• DCE and CS: Decrease glutathione intracellular levels in keratinocytes
Seo et al. 2015	Costunolide, Dehydrocostuslactone, and Alantolactone	IFNγ and TNFα + lactones	• All sesquiterpene lactones: Dose-dependent reduction of TARC, MDC and IL-8 gene expression in HaCaT cells. • Costunolide and dehydrocostus lactone: weak inhibitory effects on RANTES gene expression. • Alantolactone: suppressed RANTES gene expression in HaCaT cells.	Not observed.

Table S5 (*continuation).* Summary of the main results *in vitro* obtained from all sesquiterpene lactones investigated in the studies included in the systematic review.

Studies in vitro				
Authors	**Sesquiterpene lactone**	**Challenge /Treatment**	**Positive results**	**Negative results**
Sur et al. 2009	Parthenolide	TNFα or Lipopolysaccharide + lactone	• Inhibited the activity of 5-lipoxygenase, phosphodiesterase-3 and phosphodiesterase-4, and TNF-α in macrophages. • Dose-dependent inhibition of nitrite and PGE_2 production by RAW267.4 cells.	Not observed.
Svensson D. et al. 2018	Coronopilin and damsin	Lipopolysaccharide + lactones	• Coronopilin and damsin: Low doses (1-10 μM) prevented IL-6 and MCP-1 gene expression in HDFa fibroblasts. • Damsin: Low doses (1-10 μM) reduced GROα and p-p65 and p105 gene expression, and increased IκB protein in HDFa fibroblasts. • Damsin: Low doses (1-10 μM) inhibited MCP-1 and GROα gene expression, and increased the viability of HaCaT keratinocytes cells.	• Coronopilin and damsin: reduced cell viability at high concentration (100 μM) in HDFa fibroblasts and HaCaT keratinocytes.
Takei et al. 2015	Cynaropicrin (Cyn)	TNF-α or exposed Ultraviolet B + lactone	• Induced AhR nuclear translocation, Nrf2 activation, and upregulated CYP1A1, Nrf2 and Nqo1 gene expression. • Reduced ROS, IL-6 and TNF production.	• Cytotoxic at high concentration (100 μM) on human keratinocytes.
Wang et al. *2018*	Alantolactone, Isoalantolactone	TNFα + treatment with lactones	• All the molecules: Dose-dependent inhibition of IκB phosphorylation and degradation, and p65 NF-κB phosphorylation in HaCat cells. • TSL-IHL: Dose-dependent inhibition of IL-1, IL-4 and TNF-α expression in HaCat cells.	Not observed.

Table S5 (*continuation).* Summary of the main results *in vitro* obtained from all sesquiterpene lactones investigated in the studies included in the systematic review.

Studies in vitro				
Authors	**Sesquiterpene lactone**	**Challenge /Treatment**	**Positive results**	**Negative results**
Zhang et al. 2018	Lactucopicrin	Lactone	• Dose-dependent inhibition of SKMEL-5 skin cells proliferation. • Dose-dependent apoptosis in SKMEL-5 cells. • Dose-dependent upregulation of Bax and downregulation of Bcl-2 gene expression. • Increase in G2 cell populations, leading to G2/M cell cycle arrest. • Dose-dependent reduction in p-PI3K, p-Akt and p-mTOR levels.	Not observed.

Table S6. Bias analysis in all original studies *in vivo* evaluated from the SYRCLE's toll.

Studies	*Sequence generation*	*Baseline characteristics*	*Allocation concealment*	*Random housing*	*Blinding (Performance)*	*Random outcome assessment*	*Blinding (Detection)*	*Incomplete outcome data*	*Selective outcome reporting*	*Other sources of bias*
	Was the allocation sequence adequately generated and applied?	Were the groups similar at baseline or were they adjusted for confounders in the analysis?	Was the allocation to the different groups adequately concealed during?	Were the animals randomly housed during the experiment?	Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the	Were animals selected at random for outcome assessment?	Was the outcome assessor blinded?	Were incomplete outcome data adequately addressed?	Are reports of the study free of selective outcome reporting?	Was the study apparently free of other problems that could result in high risk of
Alonso Blasi et al., 1992a	?	-	?	?	+	?	?	?	+	-
Alonso Blasi et al., 1992b	?	-	?	?	+	?	?	?	-	-
Barbier and Benezra, 1982	?	+	?	?	+	?	?	+	+	+
Cheminat et al., 1984	?	+	?	?	-	?	?	+	+	-
Dupuis et al., 1980	?	+	?	?	+	?	?	+	+	+
Fraginals et al., 1991	?	-	?	?	+	?	?	?	+	+
Gabriel-Robez et al., 1982	?	+	?	?	-	?	?	-	-	-
Lass et al., 2008	?	-	?	?	?	?	?	?	-	-
Lass et al., 2010	?	-	?	?	+	?	?	?	+	+
Lin et al., 2016	?	+	?	?	?	?	?	+	+	+
Máñez et al., 1999	?	?	?	?	+	?	?	?	-	-
Picman et al., 1982	?	+	?	?	+	?	?	+	+	+
Recio et al., 2000	?	+	?	?	+	?	?	?	+	+
Schaeffer et al., 1990	?	+	+	?	-	?	?	+	+	-
Schmidt and Chung, 1993	?	+	?	?	+	?	?	-	+	-
Sosa et al., 2001	?	-	?	?	?	?	?	?	+	+

(+) indicates low risk of bias; (-) indicates high risk of bias; (?) indicates unclear risk of bias.

Table S6. Bias analysis in all original studies *in vivo* evaluated from the SYRCLE's toll.

Studies	*Sequence generation*	*Baseline characteristics*	*Allocation concealment*	*Random housing*	*Blinding (Performance)*	*Random outcome assessment*	*Blinding (Detection)*	*Incomplete outcome data*	*Selective outcome reporting*	*Other sources of bias*
	Was the allocation sequence adequately generated and applied?	Were the groups similar at baseline or were they adjusted for confounders in the analysis?	Was the allocation to the different groups adequately concealed during?	Were the animals randomly housed during the experiment?	Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the	Were animals selected at random for outcome assessment?	Was the outcome assessor blinded?	Were incomplete outcome data adequately addressed?	Are reports of the study free of selective outcome reporting?	Was the study apparently free of other problems that could result in high risk of
Stampf et al., 1978	?	+	?	?	?	?	?	-	+	-
Stampf et al., 1982	?	-	?	?	-	?	?	-	-	-
Sur et al., 2009	?	+	?	?	-	?	?	?	-	-
Wang et al., 2018	?	+	+	+	+	?	?	+	+	+

(+) indicates low risk of bias; (-) indicates high risk of bias; (?) indicates unclear risk of bias.

2.5 *IN SILICO, IN VITRO* AND *IN VIVO* ANTI-INFLAMMATORY, ANTINOCICEPTIVE AND ANTI-MATRIX METALLOPROTEASES PROPERTIES OF TAGITININ F

Abstract

Sesquiterpene lactones (SL) are indicated as potential scaffolds for anti-inflammatory drug design. However, its anti-inflammatory applicability remains underestimated since the impact of SL on inflammatory nociception and tissue repair are overlooked. Thus, we used an integrated *in silico*, *in vitro* and *in vivo* framework to investigate the impact of tagitinin F (TAG-F) on LPS-challenge macrophages, carrageenan-induced paw edema and mechanical hyperalgesia, and excisional skin wounds in mice. RAW 264.7 macrophages in culture were challenge with LPS and treated with TAG-F (5, 10, 50 and 100 µM). The paw of BALB/c mice was injected with carrageenan, treated with 0.5% and 1% TAG-F and evaluated during 6h post-treatment. Excisional wounds were also produced in BALB/c mice and treated with 0.5% and 1% TAG-F during 7 days. Our results indicated a consistent dose-dependent downregulation in 5-lipoxygenase, cyclooxygenase (COX-1 and COX-2), matrix metalloproteinase (MMP-1 and MMP-2) activity; as well as attenuation in prostaglandin E2 (PGE2), leukotriene B4 (LTB4), and tumor necrosis factor-α (TNF-α) production in both models *in vitro* and *in vivo*. *In vivo*, TAG-F also attenuated carrageenan-induced paw oedema and mechanical hyperalgesia in mice. From the excisional skin wound, TAG-F was also effective in reducing neutrophils and macrophages infiltration and stimulating collagen deposition in the scar tissue, accelerating tissue maturation. Together, our findings indicate that the anti-inflammatory effect of TAG-F is more comprehensive than previously suggested, exerting a significant impact on the control of inflammatory pain and modulating central metabolic processes linked to skin wounds healing.

***Keywords*:** Experimental pathology, mechanical hyperalgesia, paw oedema, skin wounds, sesquiterpene lactones, wound healing.

1. Introduction

Sesquiterpene lactones (SL) are a group of secondary metabolites of the sesquiterpenoid class mainly identified in plants of the Compositae and Asteraceae family, which have a wide geographic distribution worldwide (Chagas-Paula et al., 2012, Abe at al., 2015., Tagne et al., 2018). Although ethnobotanical and ethnomedical studies has documented a systematic use of

plants rich in SL in popular medicine in eastern and western cultures, its effects, applicability, mechanisms of action and biological safety remains poorly exploited (Dupuis et al., 1980; Máñez et açl., 1999; Seca et al., 2014). Currently, at least 8000 different natural SL are recognized (Sülsen and Martino, 2018), many of which (i.e., alantolactone, coronopilin, costunolide and damsin) have traditionally been associated with systemic and skin toxicity (Warshaw and Zug, 1996; Paulsen, 2017). However, a broad spectrum of biological activities such as antiparasitic, antimicrobial, anti-inflammatory, analgesic, antioxidant, and anticancer has been attributed to SL (Chagas-Paula et al., 2015a,b; Gonçalves-Santos et al., 2019; Choudhary and Mishra, 2019); indicating a potential relevance of these molecules as scaffolds for drug design (Chagas-Paula et al., 2012, Tagne et al., 2018).

The mechanism of action of SL is not fully understood. However, there is consistent evidence indicating that the biological effects induced by these molecules are dependent of the electrophilic α-methylene-γ-lactone functional group, which is typically encountered in SL (Arantes et al., 2011; Amorim et al., 2013). From this chemical group, SL react irreversibly with nucleophiles (sulfhydryl or amino groups) of enzymes and transcription factors from alkylation mechanisms, modulating cell metabolism (Arantes et al., 2011). Interestingly, potent anti-inflammatory effects are attributed to SL, which can be even more effective than classic and commercially available anti-inflammatory drugs (Chagas-Paula et al., 2015a,b). In general, these anti-inflammatory properties are attributed to the effect of SL in inhibiting the expression and activity of enzymes involved in arachidonic acid metabolism such as cyclooxygenase (COX) and lipoxygenase (LOX) (Abe et al., 2015; Chagas-Paula et al., 2015a,b). In addition, downregulation of signaling pathways that modulate cytokine biosynthesis such as NF-κB and MAPK/ERK are consistently associated with the anti-inflammatory effect of SL (Rüngeler et al., 1999; Tagne et al., 2018).

Within the SL group, different types of tagitinins (A, C and F) have stood out for sharing antimicrobial, antiparasitic and anti-inflammatory properties (Chagas-Paula et al., 2015a,b; Gonçalves-Santos et al., 2019). In studies developed by our research group, low concentrations of tagitinin F (TAG-F) was effective in alleviating contact irritant-induced dermatitis, an effect mediated by the dual inhibition of COX-2 and 5-LOX activity, as well as prostaglandin, leukotriene and TNF-α production; which induced a marked reduction in cutaneous inflammation (Chagas-Paula et al., 2015a,b). Although these effects indicate biotechnological potential of tagitinin F for the development of anti-inflammatory therapies, the extent of these effects and the applicability of this molecule in different inflammatory conditions remains uncertain. As effector molecules of general inflammatory processes, prostaglandins,

leukotrienes and cytokines are also associated with the modulation of nociception (Sommer and Kress, 2004; De Toni et al., 2015) and tissue repair pathways (Feiken et al., 1995; Barrientos et al., 2008). Although the control of these molecules is the primary target of several analgesic and healing drugs (Davies et al., 1984; Sekiguchi et al., 2008), it is still unknown to what extent the anti-inflammatory properties of tagitinin F are effective in modifying pain sensitivity and skin wounds healing. Thus, we used an integrated *in silico*, *in vitro* and *in vivo* framework to investigate the anti-inflammatory, antinociceptive and healing properties of tagitinin F.

