



UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
Campus de Botucatu



UNIVERSIDADE ESTADUAL PAULISTA “Júlio de Mesquita Filho”

INSTITUTO DE BIOCÊNCIAS DE BOTUCATU

FERNANDO PEREIRA BESERRA

**Mecanismos envolvidos no efeito cicatrizante de feridas cutâneas do lupeol
isolado das cascas do caule de *Bowdichia virgilioides* Kunth. (Fabaceae) em
modelos experimentais *in vivo* e *in vitro***

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biotecnologia, Área de concentração: Biotecnologia aplicada à saúde humana e animal, Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor.

Orientador: Profa. Dra. Cláudia Helena Pellizzon

Co-orientador: Profa. Dra. Arianne Leite Rozza

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“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito... não sou o que deveria ser, mas graças a Deus, não sou o que era antes...”

Martin Luther King

DEDICATÓRIA

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O projeto de doutorado possibilitou a formação específica em Biotecnologia, com estudos direcionados para a caracterização da atividade cicatrizante do lupeol estudada em modelos *in vitro* e *in vivo*. Durante a execução do projeto de doutorado, foram realizadas outras atividades, no intuito de enriquecer a formação profissional e científica do aluno.

Estágios de doutorado sanduíche

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LISTA DE ABREVIATURAS E SÍMBOLOS

A	alfa
B	beta
Δ	delta
°C	graus Celsius
μL	microlitros
μm	micrometros
μm^2	micrômetros quadrados
Akt	protein kinase B
ALT	Alanine aminotransferase
ANOVA	one-way analysis of variance
AST	aspartate aminotransferase
CAT	catalase
cDNA	complementary DNA
Cq	quantification cycle
Ct	cycle threshold
DAB	3'-diaminobenzidine
ddCT	overall average of cycle threshold
EGF	fator de crescimento epidermal
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-signal-regulated kinase
EtOHE	extrato etanólico
F	<i>forward primer</i>
FGF-2	fator de crescimento de fibroblasto-2
FW	forward
GPx	glutathione peroxidase
HE	hematoxilina and eosina
HIF-1 α	fator induzível de hipóxia-1alpha
HO-1	heme oxygenase-1
HO-2	heme oxygenase-2
IL-10	interleucina 10

IL-1 β	interleucina-1beta
IL-6	interleucina 6
iNOS	óxido nítrico sintase induzível
IP	intraperitoneal
Ki-67	antígeno Ki-67
MAPK	mitogen-activated protein kinases
MIF	fator inibidor de migração de macrófago
MMP-1	metaloproteinase de matriz extracelular – 1
MMP-2	metaloproteinase de matriz extracelular – 2
MMP-8	metaloproteinase de matriz extracelular – 8
MMP-9	metaloproteinase de matriz extracelular – 9
MMPs	metaloproteinases de matriz extracelular
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
NF- κ B	factor nuclear-kappa B
nm	nanômetros
NMR	ressonância magnética nuclear
NOS-2	óxido nítrico sintase-2
PCNA	antígeno nuclear de proliferação celular
PCR	reação em cadeia da polimerase
PDGF	fator de crescimento derivado de plaqueta
pg	picogramas
Pi3k	phosphoinositide-3-kinase-protein kinase
PMNs	células polimorfonucleares
pró-MMP-2	forma inativa da MMP-2
pró-MMP-9	forma inativa da MMP-9
psi	libra por polegada quadrada
R	<i>reverse primer</i>
RNA	ácido ribonucleico
RT-qPCR	reação em cadeia da polimerase quantitativa por transcrição reversa
RV	reverse
SC	subcutâneo
SEM	erro padrão da média
SOD	superóxido dismutase

STZ	estreptozotocina
Temp	temperatura em °C
TGF- β 1	fator de crescimento transformador- β 1
TNF- α	fator de necrose tumoral- α
UI	unidade internacional
VEGF	fator de crescimento vascular endotelial

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RESUMO

A dificuldade de cicatrização de feridas é uma das principais complicações decorrentes do *Diabetes mellitus* (DM), que torna o processo cicatricial mais lento e o tratamento mais dispendioso. O presente estudo teve como objetivo investigar o efeito e os mecanismos do lupeol envolvidos no processo de cicatrização de feridas cutâneas experimentais *in vitro* e em ratos normoglicêmicos (RN) e hiperglicêmicos (RH). Foi investigado o efeito do lupeol (0,1, 1, 10 e 20 µg/mL) em ensaios *in vitro* de cicatrização de feridas utilizando queratinócitos e fibroblastos de prepúcio neonatal humano. Para os estudos *in vivo*, ratos Wistar machos (n=8) foram randomicamente divididos nos seguintes grupos experimentais usando RN: creme base, collagenase 1.2 U/g, creme à base de lupeol 0,1%, 0,2% e 0,4% tratados durante 3, 7 e 14 dias. O estudo realizado em RH, os grupos foram divididos em: creme base, insulina 0.5 U/g e creme à base de lupeol 0.2% (menor dose efetiva em RN) em RH tratados durante 14 dias. A indução experimental de feridas cutâneas foi realizada na região dorsal dos ratos com o auxílio de um *punch* de 2 cm de diâmetro. Foram investigados parâmetros macroscópicos, histológicos, imunohistoquímicos, imunoenzimáticos e moleculares. Os resultados *in vitro* mostraram que, baixas concentrações do lupeol foi capaz de estimular a migração e proliferação celular de queratinócitos, efeito contrátil em fibroblastos dérmicos embebidos em um solução gel de colágeno, possivelmente através das vias PI3k/Akt, MAPK/p38, vias sinalizadoras envolvidas na proliferação/migração celular, angiogênese e reparo tecidual. Nossos resultados *in vivo* mostraram potencial cicatricial do lupeol após 7 e 14 dias de tratamentos em feridas de ratos normoglicêmicos. Lupeol mostrou atividade anti-inflamatória, com significativa redução de TNF- α , IL-6 e NF- κ B, e aumento nos níveis de IL-10, atividade proliferativa por estimular a expressão de Ki-67, aumento do processo angiogênico por aumento da expressão de VEGF, estímulo da reepitelização observados pelo aumento de EGF e remodelação da matriz extracelular por estimular a síntese de colágenos. Os resultados em RH, mostraram um aumento na porcentagem de retração da lesão a partir do 11º dia de tratamento após da indução da lesão. As análises histopatológicas revelaram uma diminuição da infiltração de células inflamatórias, aumento da proliferação de fibroblastos, vascularização e deposição de fibras colágenas no tratamento com lupeol. Os resultados do ELISA revelaram níveis de TNF- α e IL-6 reduzidos e níveis de IL-10 sobrerregulados, mostrando atividade anti-inflamatória. Os resultados moleculares e imunohistoquímicos confirmaram o potencial anti-inflamatório do lupeol através da redução de NF- κ B e do estresse oxidativo por aumentar a expressão de enzimas antioxidantes, como HO-1 e SOD-2, bem como mecanismos de proliferação celular, angiogênese e remodelamento de matriz extracelular estimulados por FGF-2, TGF- β 1, HIF-1 α e colágeno III. Nossos achados permitem concluir que o lupeol pode servir como uma nova opção terapêutica para o tratamento de feridas cutâneas normais e em condições hiperglicêmicas, regulando os mecanismos envolvidos nas fases inflamatória, proliferativa e de remodelação tecidual.

Palavras-chave: lupeol, pele, feridas, cicatrização de feridas, hiperglicemia

ABSTRACT

The difficulty of wound healing is one of the main complications due to *Diabetes mellitus* (DM), which makes the healing process slower and the treatment more expensive. The present study aimed to investigate the effect of lupeol and mechanisms involved in the healing process of experimental skin wounds *in vitro* as well as in normoglycemic (NR) and hyperglycemic rats (HR). The effect of lupeol (0.1, 1, 10 and 20 µg/mL) on *in vitro* wound healing assays using keratinocytes and human neonatal foreskin fibroblasts was investigated. For *in vivo* studies, male Wistar rats (n=8) were randomly divided into the following experimental groups using NR: lanette, collagenase 1.2 U/g and lupeol 0.1%, 0.2% or 0.4% treated for 3, 7 and 14 days. In the study performed in HR, the groups were divided into: lanette, insulin 0.5 U/g and lupeol 0.2% cream (lowest effective dose in NR) in HR treated for 14 days. Experimental induction of cutaneous wounds was performed on dorsal region of the rats using a punch 2 cm in diameter. Macroscopic, histological, immunohistochemical, immunoenzymatic and molecular parameters were investigated. The *in vitro* results showed that low concentrations of lupeol were able to stimulate cell migration and proliferation of keratinocytes, a contractile effect on dermal fibroblasts embedded in a collagen gel solution, possibly through PI3k/Akt, and MAPK/p38 signaling pathways involved in cell proliferation/migration, angiogenesis and tissue repair. Our *in vivo* results showed wound healing potential of lupeol after 7 and 14 days of treatment in normoglycemic rat wounds. Lupeol showed anti-inflammatory activity, with significant reduction of TNF- α , IL-6 and NF- κ B, and increased IL-10 levels, proliferative activity by stimulating Ki-67 expression, increased angiogenic process by increased VEGF expression, stimulus of re-epithelialization observed by the increase of EGF and extracellular matrix remodeling by increase the collagens deposition. The results in HR, showed an increase in the percentage of wound retraction from the 11th day of treatment after the lesion induction. Histopathological analyzes revealed a decrease in inflammatory cells infiltration, increased proliferation of fibroblasts, vascularization and deposition of collagen fibers in lupeol treatment. ELISA results demonstrated reduced TNF- α and IL-6 levels and increased IL-10 levels, suggesting anti-inflammatory activity. Molecular and immunohistochemical results confirmed the anti-inflammatory potential of lupeol by reducing NF- κ B and oxidative stress by increasing the expression of antioxidant enzymes, such as HO-1 and SOD-2, as well as mechanisms of cell proliferation, angiogenesis and remodeling of extracellular matrix stimulated by FGF-2, TGF- β 1, HIF-1 α and collagen III. Our findings allow us to conclude that lupeol may serve as a new therapeutic option for the treatment of normal and hyperglycemic skin wounds, regulating the mechanisms involved in the inflammatory, proliferative and tissue remodeling phases.

Key words: lupeol, skin, wounds, wound healing, hyperglycemia

PREFÁCIO

O **capítulo 1** contém uma revisão bibliográfica sobre o processo de cicatrização de feridas e suas três fases sobrepostas, inflamatória, proliferativa e de remodelamento, assim como, a terapêutica atual no tratamento de feridas. Este capítulo também faz uma abordagem sobre a cicatrização de feridas e sua relação com diabetes e/ou hiperglicemia, o uso de produtos naturais no tratamento de feridas, além dos objetivos propostos nesse trabalho com o triterpeno lupeol, isolado da espécie vegetal “*Bowdichia virgilioides*” (Sucupira-preta).

O **capítulo 2** é decorrente do projeto de doutorado sanduíche realizado na The University of Sydney, em Sydney, na Austrália, pelo Programa Ciências sem Fronteiras (Processo de nº: 202513/2015-7) e o estudo foi recentemente publicado na *Molecules*, intitulado: “Lupeol, a pentacyclic triterpene promotes migration, wound closure and contractile effect *in vitro*: possible involvement of PI3K/Akt and p38/ERK/MAPK pathways”. Esta pesquisa mostrou efeito cicatrizante *in vitro* através do estímulo do processo de migração e cicatrização de feridas em queratinócitos epidérmicos humanos e aumento da contração de fibroblastos incorporados em uma solução gel de colágeno.

No **capítulo 3** encontra-se o manuscrito no formato padrão da revista *European Journal of Pharmacology*, intitulado: “From inflammation to cutaneous repair: Topical application of lupeol improves skin wound healing in rats by modulating the cytokine levels, NF- κ B, Ki-67 and growth factors expression, and distribution of collagen fibers”. Os resultados deste estudo são oriundos do projeto de doutorado aprovado pela Fapesp (Processo de nº: 2014/23247-4). Este estudo mostrou o potencial efeito do creme de lupeol e mecanismos envolvidos na ação cicatrizante em ratos normoglicêmicos tratados após 3, 7 e 14 dias após a indução da lesão.

O **capítulo 4** mostra os resultados do efeito cicatrizante do lupeol em feridas de ratos hiperglicêmicos induzidas por estreptozotocina. Este estudo também faz parte do projeto aprovado pela Fapesp (Processo de nº: 2014/23247-4): “Avaliação dos mecanismos celulares e moleculares do lupeol no processo de cicatrização de feridas cutâneas em ratos”. Os resultados são promissores e nos encorajaram a escrever o manuscrito, intitulado: “Lupeol enhances wound healing in streptozotocin-induced hyperglycemic rats with modulatory effects on inflammation, oxidative stress, angiogenesis and extracellular matrix components”, no formato padrão da revista: *Jornal of Diabetes and its Complication*.

O **capítulo 5** descreve as considerações finais da tese, concluindo de uma forma geral, todos os achados deste estudo desenvolvidos durante o doutorado.

Considerações gerais

Capítulo I

1. INTRODUÇÃO

As feridas cutâneas têm impacto significativo na saúde pública e no investimento de recursos públicos. Atualmente, devido à sua complexidade de tratamento, muitos protocolos e pesquisas vêm sendo estabelecidos visando acelerar o processo cicatricial, fenômeno pelo qual o organismo tende a reparar uma porção lesada, de modo a guiar ou gerir este processo, para que sejam obtidos resultados mais eficientes em relação ao tempo de resolução e qualidade da cicatriz do tecido lesado.

O sucesso da reestruturação da pele depende de uma cascata de eventos ordenados, que envolvem respostas e/ou interações celulares e moleculares. A cicatrização de feridas é um processo importante, mas complexo, afetado por uma série de fatores, incluindo a coagulação do sangue, inflamação, fibroplasia, deposição de colágeno e contração da ferida (Lindley *et al.*, 2016). Por questões didáticas, o processo cicatricial é analisado a partir de três fases que se sobrepõem: fase inflamatória, formada por eventos como hemostasia e inflamação; fase proliferativa, caracterizada pela formação do tecido de granulação e reepitelização; e a fase de remodelamento da matriz (Morin *et al.*, 2012).

A descoberta de protótipos para o desenvolvimento de novos fármacos ou de novos agentes terapêuticos representa uma possibilidade para o tratamento de diferentes patologias, incluindo as feridas cutâneas. Os produtos naturais apresentam-se como fonte de compostos com potencial atividade biológica e determinadas espécies de plantas ou substâncias naturais isoladas das mesmas têm sido utilizados com sucesso em estudos recentes sobre o tratamento de feridas cutâneas (Cetin *et al.*, 2013; Upadhyay *et al.*, 2013). Metabólitos secundários ou compostos ativos isolados de muitas fontes naturais, além das plantas, também têm se mostrado responsáveis pela indução da cicatrização de feridas cutâneas em modelos animais (Fikru *et al.*, 2012; Thangapazham *et al.*, 2013).

Em função da busca por novos compostos e suas aplicações, usamos a *Bowdichia virgilioides* Kunth (Sucupira-preta), uma árvore bastante dispersa no cerrado e em regiões amazônicas, que pertence a ecossistemas latino-americanos e está classificada na família Fabaceae. Esta espécie, está presente em vários países da América Latina, sendo que no Brasil, se encontra distribuída pelas Regiões Sudeste, Centro Oeste, Norte e Nordeste (Barros *et al.*, 2010). O seu uso medicinal é empregado através de infusões obtidas a partir de diferentes partes da planta (semente, casca da árvore e casca da raiz), utilizadas

popularmente para o tratamento de algumas doenças como diabetes, artrite reumatoide, dermatoses, infecções em geral e feridas cutâneas (Agra *et al.*, 2013).

O lupeol ($C_{30}H_{50}O$) é um triterpeno pentacíclico encontrado em diversas plantas comestíveis, como azeitona, figo, manga, cenoura, soja, semente de melão, uvas, própolis, e também em plantas medicinais, como a *Bowdichia virgilioides* (Saleem *et al.*, 2001). As pesquisas têm mostrado potencial farmacológico do lupeol, incluindo efeitos antioxidantes, anti-inflamatórios, anti-hiperglicêmicos, anti-dislipidêmicos e anti-mutagênicos, anti-asma, anti-artrítica, cardioprotetora, hepatoprotetora, nefroprotetora, neuroprotetora e anticancerígena sob várias vias de administração, tais como tópica, oral, subcutânea, intraperitoneal e intravenosa (Badshah *et al.*, 2016; Alqahtani *et al.*, 2013; Sudhahar *et al.*, 2008; Siddique and Saleem, 2011). Destaca-se também que, os ensaios clínicos com este triterpeno têm sido realizados para tratar melanoma maligno oral canino e acne de pele moderada humana no Japão e na Coreia (Yokoe *et al.*, 2015).

Estas propriedades terapêuticas do lupeol já descritas na literatura, fazem dessa substância um elemento substancialmente promissor para uso na cicatrização de feridas cutâneas em geral. Contudo, o objetivo do presente estudo foi avaliar os mecanismos celulares e moleculares do triterpeno lupeol na cicatrização de feridas cutâneas em ratos normo e hiperglicêmicos, além de determinar seu efeito *in vitro* sobre queratinócitos e fibroblastos humanos e seus mecanismos de sinalização envolvidos.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar os mecanismos celulares e moleculares do lupeol envolvidos no efeito cicatrizante de feridas cutâneas *in vivo* e *in vitro*.

2.2 Objetivos Específicos

- Avaliar o efeito do lupeol em modelo de cicatrização de feridas *in vitro* e seus mecanismos de sinalização envolvidos;
- Investigar o potencial efeito cicatrizante do triterpeno lupeol nas três concentrações avaliadas *in vivo*;
- Caracterizar a histopatologia das lesões em ratos normo e hiperglicêmicos tratados com creme de lupeol 0.2%;
- Avaliar o efeito anti-inflamatório do lupeol no processo cicatricial;
- Determinar o envolvimento do lupeol na angiogênese;
- Analisar o envolvimento de enzimas antioxidante envolvidas no efeito cicatrizante do triterpeno lupeol;

3. REVISÃO BIBLIOGRÁFICA

3.1 Morfofisiologia da pele

A pele é o maior órgão do corpo, correspondendo a 16 % do seu peso, sendo responsável por inúmeras funções vitais, tais como, proteção química e física, termorregulação, atividade secretora e sensorial. As secreções sebáceas ácidas e a estrutura de superfície da pele são agressivas para muitos agentes patógenos (Mabona e Van Vuuren, 2013). A pele é constituída por duas camadas denominadas epiderme e derme mantendo-se unida aos órgãos subjacentes através da hipoderme, que não faz parte da pele, conforme mostra a figura 1.

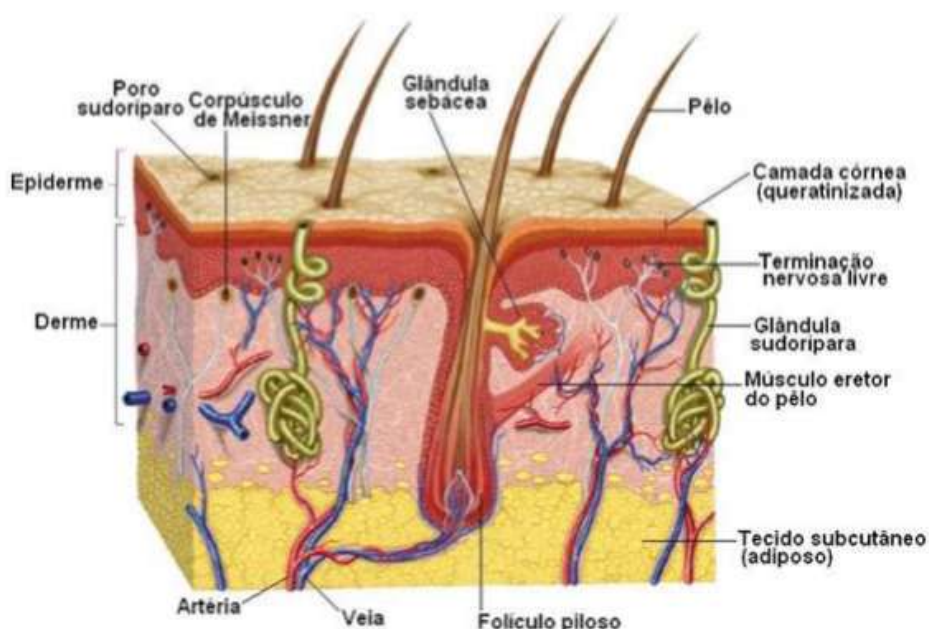


Figura 1: Esquema representativo demonstrando as estruturas da pele. Adaptado de Kendall and Nicolaou (2013).

A epiderme, camada mais externa da pele, é constituída por células epiteliais (queratinócitos, melanócitos, células de Merkel e Langerhans) organizadas em camadas. Essas células são produzidas de forma contínua na camada mais profunda da epiderme (camada basal ou germinativa) e, durante sua diferenciação/maturação, passam por alterações morfológicas e funcionais, tais como achatamento e produção acentuada de queratina, em um processo conhecido como corneificação, originando a camada córnea (a camada mais superficial da epiderme), composta basicamente de células anucleadas e repletas de queratina. A renovação celular constante da epiderme faz com que as células

da camada córnea sejam gradativamente eliminadas e substituídas por outras (Eming *et al.*, 2014; Pasparakis *et al.*, 2014).

Separada da epiderme pela membrana basal, a derme, camada localizada entre a epiderme e a hipoderme, é responsável pela resistência e elasticidade da pele. A derme é originária do folheto mesodérmico, confere sustentação à epiderme e envolve anexos cutâneos, vasos sanguíneos e linfáticos, terminações nervosas sensoriais e músculos. Sua espessura é variável e atinge um máximo de 3 mm na planta dos pés. Ao contrário da epiderme, a derme não possui uma organização regular e sua matriz extracelular é constituída por líquido tecidual e glicosaminoglicanos, proteoglicanos e glicoproteínas multiadesivas que se ligam a proteínas receptores (integrinas) nas superfícies das células, que confere resistência mecânica à pele, e fibras elásticas, colágenas e reticulares (Kamel *et al.*, 2013). Fibroblastos, células essenciais ao processo cicatricial, estão também presentes na derme, e os mesmos sintetizam e secretam colágeno e elastina, fundamentais ao processo cicatricial, além disso, desempenham papel crucial na contração e retração da ferida (Bainbridge, 2013).

A perda da integridade da pele causada por injúria, resulta em abertura ou ruptura do tecido, chamada de ferida ou úlcera (Fikru *et al.*, 2012). Tais feridas podem ser causadas por uma variedade de eventos traumáticos, tais como exposição ao calor, frio, materiais corrosivos, radiação ou problemas circulatórios (Morin *et al.*, 2012). Em resposta a essas injúrias, inicia-se a cicatrização, um processo dinâmico que restabelece a função e a integridade do tecido lesado (Guo e DiPietro, 2010; Pereira e Bártolo, 2016).

3.2 Processo de cicatrização de feridas

A cicatrização ou reparação de feridas ocorre pela regeneração espontânea da epiderme e/ou derme. Ocorre independentemente da forma de como aconteceu a lesão, seja elas traumáticas, cirúrgicas e patológicas. A profundidade é o que determina forma de cicatrização e a classificação ocorre em três categorias: primeira intenção, segunda intenção e terceira intenção (Beldon, 2010). A intenção primária envolve a aproximação das bordas da ferida de uma laceração ou ferida cirúrgica por suturas, grampos ou fitas adesivas e a regeneração ocorre dentro de 7 a 10 dias. A intenção secundária ocorre quando as bordas não podem ser aproximadas e a reconstituição se dá lentamente por tecidos conjuntivo e epitelial. Normalmente, este tipo de cicatrização de segunda intenção, ocorre em pacientes com co-morbidades subjacentes (úlceras vasculares ou de

pressão) ou em pacientes com pós-operatório que desenvolveram deiscência (devido à infecção ou hematoma). A cicatrização de feridas por terceira intenção, muitas vezes chamada de "intenção primária tardia", ocorre após a abertura da ferida devido à infecção ou contaminação. Os tecidos não viáveis são removidos, e na sequência as bordas são aproximadas e a cura continua como por intenção primária (Beldon, 2010).

A cicatrização de feridas é um processo biológico extremamente dinâmico e interativo, que envolve interações complexas de matriz extracelular, moléculas extracelulares, de mediadores solúveis, várias células residentes (fibroblastos e queratinócitos) e subtipos de leucócitos infiltrantes que, em conjunto, atuam para restabelecer a integridade do tecido danificado e substituir os perdidos. Esse processo compreende três etapas sequenciais e sobrepostas, independentemente da quantidade de tecido lesionado, são elas: hemostasia/inflamação; proliferação de células e reparação da matriz; epitelização e remodelação do tecido cicatricial, conforme é possível observar na figura 2. Envolve complexos mecanismos bioquímicos e celulares (Boateng *et al.*, 2008; Beitz, 2012).

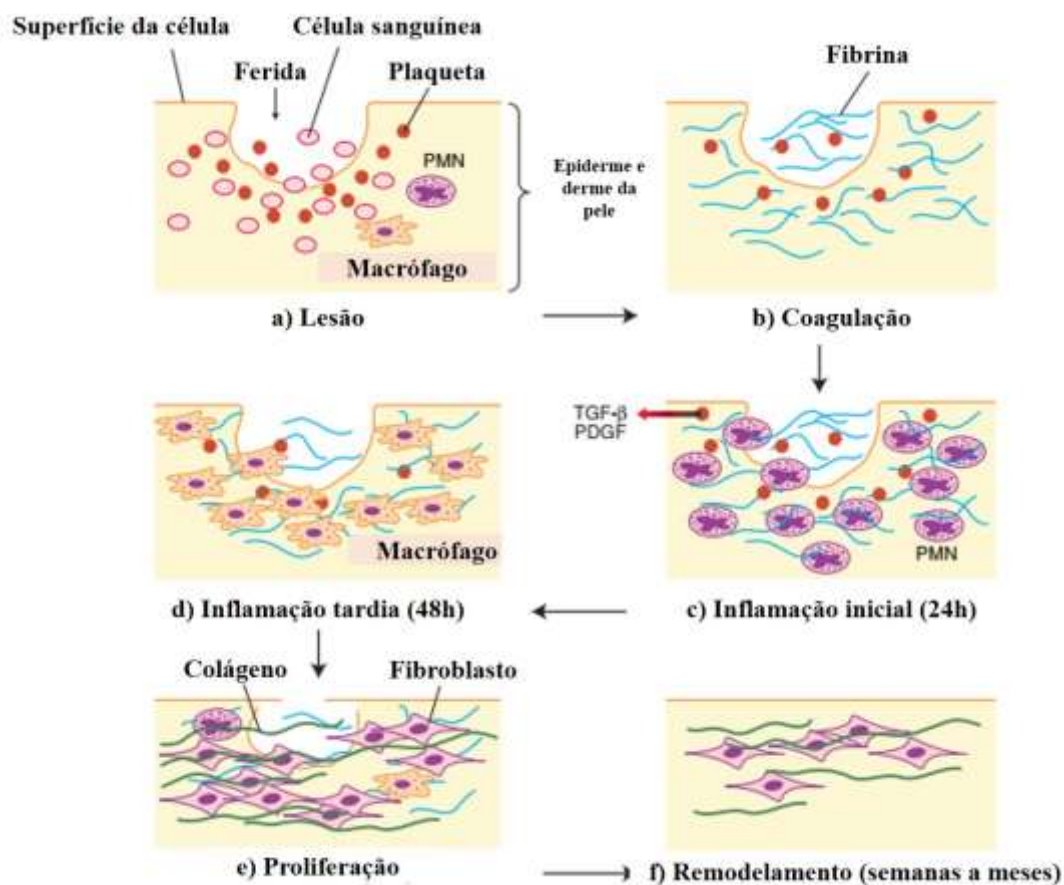


Figura 2: Esquema representativo das fases da cicatrização de feridas. (A) Imediatamente após a lesão na pele, elementos sanguíneos e aminas vasoativas extravasam dos vasos sanguíneos da derme danificados. (B) Ocorre coagulação quando as plaquetas se agregam com fibrina, a qual é depositada na ferida após conversão do fibrinogênio. (C) As plaquetas liberam PDGF e TGF- β que atraem PMNs para ferida, sinalizando o início da inflamação. (D) 48h após a lesão, macrófagos substituem as PMNs, sendo a principal célula inflamatória presente na lesão. Esses dois tipos celulares juntos removem os restos celulares da ferida, liberando fatores de crescimento e começam a reorganizar a matriz extracelular. (E) A fase proliferativa começa por volta de 72h após a lesão com os fibroblastos, recrutados para ferida pelos fatores de crescimento liberados pelas células inflamatórias, iniciando a síntese de colágeno. (F) Apesar da taxa de síntese de colágeno diminuir após aproximadamente três semanas, a reorganização do colágeno ocorre por meses após a lesão na fase de remodelamento do reparo. Adaptado de Beanes *et al.*, (2003)

Durante a primeira fase (inflamatória), ocorre hemostasia, migração de leucócitos e início da cascata de reparo tecidual. Inicialmente, em resposta à mediadores inflamatórios (Tabela 1), há redução do fluxo sanguíneo pela vasoconstrição. Com extravasamento de sangue dos vasos lesados, plaquetas são ativadas fazendo com que tenha início o processo de coagulação (Martin e Feng, 2009; Shaw e Martin, 2009). Durante este processo, há um aumento progressivo da permeabilidade vascular às células migrantes e substâncias biologicamente ativas. Deste processo surgem elementos essenciais para a continuação fisiológica da cicatrização: um arcabouço de fibrina, necessário para a migração das células que chegarão ao local da lesão, e as quimio/citocinas pró e anti-inflamatórias que irão auxiliar na ativação e migração celular (Werner *et al.*, 2007). Dentre os fatores que são secretados pelas plaquetas por degranulação, se destacam PDGF (fator de crescimento derivado de plaquetas) e TGF- β (fator transformador do crescimento beta), que neste primeiro momento terão como função atrair neutrófilos e monócitos, e o EGF (fator de crescimento epidérmico), que será mais ativo na fase proliferativa, mostrados na tabela 2 (Isaac *et al.*, 2010).

No âmbito molecular, as citocinas (mais de 30 tipos estão envolvidas) possuem tanto ações estimulantes quanto inibitórias sobre as células inflamatórias (Kujath e Michelsen, 2008). Na tabela 1 estão apresentadas as propriedades biológicas das principais citocinas envolvidas na cicatrização.

CITOCINA	FONTE CELULAR	ATIVIDADE BIOLÓGICA
<i>Citocinas pró-inflamatórias</i>		
TNF- α	Macrófagos	Promove quimiotaxia de PMN e citotoxicidade, e ativação de outras citocinas, como IL-1 β e IL-6.
IL-1 β	Macrófagos Queratinócitos	Promove quimiotaxia para fibroblastos e queratinócitos que sintetizam o colágeno.
IL-2	Linfócitos T	Aumenta a infiltração fibroblástica e o metabolismo celular.
IL-6	Macrófagos PMN Fibroblastos	Estimula a proliferação de fibroblastos.
IL-8	Macrófagos Fibroblastos	Promove a quimiotaxia de macrófagos e PMN e a maturação de queratinócitos.
IFN- γ	Macrófagos LinfócitosT	Estimula a ativação de macrófagos e PMN e atividade da collagenase
<i>Citocinas anti-inflamatórias</i>		
IL-4	Linfócitos T Basófilos Mastócitos	Inibe a ativação de TNF, IL-1 β e IL-6, a proliferação de fibroblastos, e síntese de colágeno.
IL-10	Linfócitos T Macrófagos Queratinócitos	Inibe a ativação de TNF- α , IL-1 β e IL-6; inibe a ativação dos macrófagos e PMN.

Tabela 1: Citocinas e suas propriedades biológicas na fase inflamatória. PMNs: células polimorfonucleares. Fonte: Adaptado de Schultz *et al.*, (2003).

Visando degradar elementos da matriz extracelular e tecido necrótico a fim de facilitar o trânsito na região de lesão, além da fagocitose, os neutrófilos e macrófagos secretam metaloproteinasas (MMPs) (Eming *et al.*, 2007). Os macrófagos secretam diversos fatores de crescimento, dentre os quais se incluem: EGF, FGF (fator de crescimento dos fibroblastos), PDGF, TGF- β e VEGF (fator de crescimento endotelial vascular) (Reinke e Sorg, 2012).

O segundo estágio didático do processo de cicatrização é a fase de proliferação, que se caracteriza por fibroplasia, angiogênese e re-epitelização. A migração e proliferação dos fibroblastos ocorrem a partir das margens livres da ferida. Uma vez na região de lesão e sob estímulo do TGF- β , há diferenciação de fibroblastos em miofibroblastos, os quais possuem capacidade de migração pela ferida. Durante essa

movimentação, ocorre deposição de fibronectina sobre o arcabouço de fibrina (Ariel e Timor, 2013; Artlett, 2013).

Fator de crescimento	Mecanismo envolvido	Origem	Efeito na cicatrização
PDGF	Inflamação, remodelamento de matriz extracelular	Plaquetas, macrófagos	Atração de neutrófilos e fibroblastos
FGF-2	Síntese e remodelamento de matriz extracelular	Fibroblastos, células endoteliais	Proliferação de fibroblastos, células endoteliais e queratinócitos
KGF	Reepitelização	Fibroblastos	Proliferação e migração de queratinócitos
TGF- β 1	Inflamação, síntese e remodelamento de matriz extracelular	Plaquetas, macrófagos, fibroblastos e queratinócitos	Estímulo da angiogênese e síntese de matriz extracelular/ Proliferação e diferenciação de fibroblastos
VEGF	Síntese de matriz extracelular	Neutrófilos, plaquetas, células endoteliais	Estímulo da angiogênese
EGF	Reepitelização	Queratinócitos e macrófagos	Proliferação de queratinócitos

Tabela 2: Fatores de crescimento que atuam na cicatrização de lesões de pele. Fonte: Barriento *et al.*, (2008), com modificações.

Frente à formação desse arcabouço, dá-se início a síntese de fibras colágenas na ferida (Eming *et al.*, 2014). Na matriz dérmica, há essencialmente dois tipos de colágeno: tipo I e tipo III, correspondendo a cerca de 80-85% e 15-20% do total dessa proteína, respectivamente. O tipo I é mais espesso e está localizado, principalmente, na camada mais profunda da pele. O colágeno tipo III é mais fino, e nessa fase do processo encontra-se abundantemente localizado na epiderme (membrana basal) da pele. Durante o processo de reparo da pele ferida, ao contrário da derme íntegra, há uma maior proporção de colágeno III em relação ao tipo I (Delavary *et al.*, 2011). Neste contexto, os miofibroblastos alinham-se e ligam-se às fibras de colágeno de maior espessura puxando-as em direção a eles, sendo este fenômeno responsável pela contração da ferida (Eming *et al.*, 2014). Assim, quanto maior o número de fibras de grande diâmetro, maior será a

retração da ferida. Associado ao processo de reparo há formação de novos vasos a partir dos adjacentes à ferida, fenômeno esse conhecido por angiogênese, sendo o VEGF e o TGF- β os principais agentes angiogênicos envolvidos (Isaac *et al.*, 2010).

O processo de reepitelização promove o reestabelecimento da integridade da pele. Dessa forma, os queratinócitos adjacentes à borda da lesão apresentam uma reorganização de suas moléculas de adesão, o que os permite alterar as conexões com a membrana basal e com os queratinócitos vizinhos, possibilitando que tais células migrem pela superfície da lesão, colaborando para o fechamento da ferida cutânea (Gurtner e Chapman, 2016; Tziotzios *et al.*, 2012).