2. Material and Methods

2.1 In silico Approach: Molecular Docking

Molecular docking with TAG-F was performed in the Schrödinger software suite Maestro version 10.2.010 (Schrödinger, New York, USA, 2015a), using the crystal structure of 5-lipoxygenase (5-LOX, PDB code: 3V98), cyclooxygenase-2 (COX-2, PDB code: 5KIR), matrix metalloproteinase-1 (MMP-1, PDB code: 4AUO), and matrix metalloproteinase-2 (MMP-2, PDB code: 1CK7). The drugs zileutron, celecoxib and batimastat were used as specific controls of the molecular docking, since are potent inhibitors of 5-LOX (Carter et al., 1991), COX-2 (Geis, 1999) and MMPs (1 and 2) (Botos et al., 1996), respectively. For ligand preparation, the LigPrep program was used with the OPLS_3 force field (Schrödinger, New York, USA, 2015b) and ionization state for pH 7.0 ± 2.0 (using Epik) (Schrödinger, New York, USA, 2015c). The Protein Preparation Wizard program realized the protein structures preparation, with hydrogen bonding network optimization in pH 7.0 and minimization performed using the OPLS-3 force field in the Macromodel module (Schrödinger, New York, USA, 2015d). For the docking analysis, the Induced Fit Docking (IFD) protocol was used, which performed the prediction of the protein structure and the refinement of the compounds by the Prime program, as well as the docking and provides the score by the Glide program, considering the protein and the ligand flexible (Schrödinger, New York, USA, 2015d). The grid box area was defined as 20×20×20 Å in the active site region of each enzyme analyzed, and the OPLS_3 force field was used. The final ligand protein complexes were visualized using the Maestro interface, and all figures were generated using its graphical module (Schrödinger, New York, USA, 2015a).

2.2 Cell Culture

Murine macrophages (RAW 264.7 cell line) were used to estimate the anti-inflammatory effects of TAG-F *in vitro*. Cell lineage was obtained from ATCC (American type culture collection) and maintained in DMEM culture medium (Invitrogen, Carlsbad, CA, USA) containing 100 KU/L streptomycin, 100 KU/L penicillin, and 10% heat inactivated fetal bovine serum - FBS (v/v) (Invitrogen), in a 5% CO_2 humidified incubator at 37 ºC.

2.2.1 Macrophages and molecular challenge

RAW264.7 macrophages were cultured at 37 ºC for 24h in DMEM medium with 0.1% FBS. Cells were seeded in 24-wells polystyrene plates at 2.5×10^5 macrophages and 1 mL of culture medium per well. After 24h, culture medium was replaced and RAW264.7 cells were incubated for 24h with a fresh medium containing 10% FBS with or without 100 ng/mL LPS (Sigma-Aldrich, St. Louis, Missouri, USA) and different concentrations (5, 10, 50 and 100 µM) of TAG-F (BioCrick BioTech, Chengdu, Sichuan, China) diluted in culture medium containing 0.06% dimethyl sulfoxide (TAG-F vehicle). Control cells were treated with fresh culture medium and TAG-F vehicle (VE). Then, cell cultures were harvest, centrifuged (1000 ×g for 15 min at 4ºC), and the supernatant and cell pellet were separately collected.

2.2.2 Cyclooxygenase and lipoxygenase activity in vitro

Enzymatic activity in cell lysates was measured using a biochemical kit and the manufacturer's instructions for cyclooxygenase (Cayman Chemical, Ann Arbor, MI, USA) and lipoxygenase (ABCAM, Cambridge, MA, USA). Briefly, 100 µl of RAW264.7 cell pellets were sonicated for 1 min in 300 µl cold buffer (0.1 M Tris-HCl, 1 mM EDTA, pH 7.8). Cell lysate was centrifuged at 10000 ×*g* and 4°C for 15 min, and the supernatant was used to measure COX and LOX activity. The assay for COX activity was based on the peroxidase component of these enzymes, in which peroxidase activity was spectrophotometrically measured by monitoring the production of oxidized N,N,N',N'-tetramethyl-*p*-phenylenediamine at 590nm. The activity of COX-1 and COX-2 isozymes was respectively distinguished by using the enzymatic inhibitors SC-560 and DuP-697. COX activity assay ranged from 13-63 nmol/min/mL. The assay for LOX activity was based on the conversion of LOX substrate to a lipid intermediate that reacts with the detection probe, emitting fluorescence. The increase in fluorescent was proportional to LOX activity, which was recorded at 500/536nm excitation/emission. The enzyme 5-lipoxygenase (5-LOX) was used as positive control and its specific inhibitor zileutron was used to estimate the specific activity of 5-LOX. The kit can detect as low as 0.004 mU/mg protein.

2.2.3 Prostaglandin and leukotriene production in vitro

Prostaglandin E2 (PGE_2) and leukotriene B4 (LTB_4) levels were quantified from specific enzyme-linked immunosorbent assay (ELISA) kits and the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Briefly, 10 µL culture supernatant were add to 96-wells microplates previously sensitized with specific antibodies against PGE2 and LTB_4. Prostaglandin and leukotriene levels were determined at 412nm by spectrophotometry. The assays ranging from 7.8-1,000 pg/mL (PGE2) and 3.9-500 pg/mL (LTB4). The results were normalized according protein concentration in the supernatant as previously described (Santos et al. 2015).

2.2.4 Matrix metalloproteinases activity in vitro

Matrix metalloproteinases (MMPs) activity in cell lysates was measured using a fluorometric enzymatic kit and the manufacturer's instructions (ABCAM, Cambridge, MA, USA). Briefly, 100 µl cell pellets were homogenized in 500 µl Tris-HCl buffer (pH 7.4, 5 mM) containing 0.02% NaN_3, 10mM $CaCl_2$, and 0.15M NaCl. The supernatant was collected after centrifugation at 10000 ×*g* for 30 min. Supernatant samples were used to measure MMP-1 and MMP-2 activity, which were calculated by using the respective enzyme inhibitors (N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide) (ABCAM, Cambridge, MA, USA) and (cis-9-Octadecenoyl-N-hydroxylamide) (Sigma-Aldrich, St. Louis, Missouri, USA). MMP activity was measured at 490nm/525 nm (excitation/emission) (Dias et al., 2019).

2.2.5 Tumor necrosis factor-α (*TNF-α) production in vitro*

The same supernatant obtained from cell lysates was used to measure TNF-α production. This cytokine was measured by sandwich ELISA, using commercial kit and the manufacturer's instructions (USCN Life Science Inc., Wuhan, China). The optical densities (OD) of the samples were detected in a microplate reader at 450 nm, and cytokine concentrations were determined by extrapolating the OD obtained from a standard curve for recombinant cytokine (Novaes et al., 2017).

2.3 Animal model

Eight-week-old male BALB/c mice weighing 27.39±2.81g were used in all *in vivo* experiments. Mice were randomized in individual polypropylene boxes kept in animal facility with controlled environment (12/12 h dark/light cycle, humidity 60-70% and temperature 22±2

ºC). Water and food were provided *ad libitum*. The Institutional Ethics Committee approved all experimental procedures involving animal care (protocol 72/2017).

2.3.1 Carrageenan-induced paw oedema and mechanical hyperalgesia

The animals were randomized into 5 groups with 7 animals in each group: TAG 1, 0.5% tagitinin F; TAG 2, 1% tagitinin F; SAL, 0.9% NaCl solution; VE, 5% DMSO solution (vehicle); SHAM, animals without any treatment (needle inserted without injection of any substance). In the groups TAG1 and TAG2, the right hind paw was injected with 20 µl of a solution containing 300 µg carrageenan (Trivellato Grassi et al., 2013), 0.5% and 1% TAG-F dissolved in 5% DMSO solution. Animals in the groups SAL and DMSO were injected with 20 µl saline solution or 5% DMSO, respectively. Mechanical hyperalgesia was measured by a von Frey type digital analgesimeter (Insight, Ribeirao Preto, SP, Brazil). The plantar surface of the hind paws was stimulated with a polypropylene monofilament with increasing force until the animal withdrew the stimulated limb. The withdrawal threshold was obtained as the force at which each animal withdrew the paw from at least 3 out of 5 consecutive stimuli. Paw oedema was measured from a paw plethysmometer (EF370, Insight, Ribeirao Preto, Brazil). Withdrawal threshold and oedema were measured 24h and 12h before (basal data), and 1h, 2h, 4h and 6h after paw injection with each treatment.

2.3.2 Lipoxygenase and cyclooxygenase activity, prostaglandin, leukotriene and TNF-α levels in carrageenan-induced paw oedema

After 6h of carrageenan injection, the animals were euthanized under deep anesthesia (50 mg/kg xylazine and 150 mg/kg ketamine) followed by exsanguination. The right hind paw was collected, and 100 mg tissue was homogenized in 600 µL Na2EDTA/NaCl buffer (pH 4.7), and centrifuged 2500 ×*g* for 15 min at 4 °C (Santos et al., 2019). The supernatant was collected and used to measure 5-LOX and COX activity, PGE2, LTB4 and TNF-α levels by using the same commercial kits previously described in the *in vitro* assays. In the SHAM group, the homogenate was 70% concentrated by evaporation at 4ºC to reach the detection limit of COX biochemical kit. This rate was used to correct the enzymatic activity obtained from SHAM samples.

2.3.3 Excisional wound

After intraperitoneal anesthesia (20 mg/kg xylazine and 50 mg/kg ketamine), dorsolateral hair of BALB/c mice was removed with electric shaver followed by depilatory cream (Veet,

Sao Paulo, SP, Brazil). The shaved area was degreased with ethyl ether (Merck, Rio de Janeiro, RJ, Brazil), followed by antisepsis with 10% povidone-iodine (Johnson Diversey, Rio de Janeiro, RJ, Brazil). A circular wound with 12 mm diameter was produced by skin removal with a scalpel until the dorsal muscular fascia was exposed. The animals were randomized into 4 treatment groups with 7 animals in each group: TAG 1, 0.5% tagitinin F; TAG 2, 1% tagitinin F; SAL, 0.9% NaCl solution; and VE, 5% DMSO solution. The intact skin (IS) collected during the excisional procedure was used as morphological and biochemical control. Each treatment was topically administered in a final volume of 200 µl, and lactones were dissolved in 5% DMSO solution (vehicle). All treatments were initiated 2h after the wounds were made and were administered twice a day (8 a.m. and 18 p.m.) for 7 days. Two hours after the last treatment, the entire scar tissue was collected using a scalpel and the same anesthetic procedure previously described. The scar tissue was used for molecular and microstructural analysis.

2.3.4 Lipoxygenase, cyclooxygenase and matrix metalloproteinases activity, prostaglandin, leukotriene and TNF-α levels in the scar tissue

To evaluate tissue levels of inflammatory mediators, a scar tissue fragment was homogenized in 600 µL Na2EDTA/NaCl buffer (pH 4.7) and centrifuged 2500 ×*g* for 15 min at 4 °C (Santos et al., 2019). The supernatant was collected and used to measure 5-LOX, COX-1 and 2 and MMP-1 and 2 activity, as well as PGE2, LTB4 and TNF-α levels. All measures were obtained by using the same commercial kits previously described in the *in vitro* assays. In the intact skin, the homogenate was 70% concentrated by evaporation at 4ºC to reach the detection limit of COX biochemical kit.

2.3.5 Collagen content in the scar tissue

Collagenous proteins were quantified from a biochemical method previously described (Moraes et al., 2010; Novaes et al., 2015). Scar tissue samples were homogenized in sodium phosphate buffer (pH 7.4) and centrifuged at 25000 ×*g* at 4ºC. Tissue pellets (20 mg) were dehydrated at 80ºC for 16 h and immersed in an acidic digestion solution at 380ºC for 3 h. After digestion, 50 µL samples were diluted in 2.7% H_2SO_4 solution and incubated with an alkaline developer solution containing sodium nitroprussate. Solution samples (100 µL) were analyzed at 630nm by spectrophotometry and compared with a blank solution containing 25 mg $(NH_4)_2SO_4$ and 1 mL H_2SO_4 in 37.5 mL deionized water. Collagen content was estimated multiplying the results by nitrogen correction factor (NCF= 6.25).

2.3.6 Microstructural analysis of the scar tissue

Scar tissue fragments were fixed in 4% formalin solution (pH 7.2), dehydrated in ethanol, cleared in xylene and embedded in paraffin. Cuts with 4-μm thickness were obtained in a rotary microtome, using 1 of every 20 sections to avoid analyses the same histological area. Histological sections were stained with hematoxylin and eosin (H&E) for cell counting and Sirius red (Sirius red F3B, Mobay Chemical Co., Union, N.J., USA) for collagen quantification. Ten histological fields were randomly sampled for each animal using a 40 objective lens (×400 magnification), and a total tissue area of 6.21×10^6 μm^2 was analyzed in each skin section and staining method. The number of cells in the scar tissue (cell/mm^2) was quantified in images captured in a bright field microscope (AxioScope A1, Carl Zeiss, Germany) from sections stained with H&E. The image analysis software Image-Pro Plus (Media Cybernetics, Rockville, MD, USA) was used for mononuclear (MN) and polymorphonuclear (PMN) cells counting (Novaes et al., 2016). Collagen content was evaluated by polarizing microscopy in skin sections stained with Sirius red. Tissue area occupied by collagen fibers was estimated from a two-dimension color segmentation computational method (Novaes et al., 2015) operationalized from the ImageJ software (Gonçalves et al., 2019).

2.3.7 Myeloperoxidase and N-acetylglucosaminidase assay in the scar tissue

Neutrophils accumulation in the scar tissue was measured from myeloperoxidase (MPO) activity as previously described (Guedes-da-Silva et al., 2015). Fresh skin samples were homogenized in pH 4.7 buffer (0.015 M Na2-EDTA, 0.02 M Na_3PO_4, and 0.1 M NaCl) and centrifuged for 10 min at 12,000 ×*g* (4 °C). The pellets were suspended in sodium phosphate buffer (0.05 M, pH 5.4) containing 0.5% hexa-1,6-*bis*-decyltrimethylammonium bromide. MPO activity was determined by measuring the absorbance change at 450 nm using 1.6 mM 3,3′-5,5′-tetramethylbenzidine dissolved in DMSO and 0.3 mM H_2O_2 prepared in sodium phosphate buffer (pH 6.0).

Macrophages accumulation in the scar tissue was estimated from N-acetyl-β-D-glucosaminidase (NAG) activity, which is a lysosomal enzyme intensely produced by activated monocytes/macrophages (Guedes-da-Silva et al., 2015). N-acetyl-β-D-glucosaminidase activity was measured in skin homogenate by using a 96-wells biochemical colorimetric kit and the manufacturer's instructions (Abcam, Cambridge, UK). This assay uses a synthetic p-nitrophenol derivative (R-*p*NP) as a NAG substrate and releases *p*NP, which is measured at 400nm by spectrophotometry.

2.4. Statistical treatment

The results *in vitro* and *in vivo* were expressed as the mean and standard deviation. Data distribution was verified by the Kolmogorov-Smirnov test. Parametric data were compared using one-way analysis of variance (one-way ANOVA) followed by Student-Newman-Keuls *post-hoc* test. Non-parametric data were compared using the Kruskal-Wallis one-way ANOVA on Ranks followed by the Student-Newman-Keuls method for all pairwise multiple comparisons. Results with $P \leq 0.05$ (95% confidence index) were considered statistically different.

3. Results

3.1 Predicted enzyme-ligand interactions

Molecular docking investigated potential interaction of TAG-F with the enzymes 5-LOX, COX-2, MMP-1 and MMP-2, and the results were compared with the standard inhibitory drugs. The interaction between all enzymes with TAG-F and the control drugs followed a similar profile of hydrogen bond interaction (HBond) with the same amino acid residues. Analyzing the results for 5-LOX and COX-2, TAG-F presented a greater number of hydrogen bond interactions (4 HBond with 5-LOX and 5 HBond with COX-2) compared to zileutron (2 HBond with 5-LOX) and celecoxib (1 HBond with COX-2), respectively. The opposite was observed in relation to the lower number of hydrogen bond interactions between TAG-F with the enzymes MMP-1 (1 HBond), and MMP-2 (2 HBond) in relation to Batimastat (6 HBond with MMP-1, 4 HBond with MMP-2) (Figures 1 and 2, and Table 1).

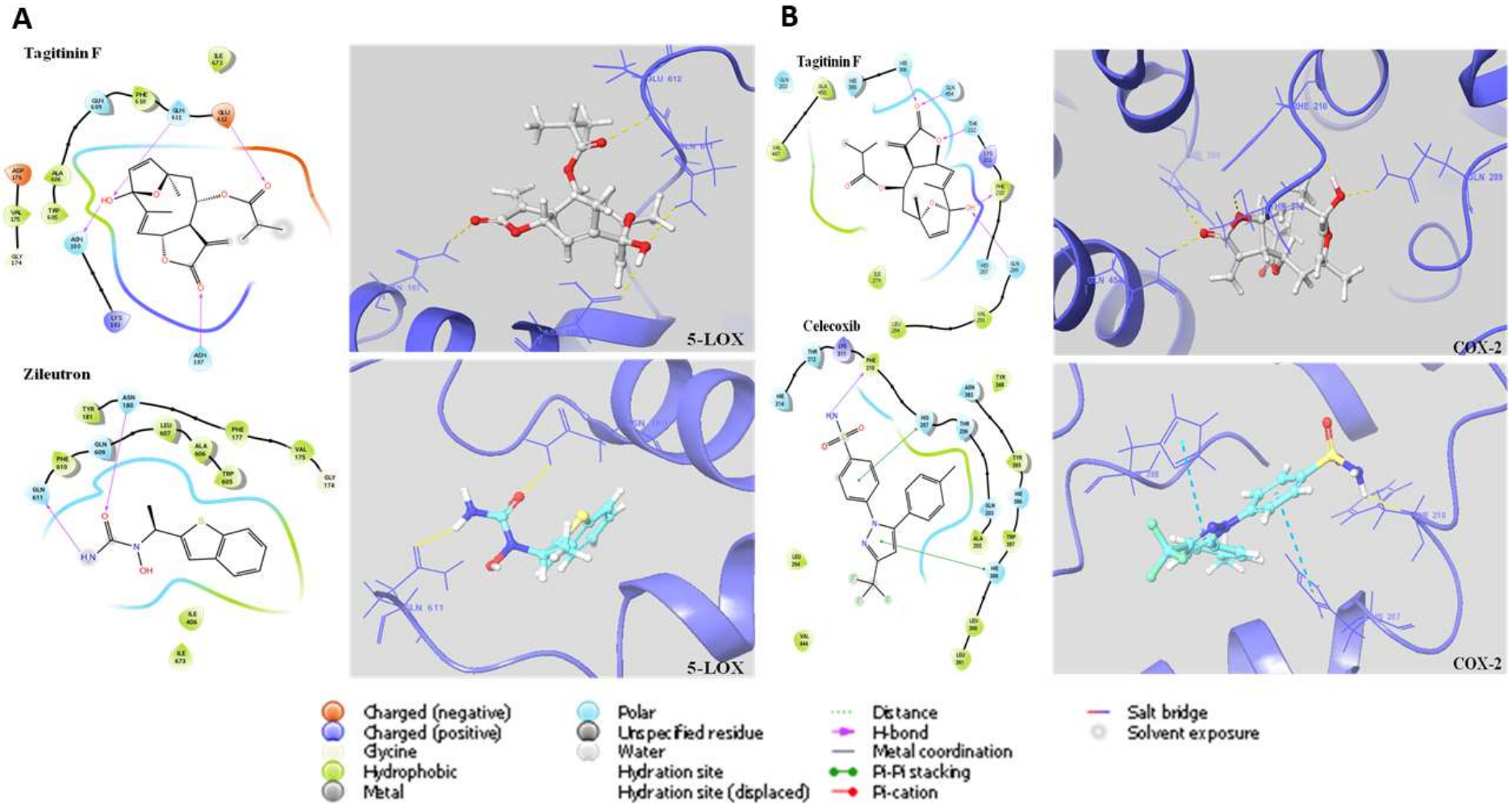

Figure 1. Representation of molecular docking results and interactions between amino acids of 5-lipoxygenase (5-LOX [A]) and cyclooxygenase 2 (COX-2 [B]) active sites and the ligands Tagitinin F, Zileutron (specific 5-LOX inhibitor) and Celecoxib (specific COX-2 inhibitor).

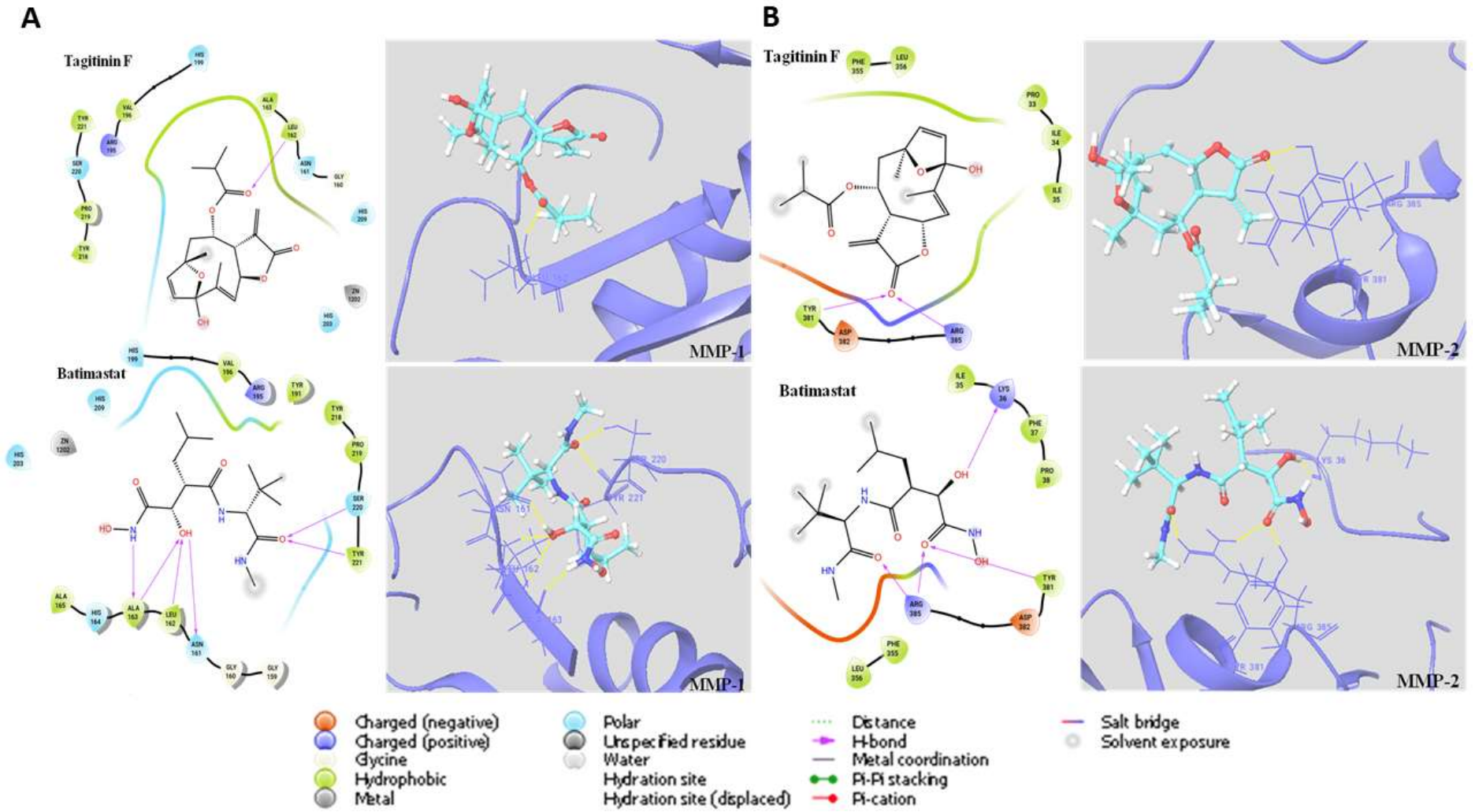

Figure 2. Representation of molecular docking results and molecular interactions between amino acids of matrix metalloproteinase 1 (MMP-1 [A]) and metalloproteinase 2 (MMP-2 [B]) active sites and the ligands Tagitinin F and Batimastat (specific MMP inhibitor).