Na terceira fase do processo de cicatrização, a fase de remodelamento, o colágeno apresenta um papel de protagonista, uma vez que há mudança do padrão de colágeno (Reinke e Sorg, 2012). O colágeno tipo III, inicialmente mais abundante que o tipo I, vai sendo degradado mais ativamente com o decorrer do tempo, enquanto que o colágeno I vai tendo sua produção aumentada pelos fibroblastos (Shaw e Martin, 2009). Juntamente com a substituição do tipo de colágeno, ocorre uma alteração em sua organização, a qual muda de fibras paralelas dispostas aleatoriamente para entrelaçadas e organizadas ao longo das linhas de stress (Gurtner e Chapman, 2016). Enquanto PDGF estimula maior degradação de colágeno I e síntese de colágeno III, TGF- β induz maior secreção do primeiro e sua menor degradação por induzir a expressão de TIMPs (inibidores de metaloproteinases) e menor de MMPs, sendo o remodelamento e a contração da ferida parcialmente controlada pela relação entre estes fatores de crescimento (Sen e Roy, 2008). Em virtude de seus múltiplos pontos de ligação ao colágeno, os miofibroblastos se contraem, reduzindo a superfície da área lesada (Tziotzios *et al.*, 2012).

O restabelecimento de componentes dos anexos da pele, como folículos pilosos e glândulas sebáceas, são partes integrantes do bom funcionamento da pele, como funções sensoriais e de termorregulação, além de contribuírem com células epidérmicas para a cicatrização de feridas (Li *et al.*, 2011; Takeo *et al.*, 2015). A neoformação dessas estruturas podem ocorrer em até 3 dias após a completa reepitelização da lesão (Takeo *et al.*, 2015). Ainda pouco se sabe sobre os mecanismos moleculares da neogênese dos folículos pilosos, mas um estudo recente realizado por Myung *et al.*, (2013), mostrou que a expressão de genes, como Wnt10b, Wnt7a, Lef1 e Shh, são necessários para o restabelecimento dos folículos pilosos na pele, e o número de pêlos regenerados, aumentou significativamente em camundongos transgênicos que superexpressam Wnt7a na epiderme.

3.3 Cicatrização e Diabetes

A Organização Mundial de Saúde (OMS) define o *Diabetes mellitus* (DM) como uma síndrome de etiologia múltipla, decorrente da falta de insulina e/ou incapacidade da insulina exercer adequadamente suas ações, caracterizada pela hiperglicemia crônica e alterações no metabolismo dos carboidratos, dos lipídeos e das proteínas. A incidência dessa doença é alarmante com proporções epidêmicas crescentes no mundo inteiro. Sua prevalência em 2010 foi estimada em 5,2% da população global, acometendo mais de 285 milhões pessoas em todo o mundo. O número de pessoas afetadas ainda está em ascensão e estima-se que em 2030 esse número se eleva para 438 milhões com DM (Ighodaro and Akinloye, 2017).

Em doenças como a diabetes, o aparecimento de neuropatia diabética pode levar a ocorrência de ulcerações e o reparo tecidual é prejudicado, levando a cronificação das feridas (Apelqvist, 2012). Segundo Menke e colaboradores (2007), aproximadamente 70% das feridas crônicas são causadas pela isquemia, secundária ao DM, estase venosa e pressão, sendo em sua maioria, nas extremidades inferiores. Os pacientes diabéticos são mais propensos a desenvolver essas úlceras nos pés que não cicatrizam e que representam uma complicação séria do DM e representam um fardo clínico relevante e socioeconômico (Guo e Dipietro, 2010).

As feridas crônicas são definidas como aquelas que falharam na progressão dos estágios normais de cicatrização e, portanto, progridem para um estado de inflamação crônica. Como resultado, o processo de cicatrização é retardado, incompleto, e não procede de maneira coordenada, podendo levar a perda da função do tecido. Essas feridas são caracterizadas pela cronicidade e recorrência frequente (Eming *et al.*, 2014).

As feridas crônicas frequentemente entram em um estado de inflamação crônica (Guo e Dipietro, 2010). Alterações, tanto sistêmicas quanto locais como, por exemplo, atividade proteolítica desequilibrada, predominância de polimorfonucleares e infiltração de macrófagos, devido ao trauma continuado (Menke *et al.*, 2007), contribuem para prolongar a fase inflamatória (Badillo *et al.*, 2007; Silvestre e Lévy, 2006). A persistência de células inflamatórias desempenham um papel importante na geração de citocinas pró-inflamatórias, como o TNF- α , que também promove resistência insulínica (Schurmann *et al.*, 2012) e inibe o PDGF e o TGF- β , suprimindo assim, a atividade mitogênica na ferida diabética (Menke *et al.*, 2007). O aumento da atividade proteolítica (elastase neutrofílica e gelatinase) associado ao aumento de espécies reativas de oxigênio (ROS) leva à

degradação dos fatores de crescimento e proteínas estruturais da matriz extracelular (colágeno), prejuízo na formação do tecido de granulação e reepitelização, crucial para o reparo da lesão (Bitar e Al-Mulla, 2012; Eming *et al.*, 2014).

A hiperglicemia decorrente do DM prejudica a atividade leucocitária o que, por sua vez, também prejudica a cicatrização da ferida (Rathur e Boulton, 2007) e, por conseguinte, a eficácia do tratamento se torna limitada (Badillo *et al.*, 2007). Desse modo, existem diversos fatores fisiológicos que podem contribuir para que o reparo da lesão seja prejudicado nos pacientes diabéticos como, a deficiência na produção do fator de crescimento, da resposta angiogênica, da função dos macrófagos, da produção de colágenos, função da barreira epidérmica, quantidade de tecido de granulação, migração e proliferação de queratinócitos e fibroblastos, número de nervos epidérmicos e cicatrização óssea. Além disso, os níveis altos de MMPs são uma característica de úlceras do pé diabético, de modo que o fluido dessas feridas crônicas apresenta quantidades de MMPs quase 60 vezes maior que em feridas agudas. Este aumento da atividade dessas proteases leva à destruição dos tecidos e inibe o processo cicatricial (Eming *et al.*, 2014).

Nas feridas diabéticas, diversas funções celulares também estão desreguladas, tais como a imunidade de células T deficiente, os defeitos na quimiotaxia leucocitária, capacidade de fagocitose e bactericida, e disfunções de fibroblastos e das células epidérmicas. Estes defeitos são responsáveis pela depuração bacteriana inadequada e atrasada em indivíduos com diabetes (Guo e Dipietro, 2010). A figura 3 ilustra os mecanismos de cronificação das lesões cutâneas.

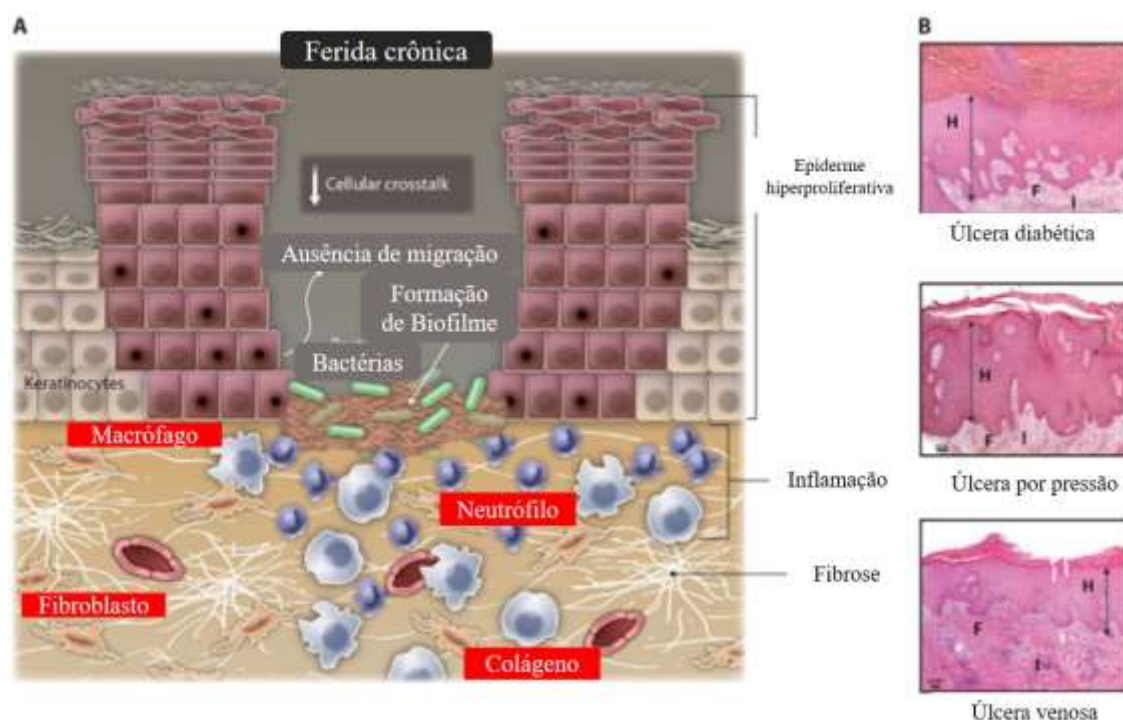


Figura 3: Esquema das principais alterações celulares de feridas crônicas. (A) Ferida crônica apresenta um perfil hiper proliferativo e não migratório da epiderme, inflamação não resolvida. (B) Fotomicrografias representativas de úlceras de pé diabético, estase venosa e úlcera de pressão. Apesar de possuírem etiologia diferente, apresentam características celulares em comum. H = epiderme proliferativa. F = fibrose. I = inflamação. Seta dupla indica o tamanho da epiderme proliferativa. Adaptado de Eming *et al.*, (2014).

3.4 Tratamentos convencionais de feridas cutâneas e o uso de produtos naturais

Embora a reparação tecidual seja um processo sistêmico, é necessário favorecer condições locais através de terapia tópica adequada para viabilizar o processo fisiológico. A terapia tópica de feridas é fundamentada em estudos científicos sobre a fisiologia de reparação tecidual, e é norteado pelos seguintes princípios: remover tecidos necróticos e corpos estranhos do leito da ferida, eliminar processos infecciosos, obliterar espaços mortos, absorver o excesso de exsudato, manter o leito da ferida úmida, promover isolamento térmico e proteger a ferida de traumas e invasão bacteriana (Blanes, 2004).

Sabe-se que alguns tratamentos existentes no mercado podem não ser eficientes dependendo do tipo e local da ferida e, embora existam no mercado inúmeras opções de curativos e tratamentos, a maioria desses produtos possuem custos elevados para o paciente, tendo em vista a duração prolongada de alguns tratamentos (Hampton, 2015).

A utilização de produtos tópicos que auxiliam o processo de reparação tecidual tem grande valor, uma vez que, é por meio da terapia tópica que as condições ideais a cicatrização podem ser alcançadas. Existe no mercado uma diversidade de produtos com essa finalidade, sabe-se da existência de uma gama de aproximadamente 2.000 itens que se destinam ao tratamento de feridas, desde as coberturas mais simples até os mais complexos tipos de curativos, que interferem de forma ativa nas diversas fases do processo cicatricial, dos vários tipos de feridas (Silva *et al.*, 2014).

Os medicamentos utilizados para melhorar a função de cicatrização da ferida utilizam uma variedade de mecanismos diferentes. Estes incluem a manutenção de ambiente úmido (hidrogéis, hidrocolóides, alginatos); o fornecimento de fonte de energia local (maltodextrina, mel, açúcar); a redução do edema da ferida por ação hidrofílica (alginatos, acemanann, mel, açúcar); o aumento do nível de fatores de crescimento (acemanann, complexo tripeptídeo-cobre); o fornecimento de fonte de substância ativa (colágeno, membranas biológicas); o aumento da resposta inflamatória (maltodextrina); o controle da infecção (agentes antimicrobianos); o auxílio no desbridamento (enzimas tópicas); o aumento do teor de oxigênio (oxigênio hiperbárico); e, o aumento do fluxo de sangue (laser, radiação ultravioleta, estimulação elétrica (Krahwinkel e Boothe, 2006).

Um estudo descritivo, exploratório com abordagem quantitativa mostrou o uso da terapia tópica empregada no tratamento de feridas cutâneas por usuários de Unidades Básicas de Saúde da Família (UBSFs) em uma cidade do interior do Estado de Goiás, Brasil. Os resultados apontaram que os produtos tópicos mais utilizados foram os antimicrobianos, como as neomicinas e nistatinas, antisépticos como o Merthiolate® e desbridantes como a collagenase e na classe dos fitoterápicos foi observado o uso da pomada ou creme Pandermin®, composto predominantemente de ácido hialurônico (Silva *et al.*, 2014).

As feridas cutâneas têm impacto significativo na saúde pública e no investimento de recursos públicos, devido ao fato de que as feridas podem levar a algum tipo de deficiência física, psicossocial ou até a morte (Bayat *et al.*, 2003; Guo e Dipietro, 2010). Estima-se que a morbidade associada a feridas não cicatrizadas aumente a cada ano. No Brasil, não há dados oficiais, mas as feridas crônicas afetam em torno de 6,5 milhões de pessoas nos Estados Unidos, atingindo um custo de \$37 bilhões de dólares por ano para o tratamento das lesões, com gasto de aproximadamente \$5.700 por pessoa aos cofres públicos (Gurtner e Chapman, 2016). Além disso, o déficit financeiro relacionado às lesões crônicas representa um problema socioeconômico, havendo a perda de um alto

número de dias de trabalho pelos trabalhadores norte-americanos todos os anos (Sen *et al.*, 2009).

Dessa forma, considerando que existem feridas resistentes às terapias convencionais, pesquisadores e indústrias têm tentado explorar novas estratégias terapêuticas para acelerar a redução das feridas (Rennert *et al.*, 2013), incluindo o uso de plantas e produtos naturais (Gál *et al.*, 2009). As plantas têm sido usadas para o tratamento de várias doenças e problemas de saúde há milhares de anos e a dependência humana dos produtos derivados de plantas para seu tratamento de diferentes afecções é crescente (Ganeshkumar *et al.*, 2012). As plantas são importantes fontes de produtos biologicamente ativos, sendo consideradas um promissor caminho para a descoberta de novos medicamentos, atuando como fitoterápicos ou tendo seus princípios ativos utilizados como matérias-primas de fármacos ou adjuvantes (Simões e Schenkel, 2002). Cerca de 3% dos medicamentos industrializados existentes destinam-se a tratar feridas e outros problemas cutâneos. Entretanto, praticamente um terço das plantas medicinais atualmente utilizadas pelo conhecimento popular destinam-se a estes tratamentos (Mantle *et al.*, 2001). Algumas espécies de plantas ou produtos naturais derivados de plantas vêm sendo testados com sucesso na cicatrização de feridas experimentais em pesquisas recentes (Cetin *et al.*, 2013; Morin *et al.*, 2012; Roy *et al.*, 2012; Tarameshloo *et al.*, 2012; Upadhyay *et al.*, 2013; Ximenes *et al.*, 2013).

3.5 Triterpeno lupeol

Os triterpenos são compostos naturais que em grande parte são derivados de óleos vegetais, cereais e frutas. Existem vários trabalhos descritos na literatura mostrando os potenciais terapêuticos de diversos triterpenos para o tratamento de muitas doenças, incluindo doenças crônicas (Tsai *et al.*, 2016). Na pele, já foram relatados o uso dos triterpenos: asiaticosideo, astragalosideo, bacosideo, cycloastragenol, ginsenosideo, madecossideo, ácido oleanólico e ácido ursólico e lupeol (Agra *et al.*, 2016).

Lupeol (estrutura química mostrada na Figura 4), também chamado de clerodol, farganasterol, fagarasterol, lupeol e monoginol B, pertence à classe de triterpenos do tipo pentacíclico lupano. Esse triterpenóide é encontrado em muitos vegetais e frutas comestíveis, como plantas do gênero aloe, chá preto, raiz de cenoura, pepino, palmeira, gengibre, tomate, polpa de manga, sementes de melão, amoras, ervilhas, grãos de soja, morangos, uvas vermelhas, repolho branco e pimenta (Duke, 1992).

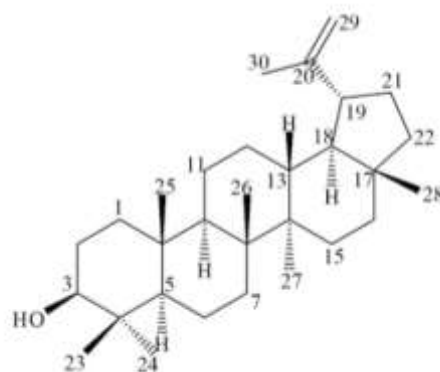


Figura 4: Estrutura química do lupeol

Fonte: Tsai *et al.*, (2016)

Lupeol apresenta diversas atividades biológicas relatadas na literatura, incluindo efeitos antimicrobianos, antioxidantes, anti-inflamatórios, anti-ateroscleróticos, hipotensivos e hipoglicêmicos (Figura 5). Esta eficácia é aplicada contra várias doenças que incluem infecções microbianas, distúrbios relacionados ao estresse oxidativo e inflamatório, artrite, hepatotoxicidade, distúrbios renais e câncer e distúrbios metabólicos, doenças cardiovasculares, diabetes e dislipidemia (Tsai *et al.*, 2016).

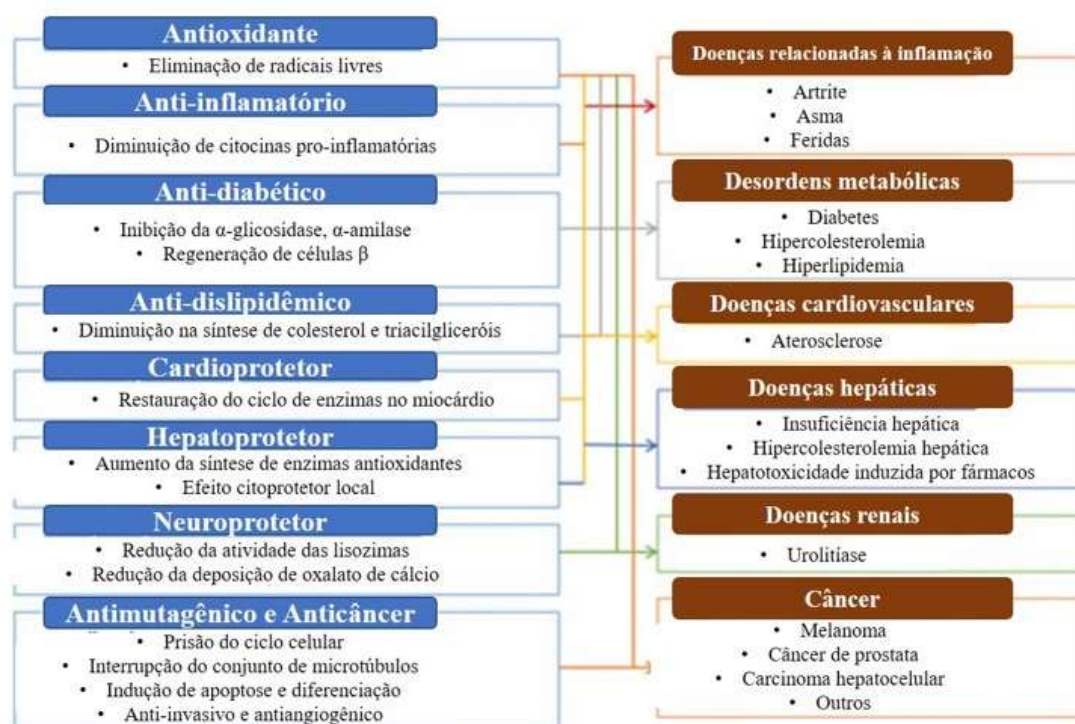


Figura 5: Diagrama representativo das atividades biológicas do lupeol e suas aplicações em doenças crônicas. Fonte: Adaptado de Tsai *et al.*, (2016).