The values of Glide Score (GScore), number of interactions by hydrogen bonds (*Hbond*), van der Waals (*good vdW*), and amino acids that perform *Hbond* between ligands and enzymes are shown in Table 1. Tagitinin F showed different degrees of interaction with all target enzymes and lower affinity values (GScore) than the standard inhibitory drugs (Table 1).

Table 1. Values of Glide Score (*GScore*), the number of interactions by Hydrogen bonds (*Hbond*) and van der Waals (*good vdW*) between Tagitinin F, Zileutron, Celecoxib and Batimastat with 5-lipoxygenase (5-LOX, PDB code: 3V98), cyclooxygenase 2 (COX-2, PDB code: 5KIR), matrix metalloproteinase 1 (MMP-1, PDB code: 4AUO), and matrix metalloproteinase 2 (MMP-2, PDB code: 1CK7) (Schrödinger Suite, Induced Fit Docking program).

Ligand	*GScore* (kcal.mol^{-1})	*H bond*	Amino acids that perform H bond	Good *vdW*
5-LOX - 3V98				
Tagitinin F	-6.352	4	Asn180, Gln611, Glu612, Asn187	224
Zileutron	-7.692	2	Asn180, Gln611	215
COX-2 - 5KIR				
Tagitinin F	-6.915	5	Gln454, Hie386, Thr212, Phe210, Gln289	231
Celecoxib	-8.406	1	Phe210	272
MMP-1 - 4AUO				
Tagitinin F	-5.067	1	Leu162	194
Batimastat	-7.780	6	Asn161, Leu162, Ala163(2), Ser220, Tyr221	279
MMP-2 - 1CK7				
Tagitinin F	-3.878	2	Tyr381, Arg385	214
Batimastat	-4.263	4	Lys36, Tyr381, Arg385(2)	179

Zileutron, celecoxib and batimastat were used as specific controls of the molecular docking, since are potent 5-LOX, COX-2, and MMP (1 and 2) inhibitors, respectively.

3.2. *In vitro findings*

Lipopolysaccharide-stimulated RAW 264.7 macrophages treated with TAG-F presented attenuated COX-1 and COX-2 activity, as well as reduced PGE2, LTB4 and TNF-α production in a dose-dependent way. COX-1 activity and PGE2 levels were reduced with the two higher doses of TAG-F compared to SAL and VE ($P < 0.05$). Conversely, COX-2 activity was attenuated in all doses of TAG-F tested, while PGE and TNF-α levels were reduced from 50 µM compared to FCM and VE ($P < 0.05$) (Fig. 3).

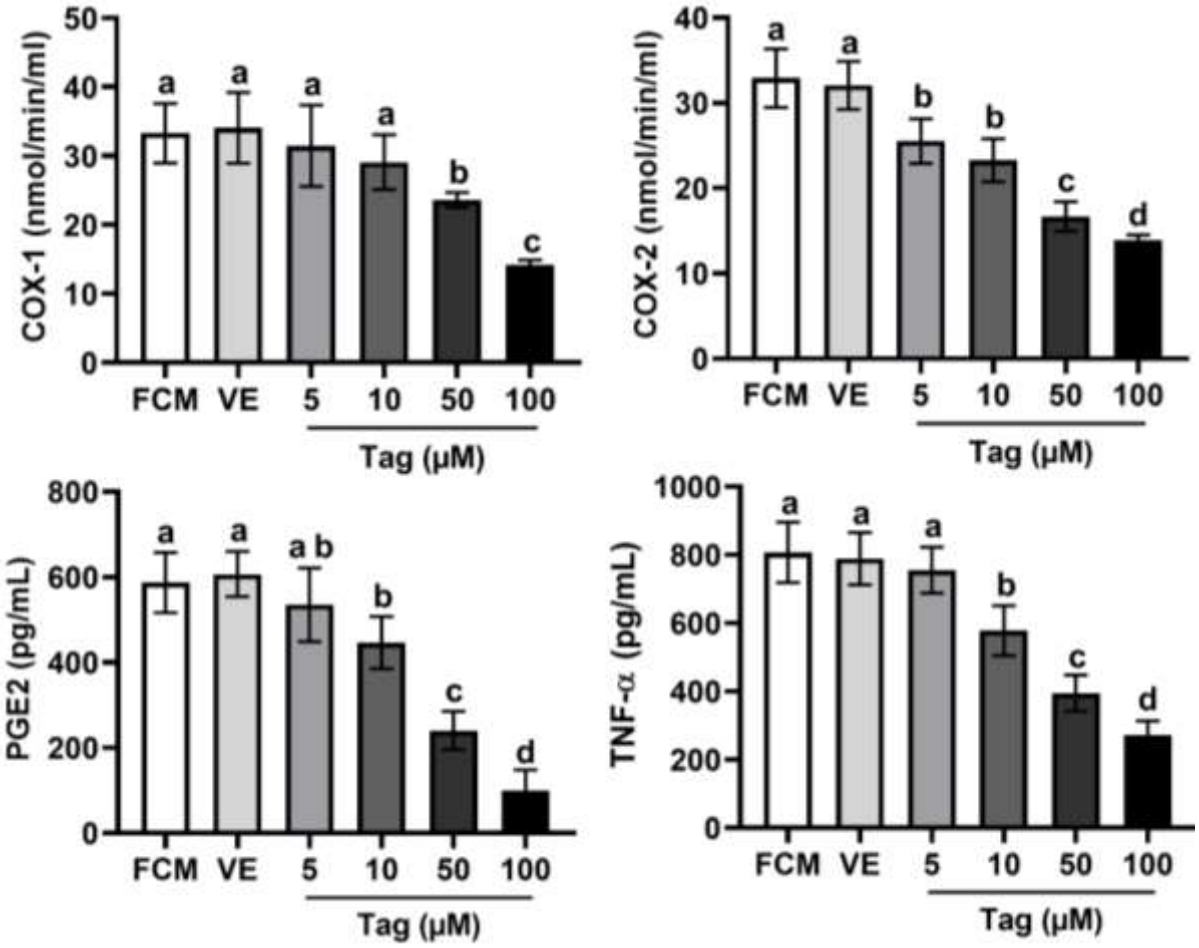

Figure 3. Cyclooxygenase (COX) 1 and 2 activity, prostaglandin E2 (PGE2) and tumor necrosis factor alpha (TNF-α) in lipopolysaccharide-stimulated RAW 264.7 macrophages treated with different doses of tagitinin F (Tag). FCM: Fresh culture medium; VE: 0.06% dimethyl sulfoxide prepared in FCM. Data are presented as mean and standard deviation. (a, b, c, d) Columns with different letters are statistically different ($P<0.05$), and columns with a common letter are statistically similar ($P>0.05$).

The activity of the enzyme 5-LOX and LTB4 levels were significantly reduced in LPS-stimulated macrophages treated with 50 and 100 µM TAG-F compared to FCM and VE ($P < 0.05$). MMP-1 activity was reduced in LPS-stimulated macrophages treated with the highest dose of TAG-F (100 µM) compared to FCM and VE ($P < 0.05$). MMP-2 activity was attenuated from 50 µM TAG-F compared to FCM and VE ($P < 0.05$), indicating a dose-dependent response (Fig. 4).

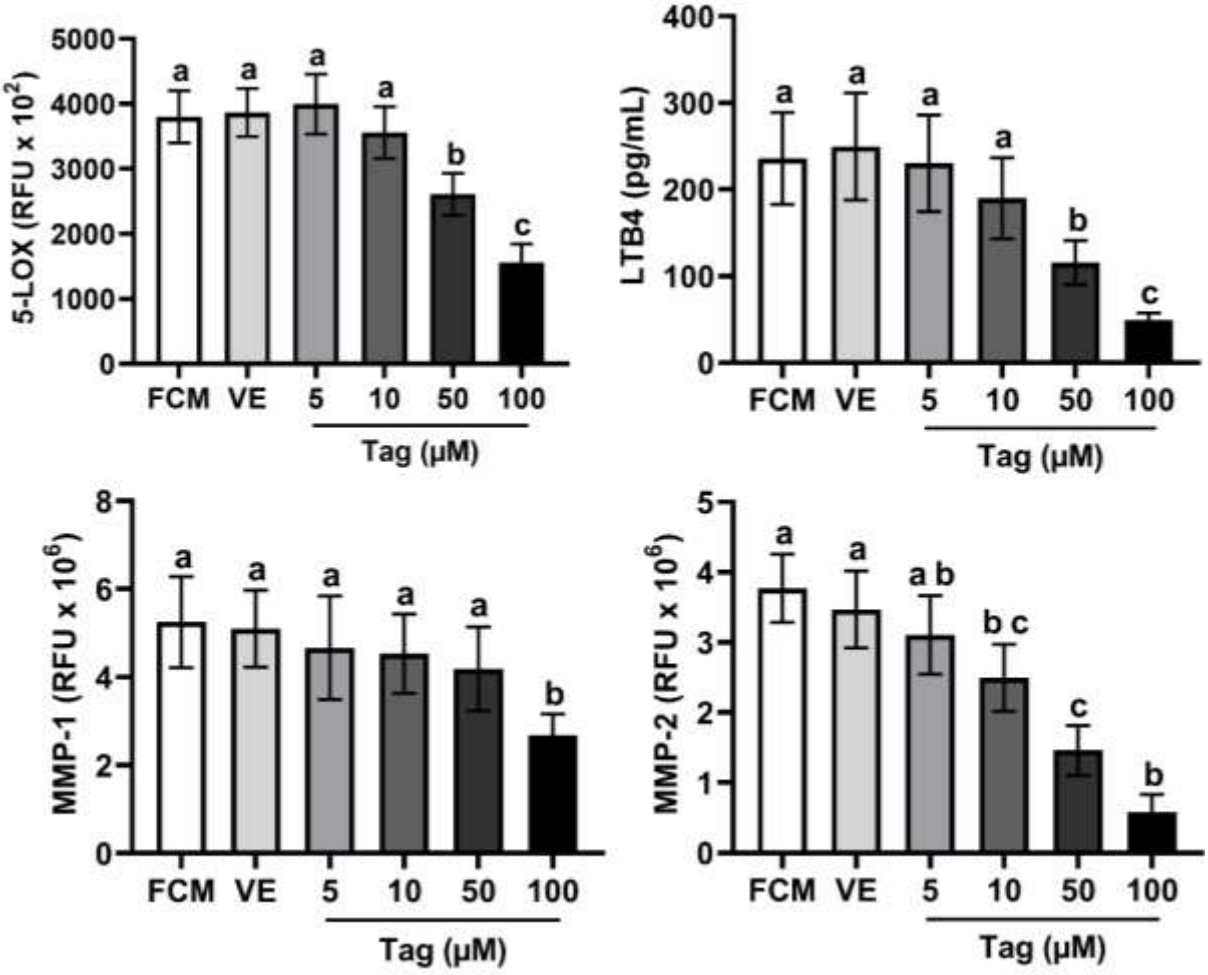

Figure 4. Lipoxygenase and matrix metalloproteinases (MMP-1 and MMP-2) activity, and leukotriene B4 (LTB4) production by lipopolysaccharide-stimulated RAW 264.7 macrophages treated with different doses of tagitinin F (Tag). FCM: Fresh culture medium; VE: 0.06% dimethyl sulfoxide prepared in FCM. Data are presented as mean and standard deviation. (a, b, c, d) Columns with different letters are statistically different ($P<0.05$), and columns with a common letter are statistically similar ($P>0.05$).

3.3. *In vivo findings*

Time-dependent carrageenan-induced paw edema and mechanical hyperalgesia was observed in all groups, especially in animals treated with saline and vehicle. Animals treated with both doses of TAG-F exhibited marked attenuation of paw edema and mechanical hyperalgesia compared to the groups SAL and VE ($P<0.05$), especially from 2h after carrageenan stimulation. The highest dose of TAG-F was more effective in reducing oedema and hyperalgesia compared to the other groups ($P<0.05$). Both doses of TAG-F were effective in reducing 5-LOX activity and LTB4 levels in paw tissue compared to the groups SAL and VE ($P<0.05$) (Figure 5).

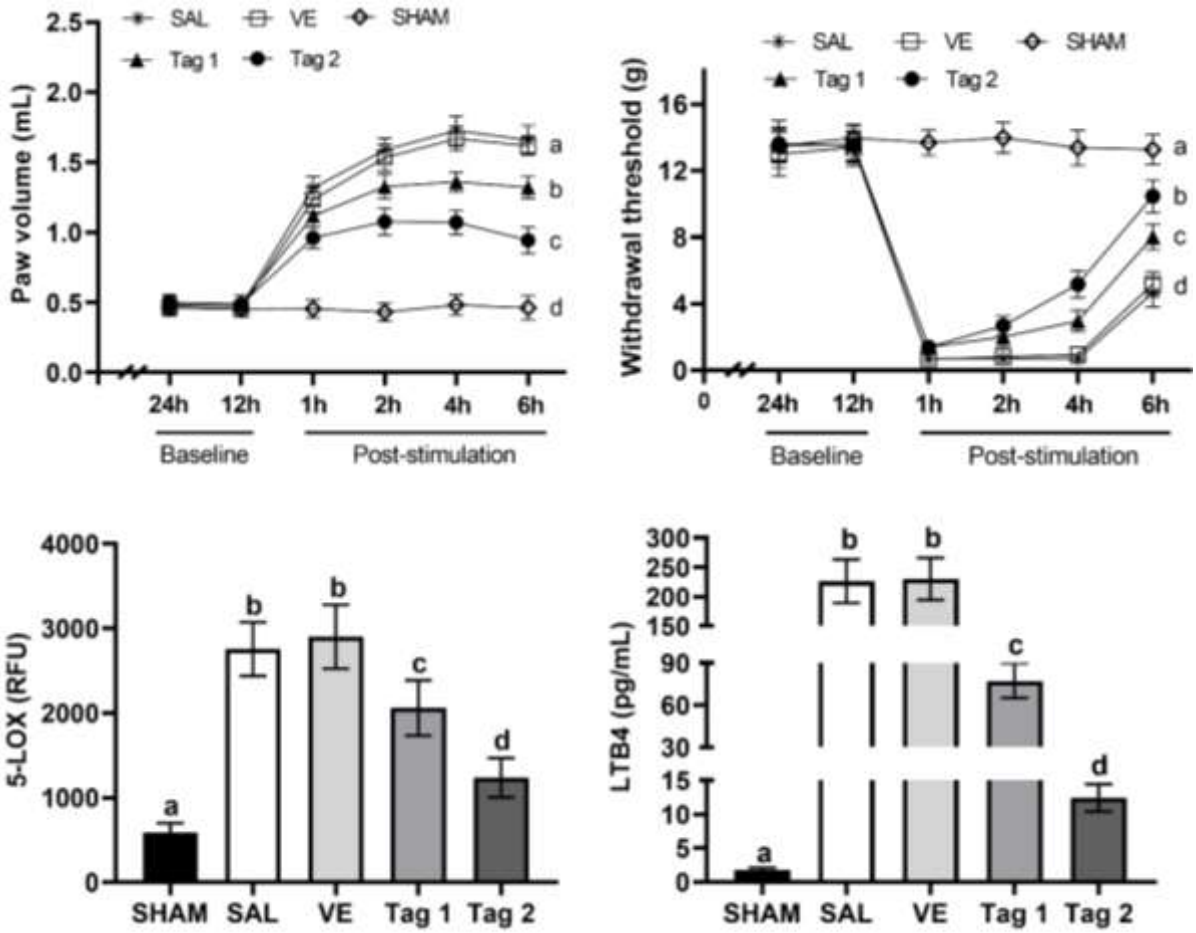

Figure 5. Paw volume, mechanical sensitivity (paw withdrawal threshold), lipoxygenase activity, and leukotriene B4 (LTB4) levels in paw tissue from mice treated with carrageenan and different doses of tagitinin F (Tag). SAL: 0.9% NaCl, VE: 5% Dimethyl sulfoxide; SHAM: animals without any treatment (needle inserted without injection of any substance); Tag 1: 0.5% Tagitinin F; Tag 2: 1% Tagitinin F. Data are presented as mean and standard deviation. (a, b, c, d) Lines followed by different letters indicate the groups with statistical difference ($P<0.05$) 4h and 6h after treatment administration. Baseline: Measures obtained 24 and 12h before treatment administration (no statistical difference was identified for paw mass and withdrawal threshold, $P>0.05$). Post-stimulation: Measures obtained 1, 2, 4 and 6h after treatment administration.

Animals in the group SHAM presented low COX-2 activity, PGE2 and TNF-α levels in paw tissue compared to the other groups ($P<0.05$). All these parameters and COX-1 activity were reduced in animals treated with TAG-F compared to the groups SAL and VE ($P<0.05$). TAG-F at 1% was more effective in reducing COX-1 and COX-2 activity, PGE2 and TNF-α in paw tissue compared to 0.5% TAG-F ($P<0.05$) (Figure 6).

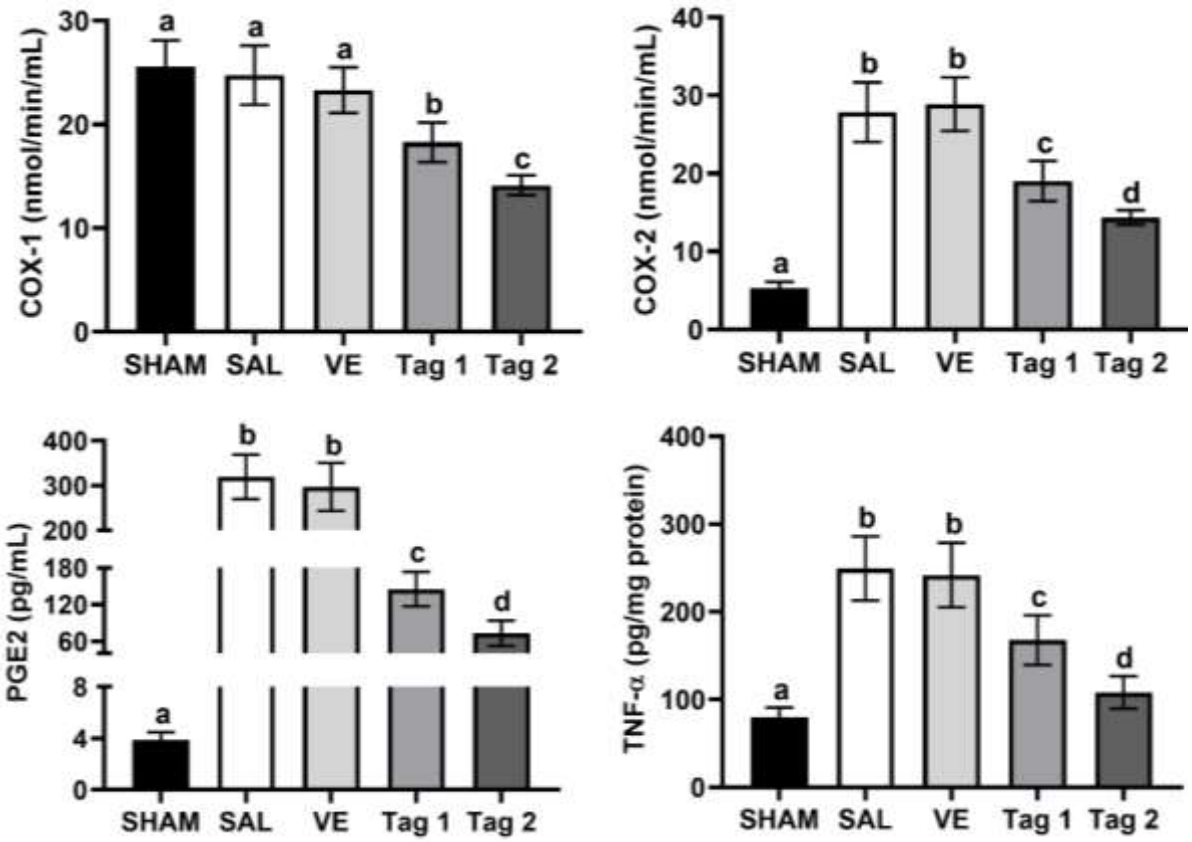

Figure 6. Cyclooxygenase (COX) 1 and 2 activity, prostaglandin E2 (PGE2) and tumor necrosis factor alpha (TNF-α) in paw tissue from mice 6 hours after treatment with carrageenan and different doses of tagitinin F (Tag). SAL: 0.9% NaCl, VE: 5% Dimethyl sulfoxide; SHAM: animals without any treatment (needle inserted without injection of any substance); Tag 1: 0.5% Tagitinin F; Tag 2: 1% Tagitinin F. Data are presented as mean and standard deviation. (a, b, c, d) Columns with different letters are statistically different ($P<0.05$), and columns with a common letter are statistically similar ($P>0.05$).

The intact skin collected from all groups presented reduced COX-2 activity, PGE2 and TNF-α levels in the scar tissue compared to the other groups ($P<0.05$). All these parameters and COX-1 activity were reduced in animals treated with both doses of TAG-F compared to the groups SAL and VE ($P<0.05$). TAG-F at 1% was more effective in reducing COX-1 and COX-2 activity, PGE2 and TNF-α levels in the scar tissue compared to 0.5% TAG-F ($P<0.05$) (Figure 7).