O lupeol, triterpeno pentacíclico do tipo lupano é uma substância que tem se tornada cada vez mais investigada pelos pesquisadores. Alguns estudos mostram a síntese de lupeol por várias rotas, como a ciclização de poliolefinas ou a via sintética enantioseletiva (Surendra e Corey, 2009; Yoder e Johnston, 2005). Como a estrutura do lupeol compreende dez centros assimétricos, isso leva ao desafio estereoquímico e o maior grau de dificuldade nos processos de síntese. Existe uma tendência na obtenção de lupeol a partir de plantas medicinais como composto majoritário, este triterpeno, como a *Crateva nurvala* e *Mangifera indica* L. ou até mesmo de resíduos industriais resultantes do processamento de cortiça (Agarwal e Kumar, 2003; Santiago e Mayor, 2014; Sousa *et al.*, 2006; Yunusov *et al.*, 2006), porque desta forma é menos poluente e mais barato.

Em nosso estudo, utilizamos o lupeol isolado das cascas de *Bowdichia virgiliodes*, popularmente conhecida como sucupira-preta, planta medicinal bastante usada na medicina popular para o tratamento de cicatrização de feridas e outras doenças, a qual vem oferecendo grande contribuição à ciência, devido a uma gama de conhecimentos e práticas médicas de caráter empírico (Agra *et al.*, 2013).

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Efeito do lupeol sobre queratinócitos e fibroblastos humanos

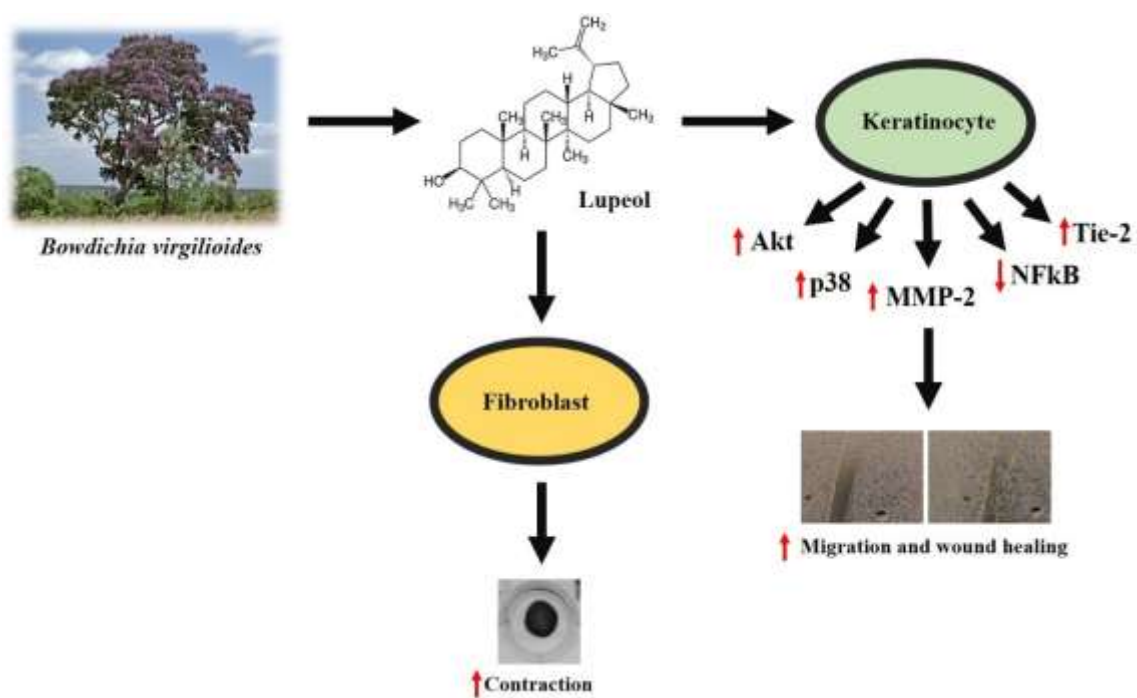
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Capítulo II

Graphical abstract



Article

Lupeol, a pentacyclic triterpene promotes migration, wound closure and contractile effect *in vitro*: possible involvement of PI3K/Akt and p38/ERK/MAPK pathways.

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Abstract: Skin wound healing is a dynamic and complex process involving several mediators at the cellular and molecular level. Lupeol, a phytoconstituent belonging to the class of triterpenes, is found in several fruit plants and medicinal plants which has been the object of study in the treatment of various diseases, including the skin wounds. Various medicinal properties of Lupeol can be found in the literature, such as anti-inflammatory, antioxidant, anti-diabetic and anti-mutagenic effects. We investigated the effects of Lupeol (0.1, 1, 10 and 20 µg/mL) on *in vitro* wound healing assays and signaling mechanisms in human neonatal foreskin keratinocytes and fibroblasts. Results showed that, at high concentrations, Lupeol reduced cell proliferation of both keratinocytes and fibroblasts, nonetheless, it increased *in vitro* wound healing in keratinocytes and promoted the contraction of dermal fibroblasts in collagen gel matrix. This triterpene positively regulated matrix metalloproteinase (MMP)-2 and inhibited the NFκB expression in keratinocytes, suggesting it has an anti-inflammatory effect. Lupeol also modulated the expression of keratin 16 according to the

concentration tested. Additionally, in keratinocytes, Lupeol treatment resulted in the activation of Akt, p38 and Tie-2, which are signaling proteins involved in cell proliferation/migration, angiogenesis and tissue repair. These findings suggest that Lupeol has therapeutic potential for accelerating wound healing.

Keywords: lupeol; keratinocytes; fibroblasts; wound healing; cell migration.

1. Introduction

The skin is one of the largest organs of the human body delimiting its limit with the external environment whose main functions are protection against external agents, thermoregulation and perception [1]. The breakdown of skin integrity caused by injury resulting from a variety of events such as trauma, heat or cold exposure, burns, or blood circulation problems which may render the patient even more susceptible to acute or chronic infections, electrolyte imbalance, and fluid loss [2]. In more severe cases, limb amputation may be necessary. Accelerating the wound healing process will assist in circumventing these complications.

Wound healing is a highly complex physiological process occurring ordered events classified into three phases that overlap: hemostasis and inflammation (inflammatory phase), granulation tissue formation and re-epithelialization (proliferative phase) and wound contraction and tissue remodeling (extracellular matrix remodeling phase) [3]. This is a very natural phenomenon that occurs through responses and interactions cellular and molecular with the main objective of reconstituting and restoring the integrity of the injured tissue [4]. During cutaneous wound healing process, re-epithelialization via keratinocyte proliferation and migration and (myo)fibroblasts contraction result in wound closure [5].

Since ancient times, there have been reports of the use of medicinal plants as a therapeutic alternative for various diseases in many countries, including developing countries. The use of plant extracts or its isolated products for acute

and chronic wounds and burns has been well described in the literature. Many of these extracts or plant-derived compounds to treat wounds contain pharmacological properties essential for injury repair, such as anti-inflammatory and antioxidants [6]. Curcumin, a polyphenol from the rhizomes of *Curcuma longa*, has shown antioxidant properties in several studies for the treatment of many diseases, including skin disorder. Topical applications of curcumin have been widely used as a research tool in the cutaneous wound healing. In a study by Manca et al. performed *in vitro* and *in vivo* with curcumin-loaded nanovesicles showed antioxidant effect on keratinocytes by reducing oxidative stress and anti-inflammatory effect through decreasing myeloperoxidase activity, edema formation and providing the stimulus of cutaneous re-epithelialization [7]. Similarly, quercetin is another well-known natural compound belonging to the class of flavonoids with strong antioxidant properties. A recent study developed from a multiphase system containing quercetin-loaded liposomes showed a significant reduction of lesion area in rats and confirmed its great potential in the skin wounds treatment [8].

In addition to phenolic compounds and flavonoids, it is known that many plant-derived secondary metabolites with antioxidant and anti-inflammatory properties, including the triterpenes, are capable of promoting these healing effects on *in vivo* and *in vitro* models [9-11]. Triterpenes chemically belong to the class of isoprenoids distributed widely in various parts of the plant, from the root to the fruits. Several studies have shown that triterpenes from medicinal plants improve the quality of healing through mechanisms ranging from the regulation of pro- and anti-inflammatory mediators, chemokines, growth factors, inducing the formation of granulation, re-epithelialization and wound contraction [12-15].

Bowdichia virgilioides (Fabaceae), is a medicinal plant widely known as "sucupira-preta" predominant from the Brazilian cerrado, found mainly in the north, northeast and central regions of the country. In popular medicine, there are reports of the use of their barks and seeds in the form of infusion to treat

various diseases, such as rheumatism, diabetes, bronchitis and skin wounds [16]. Phytochemical studies revealed that the bark and root contain a large number of alkaloids and terpenoids, such as lupeol, [17,18] volatile constituents and flavonoids [19,20] and anthocyanins [21].

Lupeol is a pentacyclic triterpene found in various species of the plant kingdom, including some vegetables and fruits, such as cucumber, tomato, white cabbage, fig and guava [22,23]. This molecule exhibits a spectrum of pharmacological activities against various acute or chronic diseases, including arthritis, arthritis, renal disorders, diabetes, hepatotoxicity, cardiovascular disease, cancer and microbial infections [24-27].

In this study we aimed to explore the effects of the triterpene lupeol on skin wound healing *in vitro* via investigation of the proliferation, migration and cell contraction as well as its signaling mechanisms involved therein using human keratinocytes and fibroblasts.

2. Results

2.1. High concentrations of lupeol decrease the proliferation and cause cytotoxicity in keratinocytes and fibroblast

Human keratinocytes or fibroblasts were treated with lupeol at various concentrations ranging from 0.1-20 $\mu\text{g/mL}$ before cell proliferation and viability were assessed by crystal violet and MTT assay, respectively. The results showed that lupeol at 10 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$, significantly inhibited keratinocyte proliferation after 24 hours treatment, by 53% and 64%, respectively (Figure 1A). Lupeol at 1 $\mu\text{g/mL}$ increased fibroblast proliferation significantly by 12%, while the higher concentration (20 $\mu\text{g/mL}$) inhibited cell proliferation in relation to control (19%) (Figure 1B). On the other hand, this triterpene did not affect keratinocyte viability, but showed cytotoxicity to fibroblasts at the higher concentration (20 $\mu\text{g/mL}$) (Figures 2A,B).

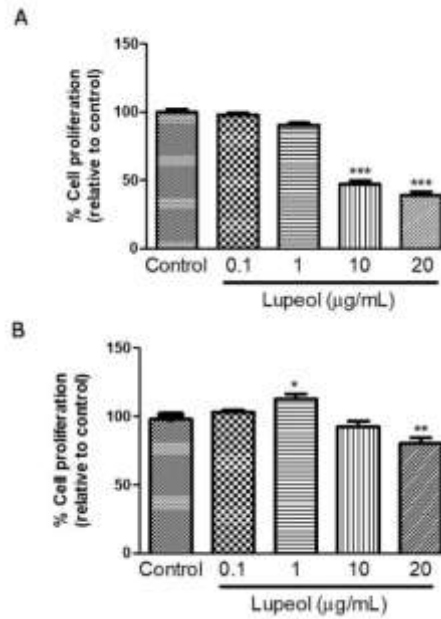


Figure 1. Cell proliferation of epidermal keratinocytes (A) and dermal fibroblasts (B) in response to lupeol. Cells were seeded in a 96-well plate and after overnight attachment, different concentrations of lupeol were added, and the cells were left for 24 hours at 37°C. Cell proliferation was measured by crystal violet assay and calculated by a comparison of the values from the lupeol treatment group with the control group. Data are expressed as mean $\pm$ standard error of the mean (S.E.M). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control group.

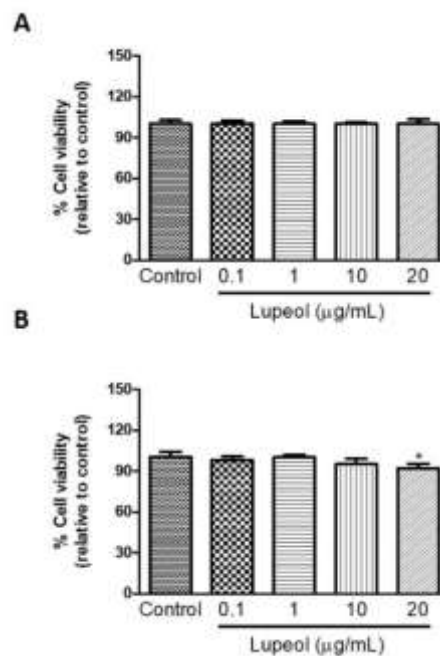


Figure 2. The viability of epidermal keratinocytes (A) and dermal fibroblasts (B) in response to lupeol. Cells were seeded in a 96-well plate and, after overnight attachment, different concentrations of lupeol were added, and the cells were left for 24 hours at 37°C. Cell viability was measured by MTT assay and calculated by a comparison of the values from the lupeol

treatment group with the control group. Data are expressed as mean $\pm$ standard error of the mean (S.E.M). * $p < 0.05$ versus control group.

2.2. Lupeol enhances migration and wound closure in human epidermal keratinocytes

The scratch wound healing assay revealed that lupeol (0.1 and 1 $\mu\text{g/mL}$) significantly increased the wound closure rate compared to the control after 24 hours (Figure 3A). The cells treated with the lowest concentration of lupeol, 0.1 $\mu\text{g/mL}$, showed a 59% increase in wound closure rate compared to control ($p < 0.001$). Lupeol at 1 $\mu\text{g/mL}$ also showed a potent wound healing effect on epidermal keratinocytes by 39% ($p < 0.05$). When used at the higher concentrations, lupeol did not cause any significant change wound closure rate.

We further examined whether lupeol stimulates migration *in vitro*. Scratch migration of human keratinocytes treated with lupeol showed a significant increase in migration at all concentrations tested (0.1 $\mu\text{g/mL}$, 93%, $p < 0.001$; 1 $\mu\text{g/mL}$ 96%, $p < 0.001$; 10 $\mu\text{g/mL}$ 94%, $p < 0.001$ and 20 $\mu\text{g/mL}$ 83%, $p < 0.05$) compared to the control group. Although, the higher concentrations are able to inhibit cell proliferation, as shown in Figure 1, these concentrations showed potent increase in epidermal keratinocytes cell migration (Figure 4B).

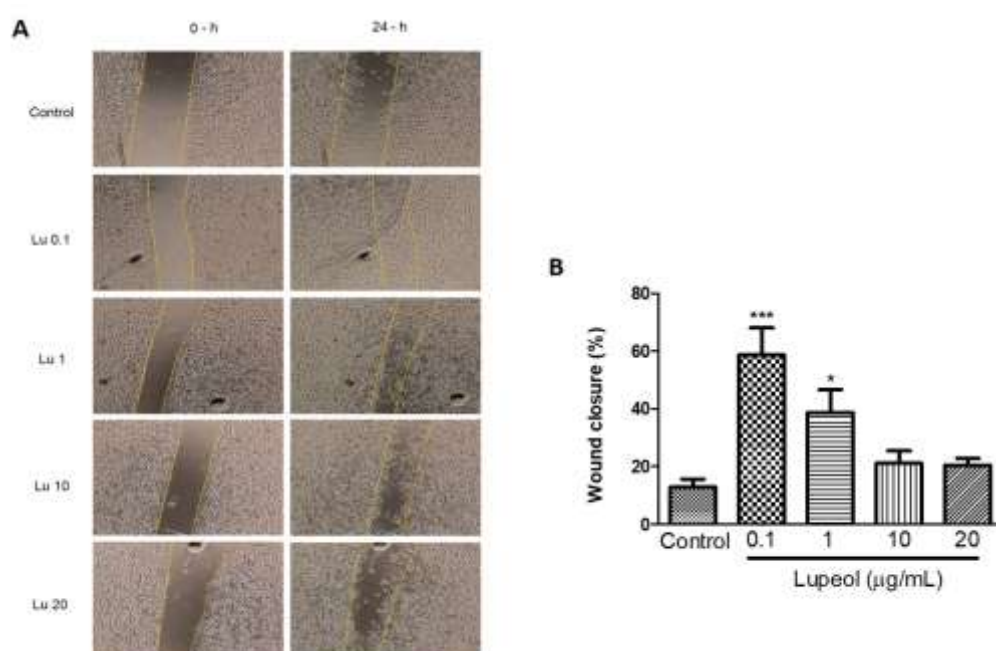


Figure 3. Wound healing effect of lupeol on human epidermal keratinocytes in the scratch assay after 24 hours of incubation. Cells were incubated with lupeol at concentrations of 0.1, 1, 10, 20 $\mu\text{g/mL}$ or DMSO at 10 $\mu\text{g/mL}$, as negative control. (A) Representative images of scratch assay at 0 and 24 hours. Lu: lupeol. (B) Dose-response effect of lupeol on wound closure. Data are expressed as mean $\pm$ standard error of the mean (S.E.M) * $p < 0.05$ and *** $p < 0.001$ versus control group.