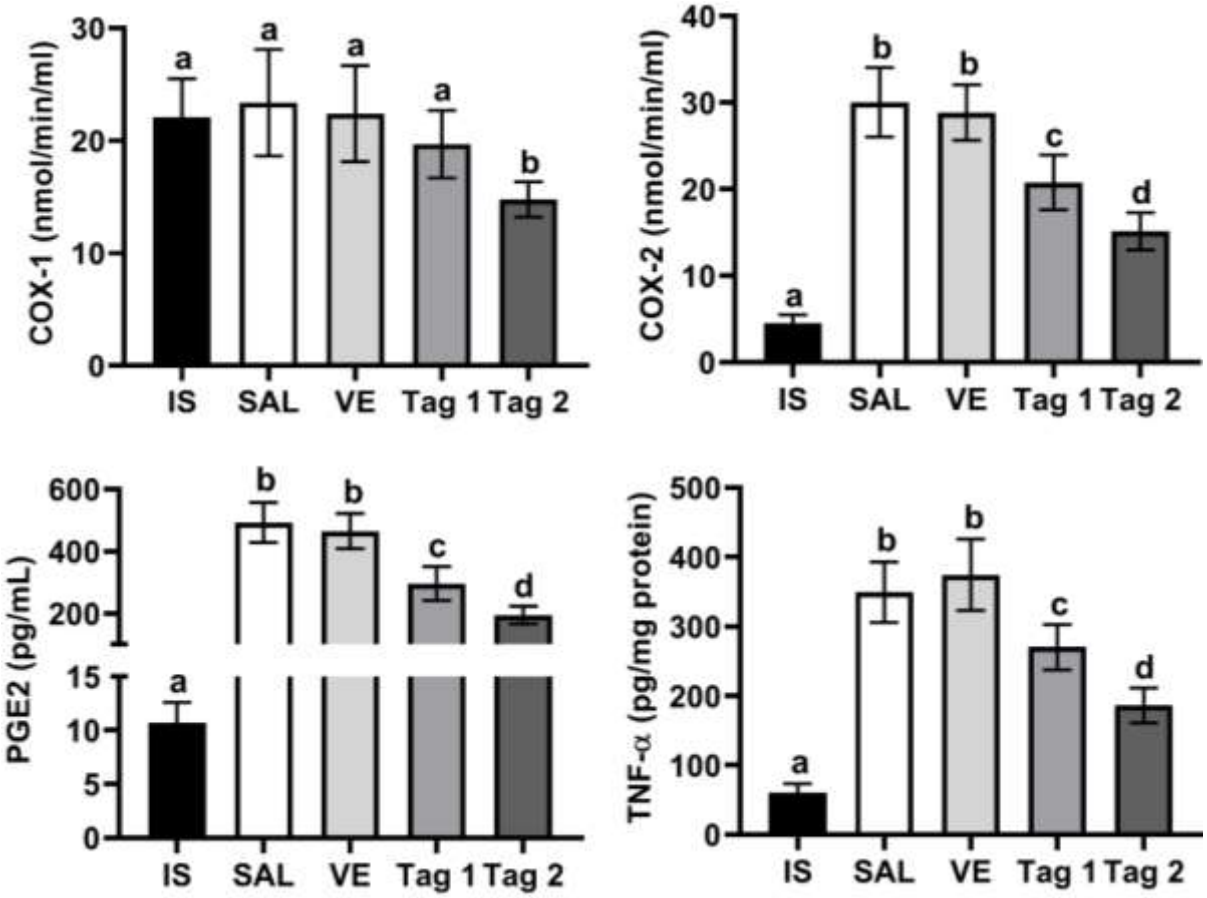

Figure 7. Cyclooxygenase (COX) 1 and 2 activity, prostaglandin E2 (PGE2) and tumor necrosis factor alpha (TNF-α) in the skin scar tissue collected from mice treated with different doses of tagitinin F (Tag). IS: intact skin (mean values considering the statistically similarity in all groups, P>0.05); SAL: 0.9% NaCl, VE: 5% Dimethyl sulfoxide; Tag 1: 0.5% Tagitinin F; Tag 2: 1% Tagitinin F. Data are presented as mean and standard deviation. Columns with different letters are statistically different (P<0.05), and columns with a common letter are statistically similar (P>0.05).

The intact skin collected from animals of all groups exhibited a similarly number of MN and PMN cells (P>0.05), which was reduced in relation to the cellularity quantified in the scar tissue of the respective groups (P>0.05). Seven days after inducing the skin wound, the scar tissue collected from SAL and VE mice exhibited a more intense inflammatory process compared to the groups treated with TAG-F, which was morphologically evidenced by intense and diffuse distribution of MN and PMN cells. This finding was corroborated by quantitative analysis of these cells, indicating that MN and PMN cellularity was significantly reduced in animals treated TAG-F, especially at 1% (P<0.05), compared to the groups SAL and VE (P<0.05) (Figure 8).

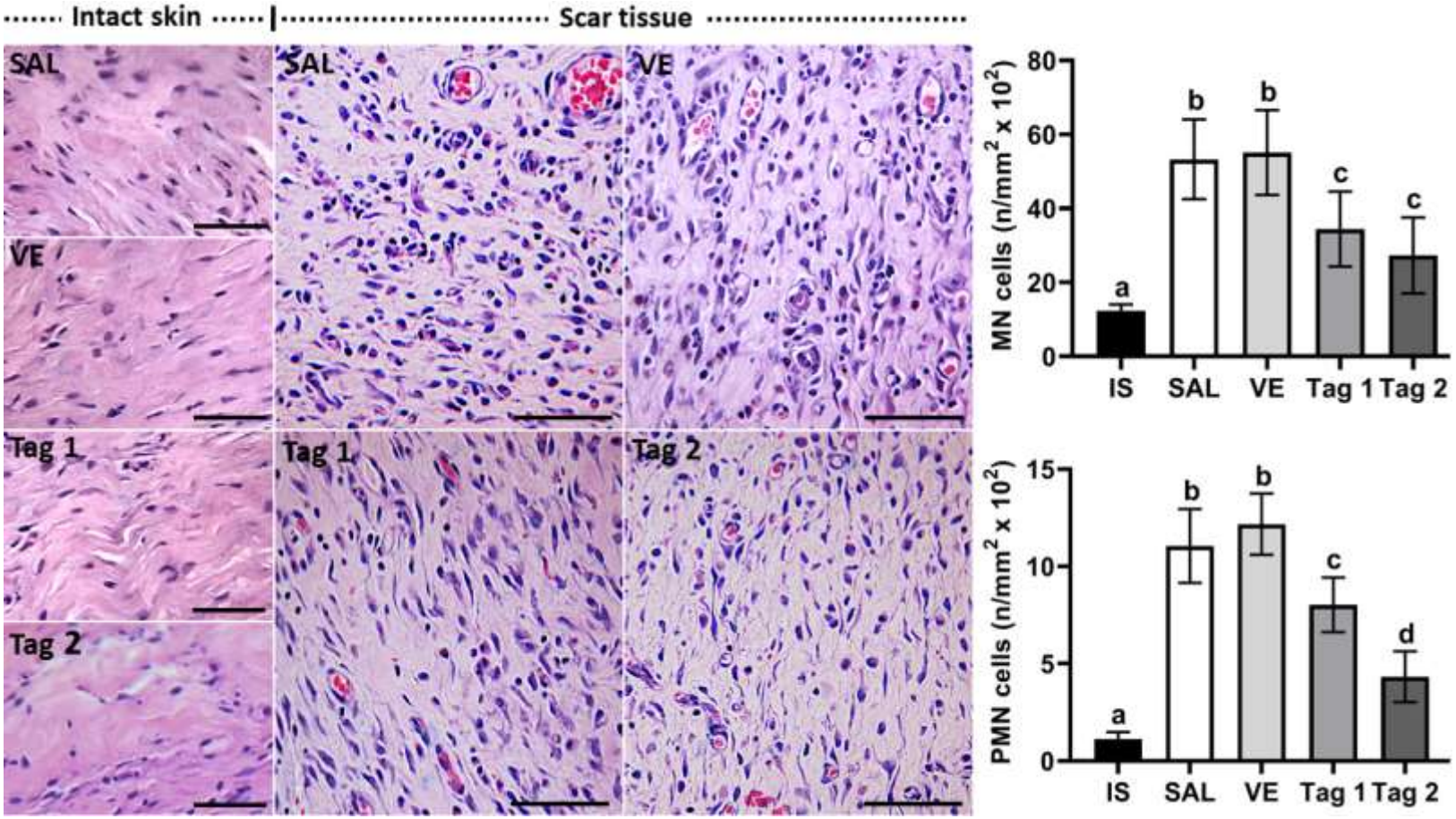

Figure 8. Microscopic images and tissue cellularity of the skin scar tissue collected from mice treated with different doses of tagitinin F (Tag). Scar tissue images (H&E staining under bright field microscopy, scale bars= 50µ). MN: Mononuclear; PMN: Polymorphonuclear. IS: Intact skin (mean values considering the statistically similarity in all groups, P>0.05). SAL: 0.9% NaCl, VE: 5% Dimethyl sulfoxide; Tag 1: 0.5% Tagitinin F; Tag 2: 1% Tagitinin F. Data are presented as mean and standard deviation. (a, b, c, d) Columns with different letters in the graphics are statistically different (P<0.05), and columns with a common letter are statistically similar (P>0.05).

As indicated in Figure 9, the scar tissue in all groups presented increased MPO and NAG activity compared to the intact skin (P>0.05). These parameters were significantly reduced in animals treated with both doses of TAG-F, especially at 1% (P<0.05), compared to the groups SAL and VE (P<0.05).

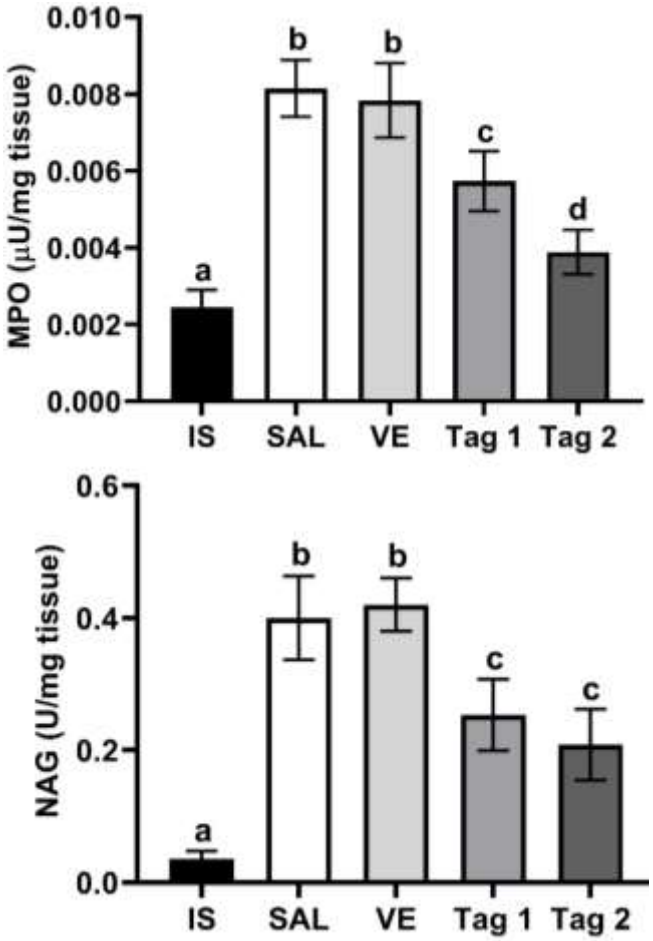

Figure 9. Myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG) activity in the scar tissue collected from mice treated with different doses of tagitinin F (Tag). IS: intact skin (mean values considering the statistically similarity in all groups, P>0.05), SAL: 0.9% NaCl, VE: 5% Dimethyl sulfoxide; Tag 1: 0.5% Tagitinin F; Tag 2: 1% Tagitinin F. Data are presented as mean and standard deviation. Columns with different letters are statistically different (P<0.05), and columns with a common letter are statistically similar (P>0.05).

As indicated in Figure 10, the scar tissue formed in response to excisional skin wound exhibited increased 5-LOX, MMP-1 and MMP-2 activity, and LTB4 levels compared to the intact skin (P<0.05). The activity of 5-LOX and LTB4 levels were markedly attenuated in the scar tissue from mice treated with both dose of TAG-F compared to the groups SAL and VE

(P<0.05). MMP-1 and MMP-2 activity was reduced with the highest dose of TAG-F compared to the groups SAL, VE and Tag 1 (P<0.05).