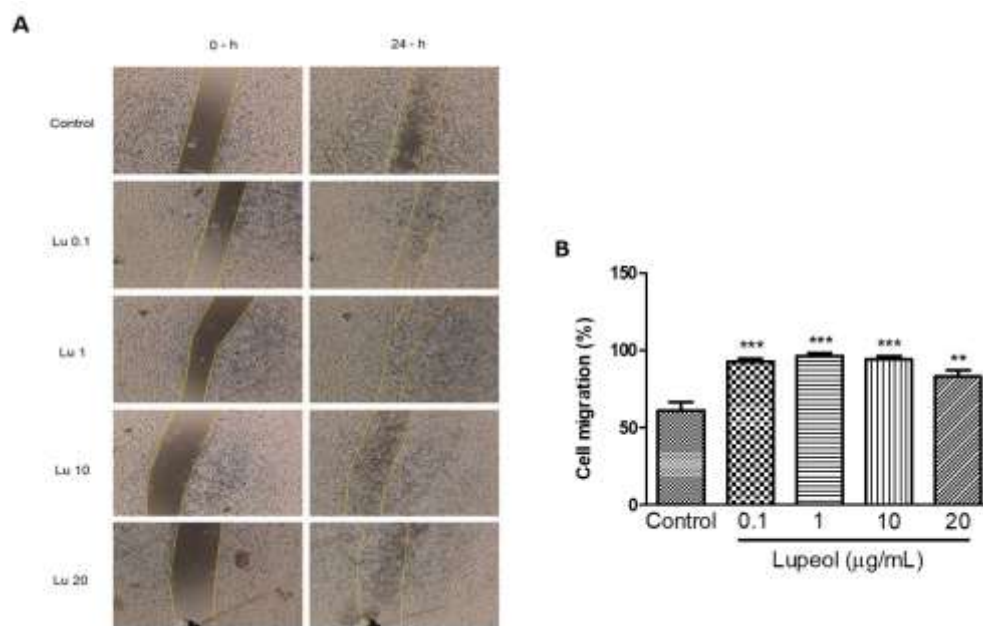


Figure 4. Keratinocyte migration using a scratch assay after 24 hours of incubation with lupeol. Cells were incubated with lupeol in different concentrations at 0.1, 1, 10, 20 $\mu\text{g/mL}$ or DMSO 0.25% at 10 $\mu\text{g/mL}$ as negative control. (A) Representative images of scratch assay at 0 and 24 hours. Lu: lupeol. (B) Effect of lupeol on migration cell. Data are expressed as mean $\pm$ standard error of the mean (S.E.M). ** $p < 0.01$ and *** $p < 0.001$ versus control group.

2.3. Lupeol promotes contractile effect on a collagen gel matrix

To obtain additional information about wound healing mechanisms, we assessed the ability of lupeol to contract a collagen gel matrix containing dermal fibroblasts (Figure 5A). Collagen gel assays provide a convenient platform to investigate a cell's contractile ability that closely represent cell behavior *in vivo*. The results showed lupeol significantly increased the contractile effect on collagen gels at 1 $\mu\text{g/mL}$ ($p < 0.01$), 10 ($p < 0.01$) and 20 $\mu\text{g/mL}$ ($p < 0.01$) compared to the control after 48 hours of treatment (Figure 5B).

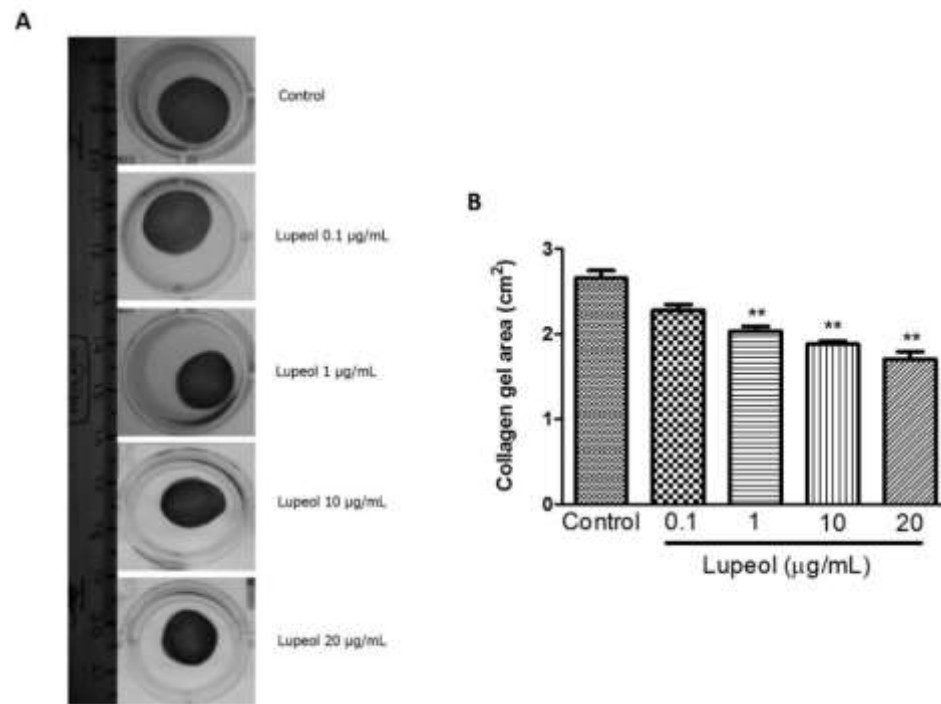


Figure 5. Contractility of lupeol on human fibroblasts. Fibroblasts were cultured within 3D-collagen matrix incubated with lupeol at concentrations of 0.1, 1, 10, 20 µg/mL or DMSO 0.25% at 10 µg/mL as negative control. The gels were carefully released from the side wall of the wells and the contractile activity of lupeol was quantified after 48 hours of treatment. (A) Representative images of collagen matrix at 48 hours. (B) Semi-quantified results of contractility of lupeol on fibroblasts, expressed as a mean $\pm$ standard error of the mean (S.E.M). ** $p < 0.01$ versus control group.

2.4. Lupeol regulates via PI3K/Akt and MAPK pathways

To identify which signaling pathways are involved in the regulation of human keratinocyte migration promoted by lupeol, we investigated the respective roles of PI3K/Akt, Tie-2, MAPK/ERK/p38, NFκB and MMP-2 in lupeol-accelerated keratinocyte migration.

As shown in Figures 6A,B, 1 µg/mL lupeol treatment sharply raised the activity of Akt compared to control ($p < 0.001$). Numerically smaller but significant increases in Akt were observed at all other concentrations (Figure 6B). Lupeol also increased Akt phosphorylation at all concentrations tested (Figure 6C). There was no change in the levels of ERK or p-ERK line response to lupeol (Figures 5D,E).

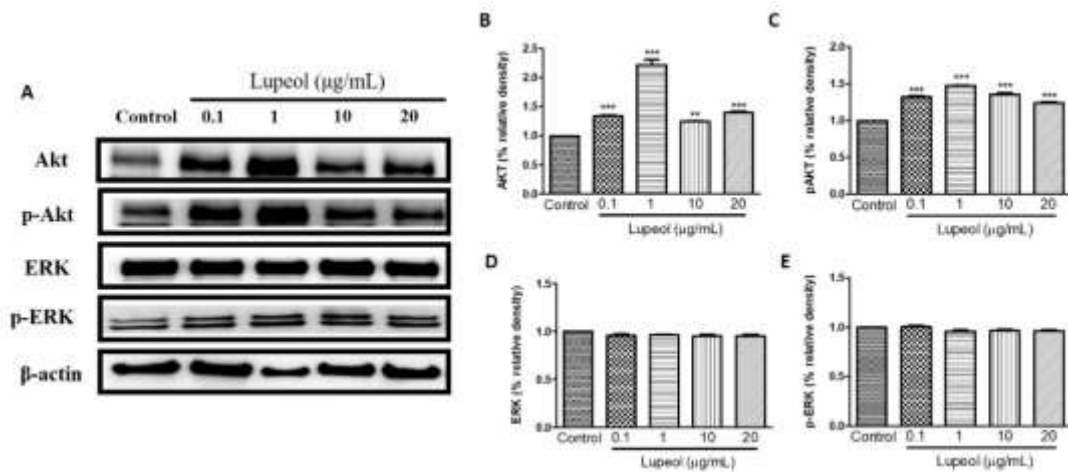


Figure 6. Lupeol regulates the expression of Akt and ERK in epidermal keratinocytes. (A) Western blot showing expression of Akt, p-Akt, ERK and p-ERK in keratinocytes after 24-hours treatment with lupeol in different concentrations. (B-E) All the proteins were semi-quantitated using Image J software and normalized to the levels of β -actin. Data are expressed as mean $\pm$ standard error of the mean (S.E.M). ** $p < 0.01$ and *** $p < 0.001$ versus control group.

The level of p-p38 and p38 proteins expressed by keratinocytes showed significant changes after treatment with lupeol. A significant increase in p38 was observed in cells treated with lupeol at 1 ($p < 0.05$), 10 ($p < 0.001$) and 20 $\mu\text{g/mL}$ ($p < 0.001$) compared to the control (Figure 7B). p-p38 showed a dose-dependent significant increase in response to all concentrations of lupeol (Figure 7C). MMP-2 activity was significantly enhanced after treatment with 1, 10 and 20 $\mu\text{g/mL}$ of lupeol (Figure 7E). The effect of treatment with lupeol on the expression of pro-inflammatory mediator, NF κ B, was also evaluated. As shown in Figure 7D, lupeol at concentrations of 10 and 20 $\mu\text{g/mL}$ significantly decreased the expression of NF κ B compared to the control.

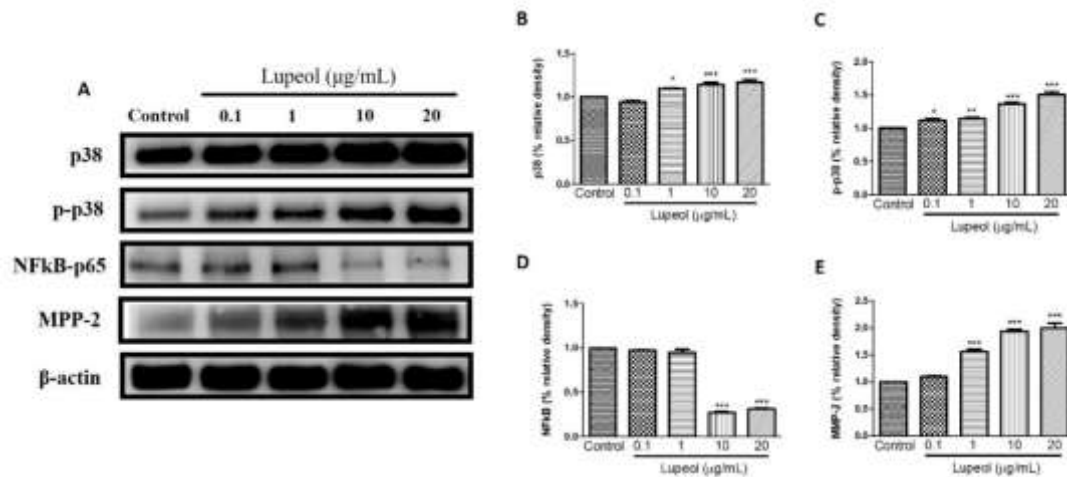


Figure 7. Lupeol regulates the expression of p-38, NFκB-p65 and MMP-2 in epidermal keratinocytes. (A) Western blot showing expression of p38, p-p38, NFκB-p65 and MMP-2 in keratinocytes after 24-hours treatment with lupeol in different concentrations. (B-E) All the proteins were semi-quantitated using Image J software and normalized to the levels of β-actin. Data are expressed as mean ± standard error of the mean (S.E.M). *p<0.05, **p<0.01 and ***p<0.001 versus control group.

We examined the influence of lupeol treatment on Tie-2 expression, a protein-tyrosine kinase receptor expressed by endothelial and epithelial cells which functions to stabilize the cell barrier. Treatment with lupeol showed a dose dependent decrease in protein expression of Tie-2, and concomitantly there was a corresponding increase in the levels of its phosphorylated isoform, p-Tie-2, clearly indicating that lupeol activates Tie-2 (Figure 8C).

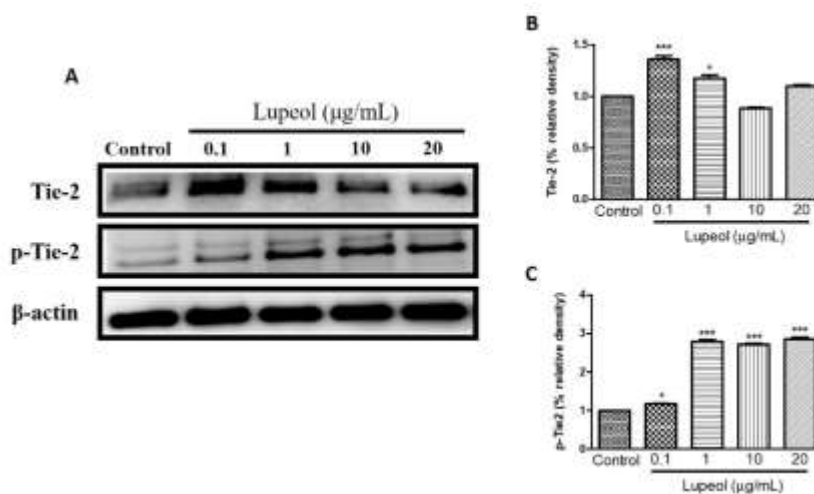


Figure 8. Lupeol regulates the expression of Tie-2 in epidermal keratinocytes. (A) Western blot showing expression of Tie-2 and p-Tie-2 in keratinocytes after 24-hours treatment with lupeol in different concentrations. (B,C) All the proteins were semi-quantitated using Image J software and normalized to the levels of β -actin. Data are expressed as mean $\pm$ standard error of the mean (S.E.M). * $p < 0.05$ and *** $p < 0.001$ versus control group.

2.5. Lupeol regulates the differentiation of cytokeratin 16

To understand the effect of lupeol on the differentiation of keratinocytes, we investigated keratin 16 expression in keratinocytes after 24-hours treatment. As shown in Figures 9A,B, 0.1 $\mu\text{g/mL}$ lupeol treatment increased the keratin 16 compared to control ($p < 0.001$). Significant decreases in keratin 16 were observed with lupeol at 1 and 10 $\mu\text{g/mL}$, with no change in response to 20 $\mu\text{g/mL}$.

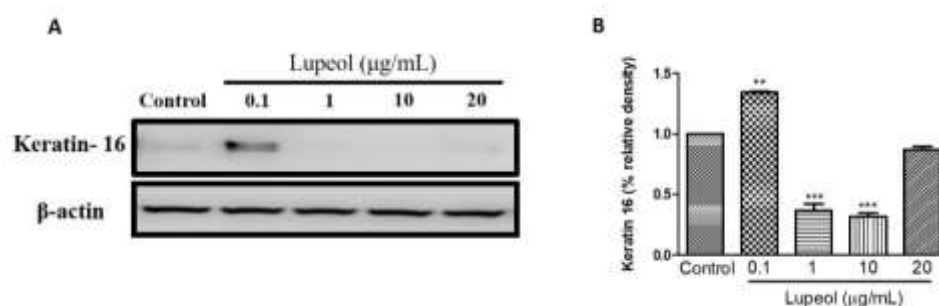


Figure 9. Lupeol regulates the expression of keratin 16 in epidermal keratinocytes. (A) Western blot showing expression of keratin 16 in keratinocytes after 24-hours treatment with lupeol. (B) All the proteins were semi-quantitated using Image J software and normalized to the levels of β -actin. Data are expressed as mean $\pm$ standard error of the mean (S.E.M). ** $p < 0.01$ and *** $p < 0.001$ versus control group.

3. Discussion

Lupeol, a natural compound isolated from *B. virgiliodes*, is known to play protective roles in a variety of cancers, skin inflammation, pancreatitis, arthritis, diabetes, hepatic and cardiovascular diseases [28-34], but little is known about its effects on *in vitro* wound healing. In this study, we demonstrate that lupeol enhances *in vitro* wound healing, possibly by stimulating the survival, migration and contraction of epidermal keratinocytes and/or dermal fibroblasts.

Wound healing is a highly complex biochemical and physiological process involving various cellular and molecular mediators, such as chemokines and

cytokines, inflammatory infiltrate, immune system cells, growth factors, and extracellular matrix proteins [2]. Keratinocyte proliferation and migration are crucial events in the wound repair. These cells migrate toward the wound surface and fill the wound area to cover the open space exposed to infections and thereby contribute to the wound healing effect [5,35]. Keratinocyte migration may occur independently of keratinocyte proliferation during re-epithelialization [36]. It is known that this cell mechanism is associated with the formation and disassembly of cell adhesion sites and cytoskeletal reorganization [35]. Using an *in vitro* migration assay, which does not involve cell proliferation, we found that lupeol had a strong effect at all concentrations, indicating that lupeol may accelerate cutaneous wound repair by stimulating the migration of keratinocytes. In contrast, we found that lupeol at low concentrations (0.1 and 1 $\mu\text{g/mL}$) exhibited a potent wound healing effect *in vitro*, a process where both proliferation and migration are involved. Our study also demonstrated that lupeol, at high concentrations, inhibited cell proliferation in both cell types and stimulated the cytotoxicity in fibroblasts. Taken together, these results suggest that cell migration, rather than cell proliferation is the more prominent pathway for lupeol to promote cutaneous re-epithelialization and wound repair in keratinocytes.

After re-epithelialization and extracellular matrix reorganization, wound contraction is an important event during the wound healing process. Our collagen gel contraction assay showed that lupeol treatment stimulated the contractile effect of fibroblasts embedded in a collagen gel solution in a dose-dependent manner. This culture system has been widely used as a model for pro-contraction remodeling of the extracellular matrix by dermal fibroblasts in situations such as wound healing [37]. This fibroblast embedded collagen gels system allows cells to develop endogenous tension through both mechanical and biochemical signals relevant for cell contractility, thus in turn triggering a signal transduction cascades capable of modulating transmembrane and/or

intracellular receptors to promote intracellular responses such as gene expression and protein synthesis [38].

It has been reported that activation of the PI3K/Akt pathway triggers mechanisms responsible for cell polarity capable of influencing the migration speed and thus leading to the migratory activity of various cell types, including keratinocytes and fibroblasts [39,40]. In addition, PI3K/Akt is a pathway that has also been implicated as important mediator in the control of survival/cell growth, malignant and metastatic oncogenic transformation, and the regulation of various diseases [41]. In our study, protein expression performed by western blot clearly showed that lupeol significantly up-regulated Akt expression and activation in epidermal keratinocytes, suggesting that Akt may be a key signaling component in lupeol-induced keratinocyte migration and wound healing.

MAPK signaling is also an important pathway formed by a chain of proteins in the cell, which is responsible for modulating the synthesis and release of growth factors involved in cell proliferation and migration during wound healing [42]. The MAPKs consist of extracellular signal-regulated protein kinase (ERK), c-Jun NH₂-terminal kinase (JNK) and p38 mitogen-activated protein kinases [43]. ERK and p38 are crucial signaling molecules in wound healing. ERK plays a crucial role in the regulation of migration, proliferation, differentiation and cell survival [44]. Although the role of the p38 MAPK signaling pathway in wound healing has not yet been totally elucidated, recent studies have suggested its involvement in the migration of keratinocytes to wounds [45]. In this study, lupeol did not significantly change ERK expression or activation, but increased the p38 activation in a concentration-dependent manner. In that way, we hypothesize that lupeol-induced migration and wound closure in human keratinocytes occurs through activation of p38 MAPK.

Inflammation is a crucial event characterized by the infiltration of inflammatory cells into the injured tissue. Successful tissue regeneration is directly related to resolution of the inflammatory phase, since low recruitment of

inflammatory cells is associated with delayed healing and excessive inflammation can result in chronic wounds making it difficult to repair formation of the scar. NF κ B pathway plays a key role in controlling the expression of various inflammatory genes including TNF- α , cell adhesion molecules such as E-selectin and vascular cell adhesion molecule-1 [46]. This protein complex is located within the cytoplasm as a pair of dimers (p50/p65) and once activated by inflammatory stimuli, including surgical lesions, NF κ B dimers migrate to the nucleus of the cell binding to the molecules of DNA, and thereby inducing the transcription of a variety of genes responsible for proliferation, migration, cell cycling, inhibition of apoptosis and most importantly inflammation [47,48]. In our study, high doses of lupeol suppressed the activation/expression of NF κ B in keratinocytes, thus contributing to the dampening of the inflammatory process in cutaneous wounds.

Another interesting finding in this study was that lupeol significantly increased the expression of MMP-2 in a concentration-dependent manner in human keratinocytes. MMPs present in keratinocytes are enzymes involved not only in the modulation of inflammation but also in the proliferative phase, in the cell proliferation/migration control and also contribute to skin barrier function and extracellular matrix remodeling [49,50]. MMP-2 in particular has strong anti-inflammatory properties [50]. Our finding showed that lupeol induced the expression of MMP-2 in keratinocytes, provides a possible explanation for the cells' increased cell migration and reduced inflammation.

Angiogenesis is a process regulated by complex mechanisms involving the proliferation and migration of endothelial cells, maturation and formation of a new basement membrane and finally the creation of new blood vessels from those existing ones [51-53]. Tie-2, a protein-tyrosine kinase cell surface receptor, plays an important role during angiogenesis. Studies have shown that both Tie-2 and its activated form, p-Tie2, are present on neonatal foreskin and adult skin epidermis [54]. Major ligands are angiopoietin 1 and 2 proteins with similar

binding affinity and its activation by Ang-1 in endothelial cells is able to enhance local vascularization by promoting blood vessels resistance and stability, and mediating angiogenesis by VEGF activating [55]. Lupeol treatment caused an increase in Tie-2 expression in human epidermal keratinocytes. Similarly, p-Tie2 was expressed in keratinocytes treated with lupeol. These findings support Tie-2 as an essential signaling component in lupeol-induced wound healing in human keratinocytes.

The epidermal layer of the skin is composed of keratinocytes in various stages of cell differentiation. After a damaging stimulus, keratinocytes are able to express different types of keratin proteins, different from those present in the healthy epidermis [56]. Cytokeratin 16 is rarely expressed under normal skin conditions, but may be found in oral mucosa and nails. This cytokeratin has a crucial role as a stimulatory keratin in skin diseases, especially in psoriasis, hypertrophic scars, other inflammatory conditions and several types of cancers including the squamous cell carcinomas [57,58]. It is important to note that reduced expression of keratin 16 contributes to the re-organization of important organelles of the cell, such as the cytoskeleton, which can directly affect the migration of keratinocyte [56]. Our study showed that Lupeol treatment is capable of up-regulating and down-regulating the keratin 16 expression according to the concentration tested.

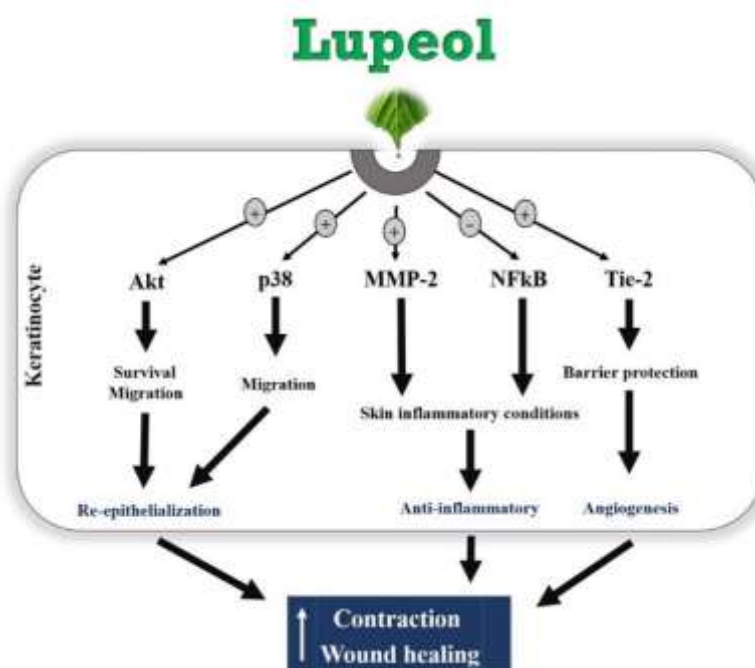


Figure 10: Hypothetical model of the regulatory mechanisms of lupeol in human keratinocytes on wound healing.

4. Materials and Methods

4.1. Plant material, extraction and isolation of lupeol

Bowdichia virgilioides Kunth. (stem bark) was collected in December 2014 in the surroundings of Santa Rita, State of Paraíba, Brazil, a coastal area around the Atlantic Forest. A Voucher specimen (*Agra et Góis* 6243) was deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), and in the reference collection of the Laboratory of Pharmaceutical Technology from Federal University of Paraíba, João Pessoa, Brazil. Three kg of air-dried ground stem bark of *Bowdichia virgilioides* were exhaustively extracted with 95% alcohol solution. The extracted solution was filtered and the solvents were subjected to the evaporation method under reduced pressure with rotary evaporation at 40°C to obtain the final ethanolic extract (tHE, 250g). The EtOHE was partitioned using solvents in increasing polarity (hexane, chloroform and methanol). The hexane residue (49g) was subjected to repeated washings with acetone under stirring followed by

filtration. The solid obtained was recrystallized from chloroform and hexane, resulting in white crystals which were performed by analyzing ^1H and ^{13}C NMR spectral data, compared with those published in the literature [59] and identified as lupeol (Figure 11) substance (3g). The comparison of ^1H and ^{13}C -NMR data of lupeol isolated and ^{13}C -NMR lupeol literature [60] are presented at Table 1 (Appendix A). A stock solution of lupeol (10 mg/mL) was prepared by dilution in DMSO (dimethyl sulfoxide) and alcohol. We defined as standard protocol for all treatments the final concentration of DMSO and alcohol 0.25 and 0.075%, respectively, which did not show cytotoxicity in previous reports [61].

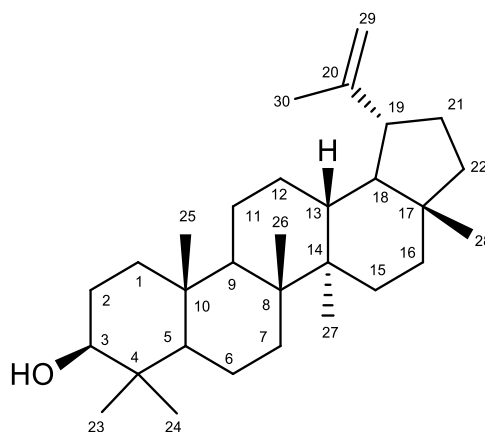


Figure 11: Chemical structure of lupeol

4.2. Cell isolation and culture

Human primary epidermal keratinocytes and dermal fibroblasts were obtained from human neonatal foreskins following the standard protocols of the local ethics committee [62]. Keratinocytes were cultured in specific culture medium (Keratinocyte serum-free medium, K-SFM) and fibroblasts were cultured in Duplecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units penicillin/streptomycin (Gibco-Life Technologies, Grand Island, NY). All cells were plated and incubated at 37°C in a 5% CO_2 atmosphere and the culture media were changed three times per week. After reaching 70% confluency, the cells were trypsinised and seeded on specific

plates for each analysis and incubated for 12 hours prior to each experimental procedure.

4.3. Cell proliferation assay

Cell proliferation test was performed using the crystal violet assay. Cells were cultured to confluency and seeded in 96-well microplates (1×10^3 cells/well) and after 24 hours of incubation, the cells were treated in the absence or presence of lupeol at different concentrations (0.1, 1, 10 or 20 $\mu\text{g/mL}$). After 24 hours of the lupeol treatment, the cells were stained for 15 minutes with a solution of crystal violet (1 $\mu\text{g/mL}$) and subsequently washed with distilled water to remove any unbound dye residue. After complete drying of each well, 0.1% sodium dodecyl sulfate (SDS) buffer diluted in PBS was added. The absorbance was read at 570nm using a microplate reader.

4.4. Cytotoxicity assay

Cell viability was assessed using MTT-based metabolic assay. Cells were cultured to confluency, and switched to DMEM with 0.1% BSA for fibroblasts and adult keratinocyte growth medium without supplements for 24 hrs. MTT was added 4 hours prior to completion of experiments. The absorbance was read at 570nm using a microplate reader. Final results were expressed as percentages of controls.

4.5. In vitro migration ("scratch") assay

Cell migration was assessed by "scratch" assay. Keratinocytes were seeded in 24-well plates and cultured to confluency. After 24 hours of incubation, a pretreatment with mitomycin (10 $\mu\text{g/mL}$) was performed to avoid any influence of cell proliferation [63]. Cells were scratched with a 100 μL -blue plastic pipette tip after 2 hours of pre-treatment with mitomycin and thereby creating a cell-free area measuring approximately 2 mm in width and photographed under phase-contrast microscopy. Cells were then immediately treated with lupeol at 0, 0.1, 1,

10 or 20 µg/mL concentrations and after 24 hours new photographs were taken. The analysis was performed by counting cells that had moved from the initial area. The percentage of cell migration was calculated by the following formula [number of moved cells after lupeol treatment/number of moved cells in the control condition] × 100.

4.6. *In vitro* wound healing ("scratch") assay

In vitro wound healing test was also evaluated by "scratch" assay. Keratinocytes were cultured to confluency using 24-well plates and incubated for 24 hours. The same procedure performed for scratch induction, as shown in section 4.5. was also used in this method, except that mitomycin C was not added. Photographs were taken immediately after scratch and the cells were then treated under the same conditions performed in the cell migration test. After 24 hours, new photographs were taken to analyze through the images the wound area remaining. The wound closure rate was determined by the initial and final wound areas during the wounding induction and wound closure percentage calculated by the following formula: [(initial - final) / initial] × 100.

4.7. *Collagen gel contraction assay*

Collagen gel contractility test was performed using a collagen solution prepared following manufacturer's protocols. Dermal fibroblasts were cultured to confluence before being embedded in a 3D collagen matrix to measure their long-term contractility. In that way, fibroblasts were added to the collagen gel solution and seeded in 24-well plates at a density of 1.7×10^5 cells/mL in each well. After 1-hour incubation to allow the collagen solution to gel, DMEM was added to each well and incubated for overnight. After the incubation, three washes with PBS were performed to remove serum and DMEM containing 0.5% of FBS was added. Cells were then treated with lupeol at 0.1, 1, 10 or 20 µg/ml concentrations and gel attachment around the side wall of the well was carefully released with

a spatula, immediately after treatment and the contractile activity was evaluated after 48 hours of treatments for each well using ImageJ™ software.

4.8. Cell lysate preparation and Western blot analysis

Keratinocytes with required confluence were pre-incubated with 0.1–20 µg/mL lupeol for 24 hours. After 24-hours incubation, cells were then lysed in ice-cold lysis buffer supplemented with protease inhibitors and phosphate inhibitors and centrifuged at 12,000×g for 5 min. An aliquot of cell lysate was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking in a 5% skim milk powder solution, the membranes were incubated with B-actin, phosphorylated (P) forms of p38 (Tyr182) and p38 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) phospho-Akt (Ser473), Akt, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), p44/42 MAPK (Erk1/2), Tie2 (D9D10), phospho-Tie2 (Tyr992), NFκB-p65 (Ser536), MMP-2 (Cell Signaling Technology, Beverly, MA, USA) and Keratin 16 (Thermo Fisher Scientific) overnight at 4°C. The next day, all membranes were washed and incubated in secondary antibody for 1 hour at room temperature. Proteins were detected by the ECL detection system, electrochemiluminescence (Amersham Biosciences) and analyzed by Image J software.

4.9. Statistical analysis

All data are expressed as the mean ± standard error of the mean (S.E.M.). Statistical significance was performed by one-way ANOVA followed by Tukey's test for all analyzes. All data were carried out using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and $p < 0.05$ was defined to be statistically significant.

5. Conclusions

In conclusion, the results of this study demonstrated the involvement of lupeol in the closure of the skin wound through the stimulation of migration of

keratinocytes and increased contraction of fibroblasts embedded in a collagen matrix. The underlying mechanism for the positive effect of lupeol on wound healing may involve the activation of PI3k/Akt and p38 MAPK, suppression of NFκB signaling and Keratin 16, as well as the cyto-protective effects of MMP-2 and Tie-2. Although these results provide important information for lupeol in promoting wound healing *in vitro*, further *in vivo* and clinical studies are required to explore these and other pathways, as well as to develop lupeol as a therapeutic potential in the treatment of cutaneous wounds.

Author Contributions: Fernando Pereira Beserra, Meilang Xue and Christopher Jackson designed the experiments. Fernando Pereira Beserra and Meilang Xue performed the experiments. Christopher Jackson and Meilang Xue helped interpreting the results. Fernando Pereira Beserra, Meilang Xue, Christopher Jackson, Cláudia Helena Pellizzon, Ariane Leite Rozza and Gabriela Lemos de Azevedo Maia contributed to the writing and the revision of this manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table 1. ¹H and ¹³C- NMR (1D, 2D) spectra data for lupeol isolated from the *B. virgilioides* (400 MHz, CDCl₃) and ¹³C-NMR data lupeol.

Lupeol (isolated)						Lupeol (literature)
C/H	$\delta^{13}\text{C}$ /ppm	DEPT	$\delta^1\text{H}$ / ppm (H multiplicity)	HMBC	COSY	$\delta^{13}\text{C}$ /ppm
1	38,75	CH ₂				38,9
2	27,48	CH ₂	1,89 (2H, m)		3.16 (H-3)	27,6
3	78,97	CH	3,16(1H,m)		1.89 (H-2)	79,2
4	38,89	C				39,0
5	55,32	CH	0,66(1H,m)		1.36 (H-6)	55,5
6	18,93	CH ₂	1,36(2 H,m)			18,5
7	34,31	CH ₂				34,4
8	40,83	C				41,0
9	50,44	CH	1,24 (1H,sl)			50,6
10	37,17	C				37,3
11	20,97	CH ₂				21,1
12	25,13	CH ₂				25,3
13	38,05	CH				38,2
14	42,09	C				43,0
15	27,40	CH ₂				27,7
16	35,62	CH ₂				35,8
17	43,10	C				43,2
18	48,30	CH			2.37 (H-19)	48,5
19	48,02	CH	2,37 (1H, m)		1.36 (H-18)	
20	150,93	C				151,2
21	29,87	CH ₂				30,0
22	40,05	CH ₂				40,2
23	28,06	CH ₃	0,94(3H, s)	55.32 (C-5); 78.97 (C-3); 15.49 (C-24)		28,2
24	15,49	CH ₃	0,74 (3H, s)	55.32 (C-5); 78.97 (C-3); 28.06 (C-23); 38,89 (C-4)		15,6
25	16,03	CH ₃	0,80(3H, s)	50.44 (C-9); 55.32 (C-5)		16,2

26	16,20	CH ₃	1,01(3H, s)	50.44 (C-9); 34.31 (C-7)		16,3
27	14,61	CH ₃	0,92(3H, s)	27.40 (C-15)		14,7
28	18,08	CH ₃	0,77(3H, s)	43.10 (C-17); 48.30 (C-18); 35.62(C-16); 40.05 (C-22)		18,1
29	109,46	CH ₂	4,67(1H, sl) 4,55(1H, sl)	48.02 (C-19); 19.38 (C-30); 48.02 (C-19); 19.38 (C-30)	4.67 (H-29 a) 4.55 (H-29 b)	109,5
30	19,38	CH ₃	1,66 (3H, s)	109.46 (C-29); 151.93(C-20); 48.02 (C-19)		19,5

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Sample Availability: Samples of the compounds are not available from the authors.