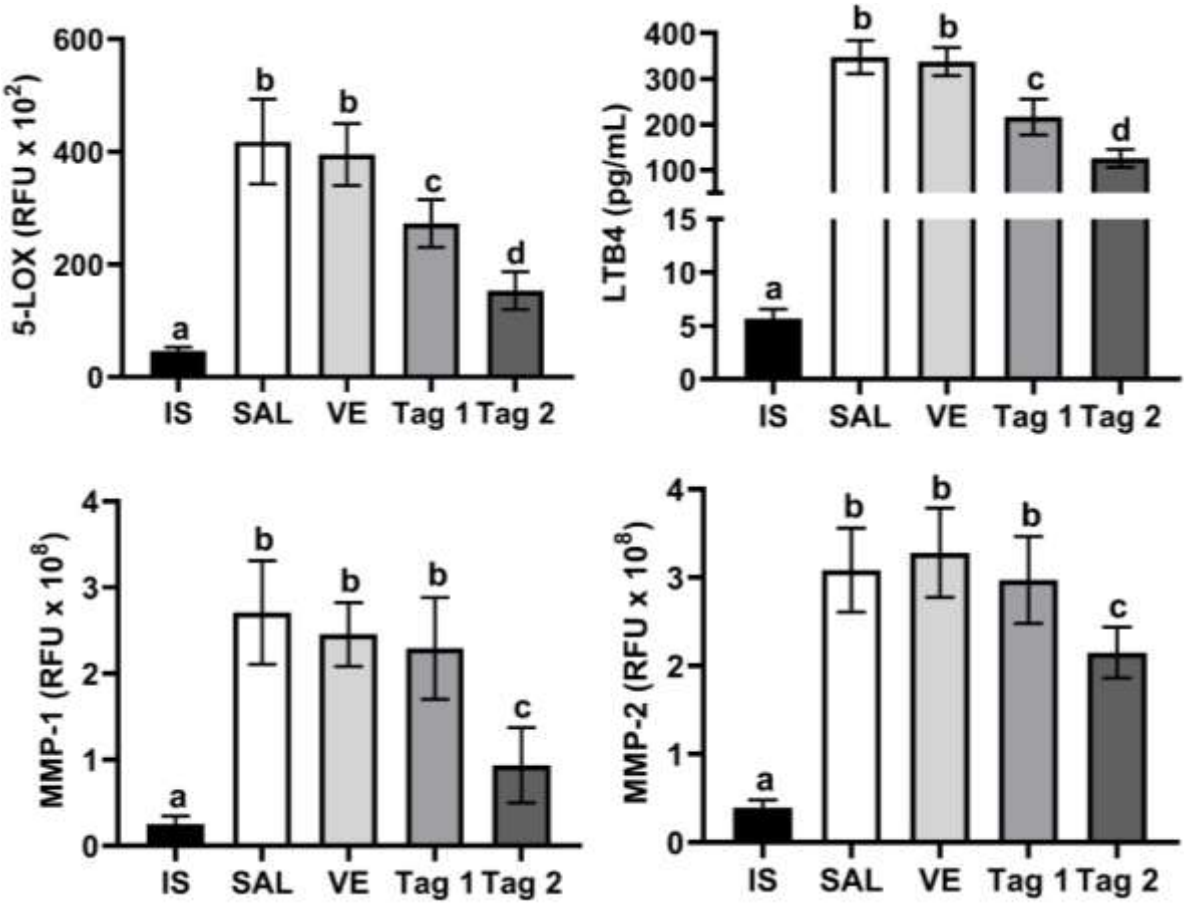

Figure 10. Lipoxygenase and matrix metalloproteinases (MMP-1 and MMP-2) activity, and leukotriene B4 (LTB4) production in the skin scar tissue collected from mice treated with different doses of tagitinin F (Tag). IS: intact skin (mean values considering the statistically similarity in all groups, P>0.05), SAL: 0.9% NaCl, VE: 5% Dimethyl sulfoxide; Tag 1: 0.5% Tagitinin F; Tag 2: 1% Tagitinin F. Data are presented as mean and standard deviation. Columns with different letters are statistically different (P<0.05), and columns with a common letter are statistically similar (P>0.05).

As expected, the intact skin in all groups exhibited a higher distribution of thick collagen fibers and collagen content compared to the scar tissue collected from all groups (P<0.05). The intact skin presented a predominance of type I collagen, while type III collagen fibers were predominant in the scar tissue of all groups. The distribution of collagen fibers and collagen content was increased in the scar tissue from mice treated with the highest dose of TAG-F compared to the other groups (P<0.05) (Figure 11).

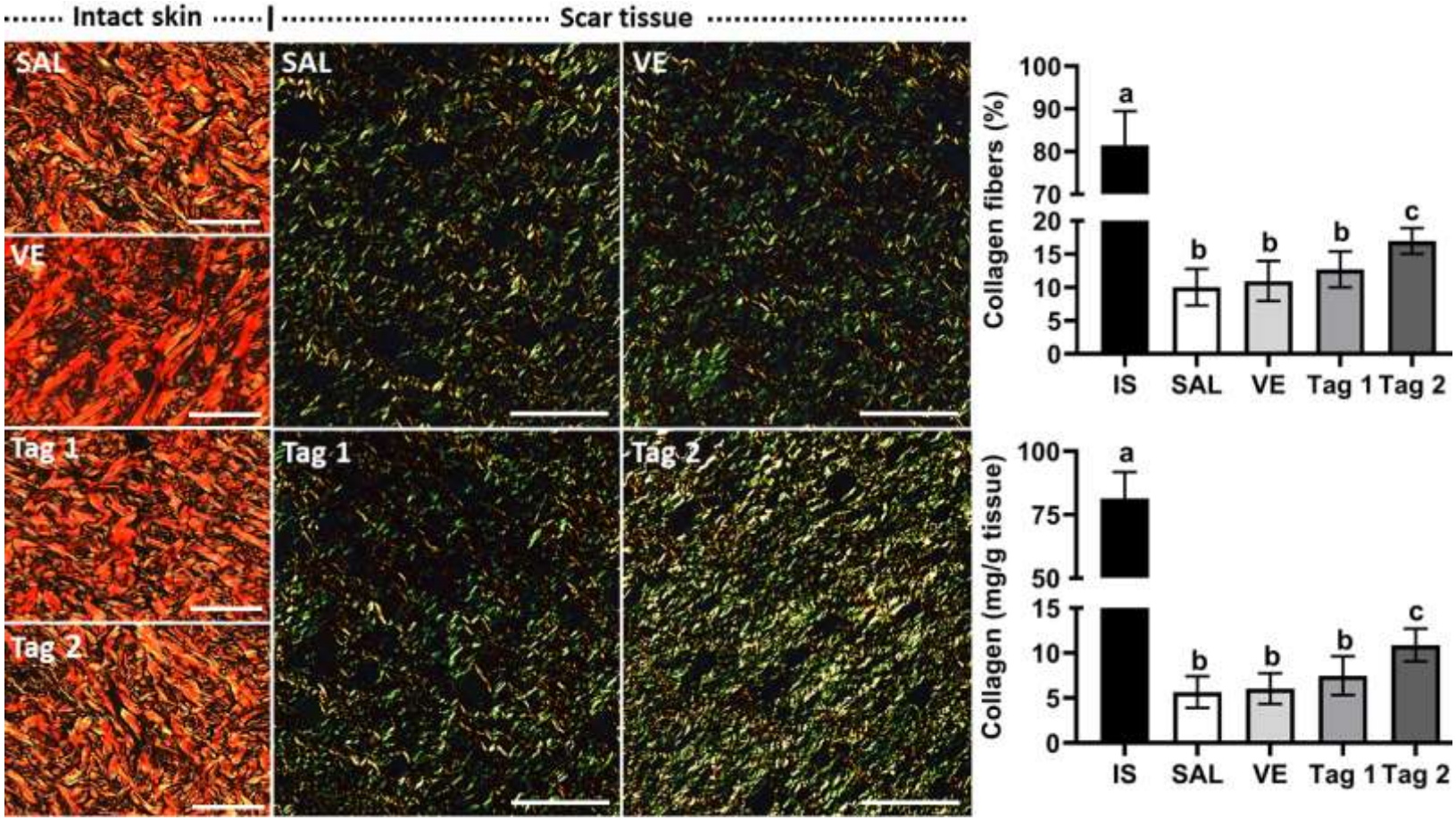

Figure 11. Microscopic images and collagen content in the skin scar tissue collected from a mice model of excisional wound treated with different doses of tagitinin F (Tag). In the scar tissue images, collagen fibers are observed in bright orange and red (type I collagen) or green and yellow (type III collagen) (Sirius red staining under polarized light, scale bars= 50µ). IS: Intact skin; SAL: 0.9% NaCl, VE: 5% Dimethyl sulfoxide; Tag 1: 0.5% Tagitinin F; Tag 2: 1% Tagitinin F. Data are presented as mean and standard deviation. (a, b, c) Columns with different letters in the graphics are statistically different ($P<0.05$), and columns with a common letter are statistically similar ($P>0.05$).

4 Discussion

In this study, we demonstrated that the anti-inflammatory effect of TAG-F is more comprehensive than previously suggested, exerting a significant impact on the control of inflammatory pain and modulation of central metabolic processes linked to skin wounds healing. Although molecular docking has identified potential interactions between TAG-F and the enzymes 5-LOX, COX-2, MMP-1 and MMP-2, predicted binding forces were not as strong compared to classic enzyme inhibitors. Thus, the effect of TAG-F *in vitro* and *in vivo* indicated a limited predictive relevance of the *in silico* model used. When evaluated on LPS-stimulated macrophages, TAG-F was effective in inhibiting the activity of COX (1 and 2 isoforms) and matrix metalloproteinases (1 and 2 isoforms), as well as the production of prostaglandin, leukotriene, and TNF-α. From a similar inhibitory effect on these inflammatory mediators, TAG-F also attenuated carrageenan-induced paw edema and mechanical hyperalgesia. Interestingly, TAG-F still exerted anti-inflammatory effects on excisional skin injuries, whose attenuation of the inflammatory infiltrate and increased collagenogenesis in the scar tissue was associated with downregulation of LOX, COX and MMPs activity, as well as prostaglandin, leukotriene and TNF-α production.

Since tagitinins were associated with potent inhibition of inflammatory effectors, these molecules have been indicated as prototypes for the development of anti-inflammatory drugs (Abe et al., 2015; Chagas-Paula et al., 2015a,b). Although macrophages are direct effectors in inflammatory processes (Fujiwara and Kobayashi, 2005), the effect of TAG-F on the response of these cells to antigenic stimulation remains overlooked. Thus, we demonstrated for the first time that by attenuating the activity of 5-LOX, COX-1 and 2; TAG-F reduces leukotriene and prostaglandin biosynthesis in LPS-activated macrophages. In fact, SL have been indicated as secondary metabolites of Asteraceae Family with potent anti-inflammatory effects (Rüngeler et al., 1999; Formisano et al., 2017). In species as *Tithonia diversifolia*, tagitinins are pointed as prominent molecules with multi-target anti-inflammatory effects. There is evidence that tagitinin A, C and F are the SL more abundant in this species, which exhibits simultaneous inhibitory effects on COX and LOX (Chagas-Paula et al., 2012, 2015a,b). In fact, the relevance of TAG-F as COX-1 and 5-LOX inhibitors was confirmed in previous metabolomic studies, which identified IC50 values of 0.001 and 18.5 µM, respectively (Chagas-Paula et al., 2015). Due to this molecular promiscuity, TAG-A, C and F demonstrates a marked potential as anti-inflammatory agents, especially considering that dual COX and LOX blockage may offer a pharmacological advantage over classical drugs that inhibit a single enzyme (Chagas-Paula et

al., 2012, 2015a,b). In this sense, although non-steroidal anti-inflammatory drugs (NSAIDs) are effective in inhibiting COX enzymes, counterregulatory mechanisms associated with LOX and leukotrienes upregulation are activated, which represent a major side effects of NSAIDs (Fiorucci et al., 2001; Chagas-Paula et al., 2015b).