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Ação do lupeol nas feridas cutâneas de ratos normoglicêmicos

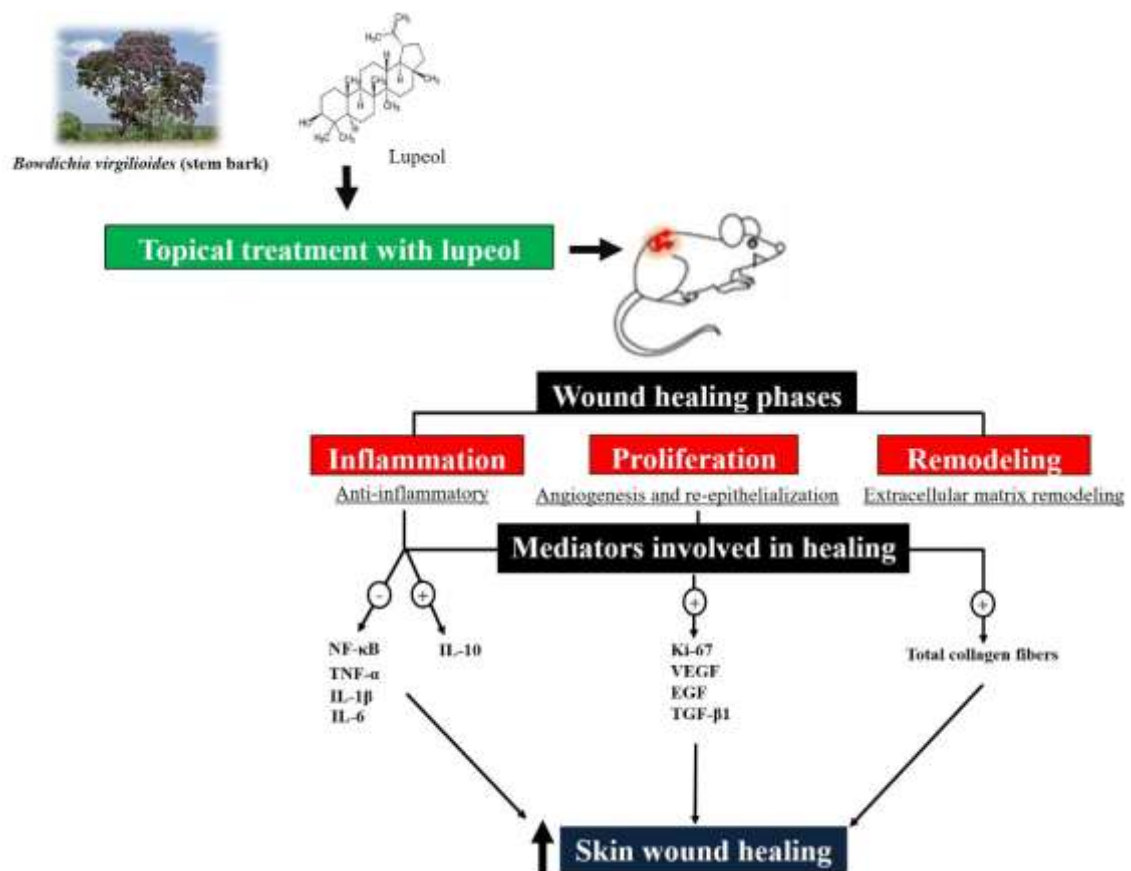
(Manuscrito referente aos resultados *in vivo* do lupeol escrito e formatado nos padrões da revista *European Journal of Pharmacology*)



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Capítulo III

Graphical abstract



ORIGINAL RESEARCH MANUSCRIPT

From inflammation to cutaneous repair: Topical application of lupeol improves skin wound healing in rats by modulating the cytokine levels, NF- κ B, Ki-67 and growth factors expression, and distribution of collagen fibers

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Abstract

Skin wound healing is a highly complex event that involves different mediators at the cellular and molecular level. Lupeol has been reported to possess different biological activities, such as anti-inflammatory, antioxidant, antimicrobial, anti-diabetic and *in vitro* wound healing properties, which motivated us to proceed with *in vivo* studies. We aimed to investigate the wound healing effect of lupeol-based cream for 3, 7 and 14 days. Wound excisions were induced on the thoraco-lumbar region of rats and topically treated for 3, 7 and 14 days after injury induction. Macroscopic analysis was performed by calculating the percentage of wound closure and clinical evaluation, as well as histopathological

analysis and immunohistochemical assay. Cytokine levels were measured by ELISA assay and the gene expression was evaluated by real-time RT-PCR method. Our results showed a strong wound healing effect of lupeol-based cream after 7 and 14 days. Lupeol treatment caused a reduction in the infiltration of inflammatory cells, cytokines (TNF- α , IL-1 β and IL-6) and NF- κ B expression, and positively altered IL-10 levels, showing anti-inflammatory effects. Lupeol treatment showed involvement in the proliferative phase by stimulating the formation of new blood vessels, increasing the expression of Ki-67, pro-angiogenic markers, such as VEGF, TGF- β 1, and a relevant marker of re-epithelialization, EGF. Lupeol was also shown to be involved in tissue regeneration phase by increasing the synthesis of collagen fibers. Our findings suggest that lupeol may serve as a novel therapeutic option to treat cutaneous wounds by regulating mechanisms involved in the inflammatory, proliferative and tissue remodeling phases.

Keywords

Cutaneous wound healing; natural product; triterpene; lupeol; skin regeneration

1. Introduction

Wound healing is a fascinating but complicated process, affected by several factors that contribute jointly to wound closure, including blood coagulation, inflammation, fibroplasia, collagen deposition, and wound contraction (Lindley et al., 2016). The success of skin restructuring is dependent on a cascade of ordered events involving cellular, biochemical and molecular responses and/or interactions. For didactic reason, this process is analyzed from three overlapping phases: inflammatory phase, formed by events such as hemostasis and inflammation; proliferative phase, characterized by granulation tissue formation and re-epithelialization; and the remodeling phase of the extracellular matrix (Morin et al., 2012).

Studies developed with biomolecules for the development of new drugs or new therapeutic agents represent a possibility for the treatment of different diseases and inflammatory conditions, including skin wounds (Cetin et al., 2013). Natural products are a source of compounds with potential biological activity and certain species of plants or natural substances isolated from them have been used successfully in recent studies on the treatment of cutaneous wounds (Upadhyay et al., 2013). Secondary metabolites or active compounds isolated from many natural sources, in addition to plants, have also

been shown to be responsible for the induction of skin wound healing in animal models (Thomé et al., 2012).

The natural triterpene, lupeol is a bioactive found in various edible plants, such as olive, fig, mango, carrot, soybean, melon seed and grapes, and also in medicinal plants such as *Bowdichia virgilioides* (Saleem et al., 2001). Several studies have shown pharmacological potential of lupeol, including antioxidant, anti-inflammatory, antihyperglycemic, anti-dyslipidemic, anti-arthritic, cardioprotective, hepatoprotective and wound healing effects in experimental models *in vivo* and *in vitro* (Sudhahar et al., 2006; Alqahtani et al., 2013; Yokoe et al., 2015; Badshah et al., 2016). We have previously reported that lupeol at low concentrations was able to stimulate the proliferation, migration and cell contraction by promoting wound healing in human keratinocytes and fibroblasts possibly through involvement of PI3K/Akt and p38/ERK/MAPK pathways (Beserra et al., 2018).

A brief study performed by Harish et al. (2008), showed wound healing activity of the lupeol-based gel applied topically on rat wounds during 16 days of treatment, only through macroscopic parameters. Our study uses lanette cream as a vehicle for the formulation, which causes greater stability to substances that can be incorporated in both the aqueous and oil phases, and being able to be used in a larger area without the risk of rapid evaporation (Gönüllü et al., 2015). Our aim was to examine the effects of this lupeol-based cream treatment on the inflammatory, proliferative and remodeling phases in skin wound healing, as well as to determine the involvement of key mediators involved in these processes.

2. Materials and methods

2.1. Plant material, extraction and isolation of lupeol

The stem bark of *Bowdichia virgilioides* Kunth. was collected in December 2014 in the surroundings of Santa Rita, State of Paraíba, Brazil, a coastal area around the Atlantic Forest. The sample was identified by comparison with the plant specimens and deposited in the Herbarium Prof. Lauro Pires Xavier, and in the reference collection of the Laboratory of Pharmaceutical Technology from Federal University of Paraíba, João Pessoa, Brazil, under number *Agra et Góis* 6243. The plant was dried (50 °C) and then powdered. The powder (3.0 Kg) was extracted with 95 % ethanol, filtered and concentrated under vacuum to obtain the crude ethanolic extract (EtOHE, 250g).

Afterwards, the EtOHE was suspended in a MeOH/H₂O mixture (2:3), and subjected to the liquid-liquid partitioning process with hexane. The hexane residue (49g) was subjected to repeated washings with acetone under stirring followed by filtration. The solid obtained was recrystallized from chloroform and hexane, resulting in white crystals which were performed by analyzing ¹H and ¹³C NMR spectral data, compared with those published in the literature (Suryati et al., 2011) and identified as lupeol (Beserra et al., 2018) substance (3g).

2.2. *Animals*

Healthy male *Wistar* rats (*Rattus norvegicus*) were procured from Central Animal House, UNESP, Botucatu. They weighed between 180 g and 220 g, and their average age was 8 weeks. The animals were housed individually in polyethylene cages in an experimental animal room with a 12 h light/dark cycle. They were maintained at a room temperature of 23 ± 2 °C and humidity at 55% ± 15%. The rats were fed a standard diet and water ad libitum, and they were acclimatized for at least one week before the experiment. All experiments were carried out in accordance with the experimental protocols (Protocol 610/2014) approved by the Ethics Committee on Animal Use (CEUA/IBB/UNESP).

2.3. *Excision wound model*

All rats were anesthetized with ketamine hydrochloride (0.08mg/100g) and xylazine (0.04mg/100g) by intraperitoneal injection. Prior to the surgical procedure, the animals received a single dose of ketoprofen (100 mg/kg, SC) as an ethical conduct to minimize postoperative discomforts. After the shaving the hair on the back of each rat, the skin was sterilized with 70% alcohol to remove any type of contamination and a lesion was created in the posterior dorsal region of each animal using a 2cm diameter punch. Thereafter, animals were housed individually and monitored in properly disinfected cages to prevent infection or further damage to the wounds after recovering from anesthesia.

2.4. *Grouping and topical treatment*

To assess the cutaneous wound healing of lupeol-based cream, wound excision models were used. The rats were divided into five groups (n=8), as shown below:

Group I - Topically treated with lanette cream (vehicle)

Group II - Topically treated with collagenase 1.2 U/g (reference drug)

Group III- Topically treated with 0.1% w/w lupeol cream (substance test)

Group IV- Topically treated with 0.2% w/w lupeol cream (substance test)

Group V- Topically treated with 0.4% w/w lupeol cream (substance test)

Group VI – Sham (without wound)

Immediately after the surgical excision, the wounds were topically treated for 3 different experimental periods: 3, 7, or 14 days. Formulations was applied topically every day, once a day during each period. The rats were placed in their respective cages with one rat per cage.

2.5. Determination of wound retraction percentage

The wound closure was analyzed daily in each treatment period group using transparency paper and a permanent marker. After scanning, the wound area was measured using specific software Adobe Photoshop C5 - version 5 (Adobe Systems Inc, San Jose, California). The area of wound retraction was calculated (%) by the following formula: % wound retraction = $\{(\text{initial area of the wound} - \text{area of wound measured}) / \text{initial area of the wound}\} * 100$. The data of the wound areas were expressed as the mean $\pm$ standard deviation.

2.6. Clinical evaluation

The clinical signs of the lesions, such as exudation, edema, local hemorrhage, presence of crust and granulation tissue, were monitored by macroscopic examination and graded on a four-point scale: 0 - absent (0%), 1 - light (30%), 2 - moderate (30-70%) and 3 - intense (> 70%) based on the method from Oliveira et al. (2014), with modifications.

2.7. Histological analysis

The animal samples were fixed with alcohol, formalin and acetic acid (8:1:1) and processed in paraffin. Number of blood vessels and deposition of collagen fibers were assessed by hematoxylin and eosin (HE) and Masson's trichrome staining, respectively. For all analyzes, three distinct regions were photographed: normal skin (skin without wound), border and the center of the wound. Ten photomicrographs of each sample were analyzed under a 40x magnifications according to the method as previously described

(Gushiken et al., 2017), being 5 for the border and 5 of the central lesion regions. The number of blood vessels, total number of inflammatory cells and collagen fibers labeled present in these regions were quantified by the marked area count, totaling an area of $100.000 \mu\text{m}^2/\text{slice}$. The photomicrographs were obtained with the software CellSens Standard (Olympus, USA) and the measurements were made using AVSoftBioView software.

2.8. Immunohistochemistry analysis

The immunohistochemical studies were carried out with sections stained with monoclonal antibodies against NF- κ B (1:100 μL), Ki-67 (1:200 μL), EGF (1:200 μL) and VEGF (1:100 μL). The same samples used for histological analysis were cut (5 μm) and fixed on silanized slides and submitted to antigen recovery by pressure (20 psi/125 °C) and the immunohistochemical reaction was performed with the polymer kit. Ten photomicrographs of each slice were analyzed with 40x magnifications software CellSens Standard (Olympus, USA), being 5 for the border and 5 of the central lesion regions (Gushiken et al., 2017). The immunolabeled area was quantified totalizing $100.000 \mu\text{m}^2/\text{slice}$. The quantification was made with the software AVSoftBioView.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Tissue homogenate was centrifuged and the supernatant was processed to determine the levels of the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 and the anti-inflammatory cytokine IL-10 using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). Measurements of total proteins were determined by the biuret assay so that the data of the parameters analyzed are expressed relative to the amount of protein in the sample in milligrams (Krohn, 2002).

2.10. RNA extraction and Reverse Transcription Quantitative PCR (RT-qPCR)

The RNA of the skin samples (*Rattus norvegicus*) was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommendations. To avoid genomic DNA contamination, the RNA samples were treated with DNase I, RNase-free kit (Invitrogen, Carlsbad, CA, USA) prior to cDNA synthesis. The cDNA synthesis was performed with random hexamers using Superscript® II (Invitrogen, Carlsbad, CA, USA) with random hexamers according to standard protocols (Nóbrega et al., 2010). The quantitative PCR reaction was carried out using designed and

specific forward and reverse primers for *Rattus norvegicus* (table 1) to evaluate the expression of the genes for *Nf- κ b*, *Ki-67*, *Egf*, *Vegf* and *Tgf- β 1*, among the different treatments used. For qPCR, Cts values were determined using SYBR Green kit (Invitrogen, Carlsbad, CA, USA). And for each gene of interest, the mRNA levels (Cts) were normalized by the reference gene, *β -actin* (table 1), and expressed with values relative to the Cts mean (point from which the system starts the quantification of the genetic material from the exponential phase threshold) of each group (ddCt - Ct normalized by means of the respective *β -actin* groups). All qPCR reactions (10 μ l) used 900 nM for each primer and 700 ng of total RNA. Each reaction was performed in duplicate in a StepOne system (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions, and relative gene expression profiles were calculated, according to the $\Delta\Delta$ Ct method as previously described (Vischer et al., 2003).

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.01 software. Parametric data were expressed as mean $\pm$ standard error of the mean and the comparison between groups was performed by ANOVA followed by Newmann-Keuls test. The nonparametric data were expressed as median (maximum and minimum) and performed by the Kruskal-Wallis test, followed by the Dunn test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Macroscopic analysis of lupeol treatment in cutaneous wounds

The wound reduction was calculated after 3, 7 and 14 days of lesion induction in rats (Fig. 1A-C). Wound closure was notably improved after 7 and 14 days of lupeol treatment. Initially, the lesions were analyzed throughout the three days of treatment, during which no significant changes were observed in wound closure rate between the treatments. After 7 days of treatment, it was possible to observe a statistically significant retraction of the lesions in the lupeol treated groups at all concentrations studied in relation to the lanette group. The lesions analyzed after 14 days of treatments with lupeol at 0.2% and 0.4% showed a significant 89% and 87% increase, respectively, in the wound reduction rate compared to lanette group (Fig. 1D). The collagenase (reference drug) group showed a 88% significant increase in wound reduction only after 14 days of

treatment in relation to the lanette group, and when compared to lupeol treated groups (in all concentrations), no significant differences were observed in the wound closure percentage.

We also evaluated the clinical parameters such as exudation, edema, hemorrhage, presence of crust and granulation in all rats (Table 2). After 3 days of treatment, we observed a significant reduction in the exudation process of the groups treated with collagenase, and lupeol 0.2% and 0.4% in relation to the lanette group. After 7 days of treatment, the groups treated with lupeol 0.2% and 0.4% also demonstrated a significant increase in the granulation process compared to lanette, but only the higher concentration of lupeol treatment (0.4%) was able to significantly increase the crust formation in relation to the lanette treatment. The lesions analyzed during 14 days of treatment showed that the lanette-treated animals still exhibited a small amount of crust adhered to the lesion, differently from the groups treated with lupeol 0.2% and 0.4%, which did not possess this crust, showing only the scar of the injured region. There were no significant changes in edema and hemorrhage parameters analyzed after 3 and 7 days of wound induction. According to the results of the macroscopic analysis, we selected the 0.2% concentration as the lowest effective concentration to perform the histopathological, immunoenzymatic and molecular analyses.

3.2. Histological examination

The HE staining allowed the counting of blood vessels and the total number of inflammatory cells in the border and the lesion center (Fig. 2A-C). It was not possible to observe significant change in vascularization of the border and the lesion center in the groups tested after 3 days of lesion induction. However, we observed a significant increase in the number of blood vessels in the central region of the lesion in the lupeol-treated groups in relation to the lanette and collagenase-treated group. After 14 days of treatment, there was a significant increase in vascularization in the border region in the collagenase treatment compared to lanette. There were significant vascular alterations in the wound center in the groups treated with collagenase and lupeol in relation to the lanette group (Fig. 2D). Regarding the total number of inflammatory cells, we observed significant changes between the treatments in all periods studied. After 3 and 7 days, lupeol treatment was able to significantly decrease the inflammatory cell infiltration in the border and center of the lesions and after 14 days of treatment only in the central region compared to the lanette group. Lupeol also showed significant reductions in the

total number of inflammatory cells in the central region of lesions treated after 3 days and in the border of the lesion after 7 days of experimentation compared to the collagenase-treated group (Fig. 2E).