Coherent with a reduced COX and LOX activity, TAG-F inhibited PGE2 and LTB4 biosynthesis. In addition, TAG-F attenuated TNF-α production in LPS-stimulated macrophages, a potent pro-inflammatory cytokine (Nakao et al., 2002; Huang et al., 2003). Currently, the effect of TAG-F on immune cells is poorly understood, especially on macrophages. However, previous studies indicated that SL-rich extracts exhibits dose-dependent inhibitory effects on lymphocyte proliferation and LTB4 production by LPS-stimulated cells *in vitro* (Lasure et al., 1995; Hiransai et al., 2016), with an IC50 of 4.42 μg/mL (Hiransai et al., 2016). In addition, there is limited evidence indicating that TAG-F induce a potent inhibition on human neutrophils, reducing myeloperoxidase activity, IL-6, CXCL8 and TNF-α production by cells challenge with LPS (Abe et al., 2015). Despite cytotoxic effects are attributed to several SL (Arantes et al., 2011; Amorim et al., 2013), TAG-F treatment was not associated with cell death, indicating that concentrations up to 100 μM are potentially safe on neutrophils (Abe et al., 2015). Similarity TAG-F seems to be well tolerated by macrophages, presenting an IC50 of 50 μg/mL (de Toledo et al., 2014).

Interestingly, we identified that the anti-inflammatory effect of TAG-F was not limited in downregulating of the arachidonic acid pathway and TNF-α production, but also inhibited MMP-1 and MMP-2 activity in LPS-stimulated macrophages. Inhibition of cytokine production represents a remarkable anti-inflammatory effect of SL, including TAG-F (Abe et al., 2015). TNF-α is a cytokine produced early by macrophages in inflammatory processes. As a potent pro-inflammatory effector, TNF-α stimulates vascular activation, leukocyte influx and activity (Feiken et al., 2995; Barrientos et al., 2008), COX-2 expression and prostaglandin release (Nakao et al., 2002; Huang et al., 2003). Thus, cytokines downregulation is a relevant anti-inflammatory property of SL, which is consistently mediated by direct inhibition of the NF-κB signaling pathway (Merfort, 2011; Abe et al., 2014). Together with arachidonic acid metabolites, cytokines play a complex regulatory role in inflammatory responses, which are influenced by MMPs (Fingleton, 2017). Although MMPs are classically known by its proteolytic functions (Nissinen and Kähär 2014; Fingleton, 2017), these enzymes are also involved with cytokines and chemokines activation. Thus, MMPs indirectly modulates leukocytes recruitment and activity in inflammatory sites (Nissinen and Kähär, 2014; Smigiel and Parks, 2017). As macrophages are important sources of proteases, the effect of TAG-F in

attenuating MMP-1 and MMP-2 after antigenic stimulation indicates an additional anti-inflammatory property of this SL, which is described for the first time in this study.

Considering our findings *in vitro*, we investigated if a similar inhibitory effect of TAG-F on prostaglandin, leukotriene and TNF-α could manifest *in vivo*, exerting morphofunctional impact on two different models of inflammatory tissue damage. As expected, intradermal administration of carrageenan was effective in inducing paw oedema and mechanical hyperalgesia, which were associated with an upregulated 5-LOX and COX (1 and 2) activity, and increased PGE2, LTB4, and TNF-α tissue levels. Interestingly, all these parameters were attenuated by TAG-F administration, reinforcing the evidence that eicosanoids and cytokines biosynthesis maintains a closed correlation with oedema (Ricciotti and FitzGerald 2011), nociception at the inflammation site (Funk 2001; Chung-Ren et al., 2006). According Guay et al. (2004), carrageenan-induced inflammation is a useful model in the screening of new anti-inflammatory molecules, especially those whose action mechanism is potentially attributed to COX and eicosanoids inhibition. As PGE2 and TNF-α released in response to tissue damage are involved in oedema and pain pathogenesis (Sommer et al., 2004; Ricciotti and FitzGerald 2011; Wittmann et al., 2014), reduction in paw volume and mechanical hyperalgesia was expected in TAG-F-treated animals. The impact of tagitinins on pain control is poorly understood. However, anti-inflammatory effects in carrageenan-induced paw oedema, and analgesic properties in pain induced by thermal and chemical stimuli were attributed to *Tithonia diversifolia* leaves extract (Owoyele et al., 2004), which is recognizably rich in tagitinins A, C and F (Chagas-Paula et al., 2015a,b). Although the analgesic mechanism induced by TAG-F requires additional detail, the attenuation of prostaglandin production cannot be disregarded. This proposition is reinforced by the evidence of receptors sensitive to PGE in the peripheral terminals of the high-threshold sensory neurons, which are involved in the perception of nociceptive stimuli in inflammatory sites (Omote et al., 2002, Trebino et al., 2003; Lin et al., 2006). As attenuation of eicosanoids production by classical anti-inflammatory drugs is accompanied by increased pain threshold during peripheral sensitization (Sekiguchi et al., 2008; De Toni et al., 2015), downregulation of COX activity and eicosanoids biosynthesis seems to be a key mechanism by which TAG-F exerts its anti-inflammatory and analgesic effects.

In addition to the effects observed in the paw inflammation model, we observed an intense inflammatory process in response to excisional skin wounds. In this model, our findings indicated for the first time that topical administration of TAG-F was also efficient in attenuating LOX, COX and MMPs activity, as well as prostaglandin, leukotriene and TNF-α production. Eicosanoid, cytokines and MMPs are early activated after skin injuries, playing an essential

role in all phases of tissue repair by modulating cell recruitment, proliferation, neoangiogenesis, synthesis and remodeling of the extracellular matrix (Mahdavian Delavary et al., 2011, Bonnans et al., 2014). As all molecular effectors analyzed exhibits a peak of production in the initial stages of wound healing (Sorg et al., 2017; Cañedo-Dorantes and Cañedo-Ayala, 2019), the impact of TAG-F was consistently analyzed 7 days after skin injury, a period aligned with the transition from the inflammatory to the proliferative phase (Gonçalves et al., 2014; 2026; Sarandy et al., 2017). Accordingly, intense PGE2, LTB4 and TNF-α level were associated with an intense inflammatory infiltrate in the scar tissue from untreated animals, reinforcing the role of these effectors in stimulating chemotaxis, cell activation and proliferation in the early healing process (Futagami et al., 2002; Trebinoet al., 2003; Mahdavian Delavary et al., 2011; Ricciotti et al., 2011). As TAG-F was efficient in attenuating eicosanoids production, a marked reduction in MN and PMN leukocytes infiltration in the scar tissue was expected. Considering that neutrophils and macrophages are early recruited after skin injuries (Lucas et al., 2010; Boering at al., 2019), our findings of MPO and NAG corroborate the inhibitory effect of TAG-F on neutrophils and macrophages recruitment in the inflammatory stage of wound healing. Since these leukocytes are direct sources of matrix proteases, the inhibitory effect of TAG-F on these cells may also have influenced MMP-1 and MMP-2downregulation in the scar tissue.

Interestingly, the interaction between TAG-F with MMP-1 and MMP-1 predicted in our *in silico* model was corroborated by our findings *in vivo*. As expected, skin injury was associated to intense MMPs activation, which was attenuated by the highest dose of TAG-F. These findings indicate an effect still unknown for TAG-F, which was potentially associated with the differential remodeling of the collagen matrix of the scar tissue in animals receiving this SL. In addition to modulating the immune response (Fingleton, 2017; Smigiel and Parks, 2017), MMPs are enzymes directly involved in controlling the balance between synthesis and degradation of collagenous and non-collagenous components of the extracellular matrix (Krejner et al., 2016; Fingleton, 2017). As indicated by our findings, these enzymes are present in intact skin, and have greater activation during in the skin healing process (Ravanti and Kähäri, 2000; Fingleton, 2017). Due to its proteolytic and gelatinolytic properties, these enzymes directly influence collagen dynamics, controlling the maturation of scar tissue through adjustments in the types and amount of collagen as the healing process progresses (Ravanti and Kähäri, 2000; Fingleton, 2017). Accordingly, reduced MMPs activity was consistent with an increased collagen deposition in the scar tissue of animals treated with the highest dose of TAG-F. These findings indicate a remarkable stimulatory effect of TAG-F on the healing process, especially considering that collagen deposition is essential to ensure adequate structural support

and mechanical resistance for the newly formed tissue (Gonçalves et al., 2014; Olczyk et al., 2014). Thus, by inhibiting MMPs and potentiating collagenogenesis, TAG-F appears to accelerate the maturation of the scar tissue while controlling tissue inflammation, an aspect that deserves detailed investigation considering a potential relevance and biotechnological applicability in regenerative medicine.

Taken together, our findings indicated that TAG-F exerts potent anti-inflammatory effects. In LPS-stimulated macrophages, this molecule attenuates LOX, COX, and MMPs activity, as well as eicosanoids and TNF-α production in a dose dependent way. Similar inhibitory effects were obtained in a murine model of carrageenan-induced paw oedema, in which the anti-inflammatory properties of TAG-F in downregulating PGE2, LTB3 and TNF-α biosynthesis was potentially associated with a higher nociceptive threshold and marked attenuation of mechanical hyperalgesia in mice. In addition, TAG-F limited eicosanoids and TNF-α biosynthesis in a murine model of excisional skin lesions, a finding closed correlated with a reduced inflammatory infiltrate in the scar tissue. Interestingly, TAG-F also potentiated scar maturation by stimulating collagen deposition in the scar tissue, a collagenogenesis process potentially mediated by the effect of TAG-F in downregulating MMP-1 and MMP-2 activity. Accordingly, TAG-F stands out as a multi-target anti-inflammatory candidate, whose applicability in regenerative medicine deserves to be better detailed in further studies.

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3 CONSIDERAÇÕES FINAIS

A partir da revisão sistemática pôde-se concluir que as lactonas sesquiterpênicas agem nas células, através do grupo α-metileno-γ-butirolactona, promovendo seu mecanismo de ação, uma vez que a presença deste anel diferencia as lactonas sesquiterpênicas de outros compostos orgânicos. Através deste mecanismo, as LSs podem exercer diversos efeitos nas células, incluindo efeitos anti e pró-inflamatórios. A maioria dos estudos em animais, incluídos na revisão sistemática, demonstraram efeitos pró-inflamatórios destas moléculas, o que pode estar relacionado com a dose administrada, enquanto os estudos *in vitro*, evidenciaram efeitos anti-inflamatórios das LSs, quando utilizadas em baixas doses, agindo por meio de diversas vias metabólicas, inibindo ou reduzindo a inflamação, levando a considerar que provavelmente, seus efeitos citotóxicos sejam influenciados pela dose administrada. No entanto, os exatos mecanismos pelos quais as LSs exercem seus efeitos por meio do grupo α-metileno-γ-butirolactona ainda não estão totalmente esclarecidos, sendo necessários mais estudos pré-clínicos e clínicos para evidenciar melhor seus efeitos. Contudo, a partir da revisão sistemática com a docagem molecular, pôde-se observar que as LSs possuem grandes afinidades com enzimas do tipo COX-2, 5-LOX, MMP-1, MMP-2 e MMP-9, com potencial inibição destas enzimas, o que sugere uma possível terapia farmacológica como anti-inflamatório das LSs, uma vez que o mecanismo pelo qual os anti-inflamatórios comercialmente disponíveis se dá pela inibição destas enzimas.

Ao avaliar os efeitos da Tagitinina F em macrófagos estimulados com LPS e em camundongos submetidos ao edema de pata com carragenina e à ferida excisional, foi observado que a Tagitinina F se mostrou como potente anti-inflamatória, antinociceptiva e reguladora de enzimas envolvidas com a cicatrização. Os achados no estudo evidenciaram que a Tagitinina F reduziu moléculas como LOX, COX e MMPs, bem como a produção de TNF-α de maneira dose dependente, nos modelos *in vitro* e *in vivo*. Além disso, Tagitinina F exerceu efeitos inibitórios no edema de pata induzido por carragenina, regulando PGE2, LTB3 e TNF-α, diminuindo o edema e aumentando o limiar nociceptivo. Enquanto na ferida excisional, também reduziu o infiltrado inflamatório no tecido cicatricial, e melhorou a maturação do colágeno do tecido.

Assim, as lactonas sesquiterpênicas, em especial a Tagitinina F, podem ser promissoras candidatas à terapia farmacológica em processos inflamatórios e imunológicos da pele, podendo agir por diferentes vias metabólicas, embora ainda sejam necessários mais estudos, a fim de elucidar por quais mecanismos e vias específicas estas moléculas podem atuar.

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