Analysis of total collagen fibers using Masson's trichrome method showed labeled area (μm^2) in the border and center regions of the lesion, as shown in Fig. 3A-C. There was a significant increase in the labeling of these proteins in the border region of the lesion of the lupeol-treated group in relation to the lanette group, after 3 days of treatment. On day 7, we observed a significant increase in the deposition of total collagens in the wound center of the lupeol treatment in relation to the lanette and collagenase-treated groups. By day 14, only the collagenase-treated group showed a significant increase in the labeled area by total collagens compared to lanette (Fig. 3D).

3.3. Effect of lupeol on the immunostaining of NF- κ B, Ki-67, EGF and VEGF

Immunohistochemical analysis for NF- κ B after 3 days of treatment, showed a significant decrease in the border and lesion center immunolabeling of lupeol-treated group compared to lanette and collagenase treatments. After 7 days of treatment, a significant decrease in immunolabeling was observed only at the wound center of lupeol-treated animals compared to the lanette group. On day 14, the lupeol-treated group showed a significant decrease of the immunolabelled area compared to the lanette or collagenase groups (Fig. 4A). Immunolabeling to localize Ki-67 performed after 7 days of treatment showed a significant increase in antibody-labeled area in the border and lesion center of the lupeol-treated group compared to lanette treatment. Analysis carried out after 3 and 14 days of treatment did not show significant changes in the groups tested (Fig. 4B). Regarding EGF, after 3 days, there was no significant change in any groups tested (Fig. 4C), however by day 7 there was a significant increase in staining of the border area in lupeol compared to control treatment. In the lesion center, both lupeol and collagenase treatments significantly increased the EGF-immunolabelled area in relation to lanette. After 14 days, only collagenase-treated rats showed a significant increase in the wound border compared to the lanette. The immunohistochemistry of VEGF showed that the lupeol treatment significantly increased the immunolabeling of the lesion border compared to the lanette group after 3 and 7 days of treatment. Collagenase treatment also showed a significant increase of the immunolabelled area in the borders compared to the lanette group after 7 days of experimentation. After 14 days, a significant increase of the immunolabeling was observed in the lupeol and collagenase-treated groups compared to

lanette. Lupeol treatment also caused a significant increase of the immunolabelled area in the lesion center after 3 and 7 days of treatment compared to lanette, while the collagenase treatment increased VEGF immunolabeling in the lesion center only after 7 days of treatment, as shown in Fig. 4D. The photomicrographs of the immunohistochemical analyzes can be found in the supplementary materials.

3.4. Effect of lupeol on pro-and anti-inflammatory cytokine levels

The results of the ELISA assay showed anti-inflammatory effect of lupeol through a significant reduction of TNF- α and IL-6 levels compared to lanette group after 7 and 14 days of treatment. After 14 days, there was also a significant increase in IL-10 levels in lupeol-treated rats compared to lanette group. Lupeol did not cause significant any changes in IL-1 β levels in the three periods studied (Fig. 5).

3.5. Effect of lupeol on *Nf- κ b*, *Ki-67*, *Egf*, *Vegf-A* and *Tgf- β 1* mRNA expression

The molecular analyzes of the real-time gene expression of *Nf- κ b*, *Ki-67*, *Egf*, *Vegf-A* and *Tgf- β 1* are shown in Fig. 6. There was a decrease in *Nf- κ b* expression in the collagenase and lupeol-treated groups compared to lanette after 3 days, and a significant decrease caused by lupeol compared to collagenase after 14 days was observed. The relative expression of *Ki-67* decreased significantly in the collagenase and lupeol-treated groups compared to lanette after 14 days of treatment. Lupeol treatment caused a significant increase in the expression of *Vegf-A* compared to lanette after 3 and 7 days of treatment. After 14 days, a reduction of the *Vegf-A* expression in the lupeol-treated rats compared to the collagenase group was observed. There was an increase in the gene expression of *Egf* and *Tgf- β 1* in the collagenase and lupeol-treated groups compared to lanette only after 7 days of treatment (Fig. 6).

4. Discussion

The use of medicinal plants and isolated bioactive substances appear as alternatives to assist in the cicatricial process, reducing wound closure time, re-epithelialization and tissue fibrosis, due to the presence of secondary metabolites such as tannins, steroids, terpenes, flavonoids, alkaloids, coumarins and saponins - that act in the different stages of cutaneous wound healing through various mechanisms of action involved (Thomé et al., 2012). The present study confirms the efficacy of lupeol isolated from *B. virgilioides* as analyzed by macroscopic, histopathological, biochemical and

molecular parameters, showing an anti-inflammatory effect, a significant increase in pro-angiogenic and re-epithelializing markers, and of stimuli in the deposition of collagen fibers in dermal wounds treated after 3, 7 and 14 days *in vivo*.

In general, natural products have been considered an important source of bioactive molecules with therapeutic potential in cutaneous wound treatment (Perini et al., 2015). Proteolytic enzymes have been used as a debriding agent in the treatment of these injuries (Tallis et al., 2013). Collagenase, an enzyme debriding agent derived from *Clostridium histolyticum*, is used in the clinic for the treatment of infected and surgical wounds (Mekkes et al., 1998). Previous studies have reported that collagenase acts by cleaning the necrotic tissue of the wound through an enzymatic method, accelerating the formation of granulation tissue and subsequent re-epithelialization, and increasing the activation of fibroblasts, myofibroblasts and collagens in dermal lesions of rats (Durmus et al., 2009). Therefore, collagenase was used in our study as a positive control for treatment of rat skin wounds.

Mechanism of retraction of the cutaneous wounds involves a series of interactions between cells and extracellular matrix molecules during the three healing phases, capable of generating mechanical forces that result in the wound closure (Agha et al., 2011). The inflammatory phase of the healing process is essential for correct tissue repair of the lesions. Inflammatory cells such as neutrophils and activated macrophages trigger the release of inflammatory mediators such as cytokines, chemokines, reactive oxygen species, and proteolytic enzymes. The production of inflammatory mediators is finely regulated and one of the key regulatory points of these is the inhibition of transcription factors, such as factor nuclear kappa B (NF- κ B). This protein complex is formed by cytoplasmic subunits that are inactive, and when activated, it translocates to the nucleus and binds to the consensus region of genes that express cytokines and oxidizing enzymes. Because of this, NF- κ B has been considered an important inflammatory marker and a target molecule for the treatment of inflammatory disorders (Karin, 2005). Our results showed a strong reduction in the gene expression of NF- κ B after 3 days of treatment and in the immunolabeling of this antibody in the three periods studied. Histological analysis supports the NF- κ B gene and protein expression data by decreasing the infiltration of inflammatory cells observed in all periods of treatments. These results corroborate with recent findings *in vitro*, where lupeol was able to suppress the activation and expression of NF- κ B in keratinocytes (Beserra et al., 2018).

TNF- α is a proinflammatory cytokine, which during the inflammatory phase of the cicatricial process of lesions is expressed in greater quantity when compared to its normal levels. IL-1 β , is proinflammatory and synthesized by different cell types, such as monocytes and macrophages, as well as other endothelial cells, and among the main functions of IL-1 β , are the contribution with local inflammatory processes in the injury and promotion of the secretion of other proinflammatory cytokines such as TNF- α and IL-6 (Wagner and Wehrmann, 2007). Interleukin IL-6 is a soluble proinflammatory mediator with pleiotropic activities in inflammation, hematopoiesis and immune responses (Tanaka et al., 2014). Together with TNF- α and IL-1 β , IL-6 is present in high concentrations in inflammatory processes (Scheller et al., 2011). Another cytokine involved in healing is anti-inflammatory interleukin (IL-10), which is related to the inhibition of the synthesis of proinflammatory cytokines such as TNF- α , IL-1 β and IL-6 in active macrophages in the inflammatory process (Sato et al., 1999), and is also involved in the angiogenesis process (Silvestre et al., 2000). Inhibition of exacerbated expression of proinflammatory cytokines and increased expression of anti-inflammatory cytokines are involved as an essential healing mechanism (Abraham and Kappas, 2008). Some studies have demonstrated that lupeol is associated with the modulation of various inflammatory agents, such as TNF- α , IL-1 β and IL-6 (Nguemfo et al., 2009). The present study confirms this potential anti-inflammatory effect of lupeol in cutaneous lesions of rats by significantly reducing the levels of proinflammatory cytokines such as TNF- α , IL-1 β and IL-6 after 7 and 14 days of treatment and increasing the IL-10 levels in these two last periods of treatment studied.

The formation of granulation tissue, constituted by macrophages, fibroblasts and neoformed vessels, is fundamental for the re-epithelialization process and reconstructing of the extracellular matrix (Profyris et al., 2012). Increased cell proliferation is a crucial aspect of wound healing in general, and the Ki-67 protein is an important marker of this cellular event. Ki-67 expression is also widely known to be an indicator of cell growth within a total cell population (Choi et al., 2012). Our results showed a reduction in Ki-67 gene expression in the lupeol-treated group after 14 days. In contrast, we also observed increased expression of this protein in the immunohistochemical analysis for the lupeol treatment after 7 days of treatment, suggesting that lupeol increases cell proliferation and the consequent stimulus for the next phase of the cicatricial process, remodeling phase.

Another essential event in the wound healing is re-epithelialization, which begins a few hours after injury, but shows more evident activity in the proliferative phase of

healing, and continues until extracellular matrix remodeling (Profyris et al., 2012). Epidermal growth factor (EGF) is a crucial marker of this phase and possess mitogenic and migratory activity on the edge keratinocytes (Werner and Grose, 2003). Our results showed increased gene expression of EGF in the lupeol-based cream treatment, as well as increased immunostaining for this growth factor after 7 and 14 of treatments analyzed in the border and lesions center, and that way, confirming the role of the lupeol on re-epithelialization in the healing process.

The angiogenic process is an important part of the proliferation phase and involves variable growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2), transforming growth factor-beta 1 (TGF- β 1), vascular endothelial growth factor (VEGF) and angiotensin (Yoshida et al., 2004). VEGF, in particular, is one of the most potent mediators of angiogenesis, considered to be a pivotal process in wound healing, contributing to the formation of new vessels by stimulating the survival, proliferation and migration of endothelial cells. This event is fundamental because it promotes nutrition through the supply of oxygen and other essential substances in the tissue repair (Chou et al., 2002; Lerman et al., 2003). The data obtained in our study showed an increase in the number of blood vessels in the border and lesion center in the lupeol-treated groups after 7 and 14 days, together with an increase in gene expression observed after 3 and 7 days of treatment and in the immunolabeling of VEGF during the three treatment periods tested. The increased number of blood vessels associated with up-regulation of VEGF, clearly demonstrates the role of lupeol on angiogenesis for cutaneous wound healing.

TGF- β 1 is reported to be one of the proteins with the broadest spectrum of activities, which displays effects on the inflammatory process, cell differentiation and extracellular matrix production, evolution of granulation tissue through the recruitment of inflammatory leukocytes and stimulation of fibroblasts and epithelial cells (Canesso et al., 2014). Our results showed an increase in the gene expression of TGF- β 1 in the lupeol-treated group after 7 days corresponding to the proliferative phase, which mediates the formation of granulation tissue and local neovascularization.

Finally, the regeneration phase, which involves an extensive tissue remodeling, is replaced by proteoglycan and collagen molecules, which organize in thicker bundles, resulting in intact and resistant tissue (Maruyama et al., 2007). It is interesting to emphasize that synthesis of collagen fibers is one of the most important events in the wound healing process (Greenhalgh, 2003). Our study showed an increase in the

deposition of collagen fibers after 3, 7 and 14 days of treatment with lupeol indicating that lupeol treatment is also effective in tissue regeneration phase by increasing the synthesis of collagen fibers.

5. Conclusion

In conclusion, our data show that lupeol from *B. virgilioides* accelerates cutaneous wound healing by involving several mechanisms, including anti-inflammation, through the modulation of NF- κ B expression and pro- and anti-inflammatory cytokines, mediators involved in the formation of granulation tissue, angiogenesis and re-epithelialization, such as Ki-67, VEGF, EGF and TGF- β 1, and stimulation of the synthesis of collagen fibers, contributing to the tissue remodeling. Our findings confirm the wound healing potential of lupeol and makes this triterpene a promising molecule for therapeutic use. Further *in vivo* studies are needed in order to evaluate the effect of lupeol on wounds associated with metabolic disorders, such as diabetes.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version.

Abbreviations

Akt: protein kinase B

ANOVA: one-way analysis of variance

cDNA: complementary DNA

Cq: quantification cycle

Ct: cycle threshold

ddCT: overall average of cycle threshold

ERK: extracellular-signal-regulated kinase
 EGF: epidermal growth factor
 ELISA: enzyme-linked immunosorbent assay
 EtOHE: ethanolic extract
 FGF-2: fibroblast growth factor-2
 FW: forward
 HE: hematoxylin and eosin
 IL-10: interleukin 10
 IL-1 β : interleukin-1beta
 IL-6: interleukin 6
 Ki-67: antigen Ki-67
 MAPK: mitogen-activated protein kinase kinase kinases
 mRNA: messenger RNA
 NCBI: National Center for Biotechnology Information
 NF- κ B: factor nuclear kappa B
 PCR: polymerase chain reaction
 PDGF: platelet-derived growth factor
 PI3K: phosphatidylinositol-3-kinase
 RT-qPCR: quantitative reverse transcription PCR
 RV: reverse
 SC: subcutaneous
 SEM: standard error of the mean
 TGF- β 1: transforming growth factor-beta 1
 TNF- α : tumor necrosis factor alpha
 VEGF: vascular endothelial growth factor

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Table legends

Table 1: Sequence of primers used in RT-qPCR.

Table 2: Clinical parameters of cutaneous lesions after topical treatment with Lanette, Collagenase 1.2 U/g and lupeol 0.1, 0.2 or 0.4% (n=8) in rats. Data are expressed as

median (minimum and maximum) and analyzed by the Kruskal-Wallis test, followed by the Dunn test. $*p < 0.05$ vs. Lanette group. (–) means parameter corresponding to the absence of observed signals.

Figure legends

Fig. 1: Effect of lupeol on the healing of excisional wounds in rats. Representative images of lesion area (initial and from the last day of experimentation) of animals treated with Lanette, Collagenase 1.2 U/g and lupeol 0.1%, 0.2% or 0.4% after 3 (A), 7 (B) and 14 (C) days and percent of wound closure (D) during the three treatment periods studied. $*p < 0.05$ and $**p < 0.01$ vs. Lanette group by ANOVA followed by the Newman-Keuls test.

Fig. 2: HE stained skin tissue sections on day 3 (A), 7 (B) and 14 (C) post wound induction. Number of blood vessels (D) and inflammatory cells infiltration (E) in HE staining of the border and central region of rats cutaneous lesions treated with Lanette, Collagenase 1.2 U/g or lupeol 0.2% for 3, 7 and 14 days. $*p < 0.05$ and $***p < 0.001$ vs. Lanette group. $###p < 0.001$ vs. Collagenase group, using ANOVA followed by the Newman-Keuls test. Bar represents 20 μ m. (black arrows) indicate the presence of inflammatory cells, (#) collagen fibers, (*) blood vessels, (+) fibroblasts and (▲) sebaceous glands.

Fig. 3: Masson's trichrome stained skin tissue sections on day 3 (A), 7 (B) and 14 (C) post wound induction. Labeling area of total collagen fibers (D) (μm^2) in the border and central region of rats cutaneous lesions treated with Lanette, Collagenase 1.2 U/g or lupeol 0.2% for 3, 7 and 14 days. $*p < 0.05$ and $**p < 0.01$ vs. Lanette group. $##p < 0.01$ vs. Collagenase group, using ANOVA followed by the Newman-Keuls test. Bar represents 20 μ m. (white arrows) indicate the presence of total collagen fibers and (▲) sebaceous glands.

Fig. 4: Immunolabeled area (μm^2) for NF- κ B (A), Ki-67 (B), EGF (C) and VEGF (D) in the border and central region of rats cutaneous lesions treated with Lanette, Collagenase 1.2 U/g or lupeol 0.2% for 3, 7 and 14 days. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs. Lanette group. $#p < 0.05$ vs. Collagenase group, using ANOVA followed by the Newman-Keuls test.

Fig. 5: Quantification of TNF- α , IL-1 β , IL-6 and IL-10 levels (pg/mg protein) in rats cutaneous lesions treated with Lanette, 1.2 U/g or lupeol 0.2% for 3, 7 or 14 days. $*p$

<0.05 and $**p < 0.01$ vs. Lanette group. $+p < 0.05$, $++p < 0.01$ and $+++p < 0.001$ vs. sham group, using ANOVA followed by Newman-Keuls test.

Fig. 6: Gene expression (RT-qPCR) of NF- κ B, Ki-67, VEGF-A, EGF and TGF- β 1 in rats cutaneous lesions treated with Lanette, 1.2 U/g or lupeol 0.2% for 3, 7 or 14 days. $*p < 0.05$ and $**p < 0.01$ vs. Lanette group. $\#p < 0.05$ vs. sham group, using ANOVA followed by Newman-Keuls test.

TABLES

Table 1

Gene	Primers sequence 5'-3'	Product Size	Melting Temperature	Access number*
<i>β-actin</i>	FW: CCCTGGCTCCTAGCACCAT RV: GATAGAGCCACCAATCCACACA	80 pb	60°C	NM_031144.3
<i>NF-κb</i>	FW: CCTCATCTTTCCCTCAGAGCC RV: CGCACTTGTAACGGAAACGC	98 pb	60°C	NM_199267.2
<i>Ki-67</i>	FW: GGGTTTCCAGACACCAGACC RV: CCAGGAAGACCAGTTAGAACC	100 pb	60°C	NM_001271366.1
<i>Egf</i>	FW: CTCAGGCCTCTGACTCCGAA RV: ATGCCGACGAGTCTGAGTTG	93 pb	60°C	NM_012842.1
<i>Vegf-A</i>	FW: TGC GGATCAAACCTCACCAA RV: GGCTCACAGTGATTTTCTGGC	115 pb	60°C	NM_001110333.2
<i>Tgf-β1</i>	FW: GGGCTACCATGCCAACTTCTG RV: GAGGGCAAGGACCTTGCTGTA	82bp	60°C	NM_021578.2

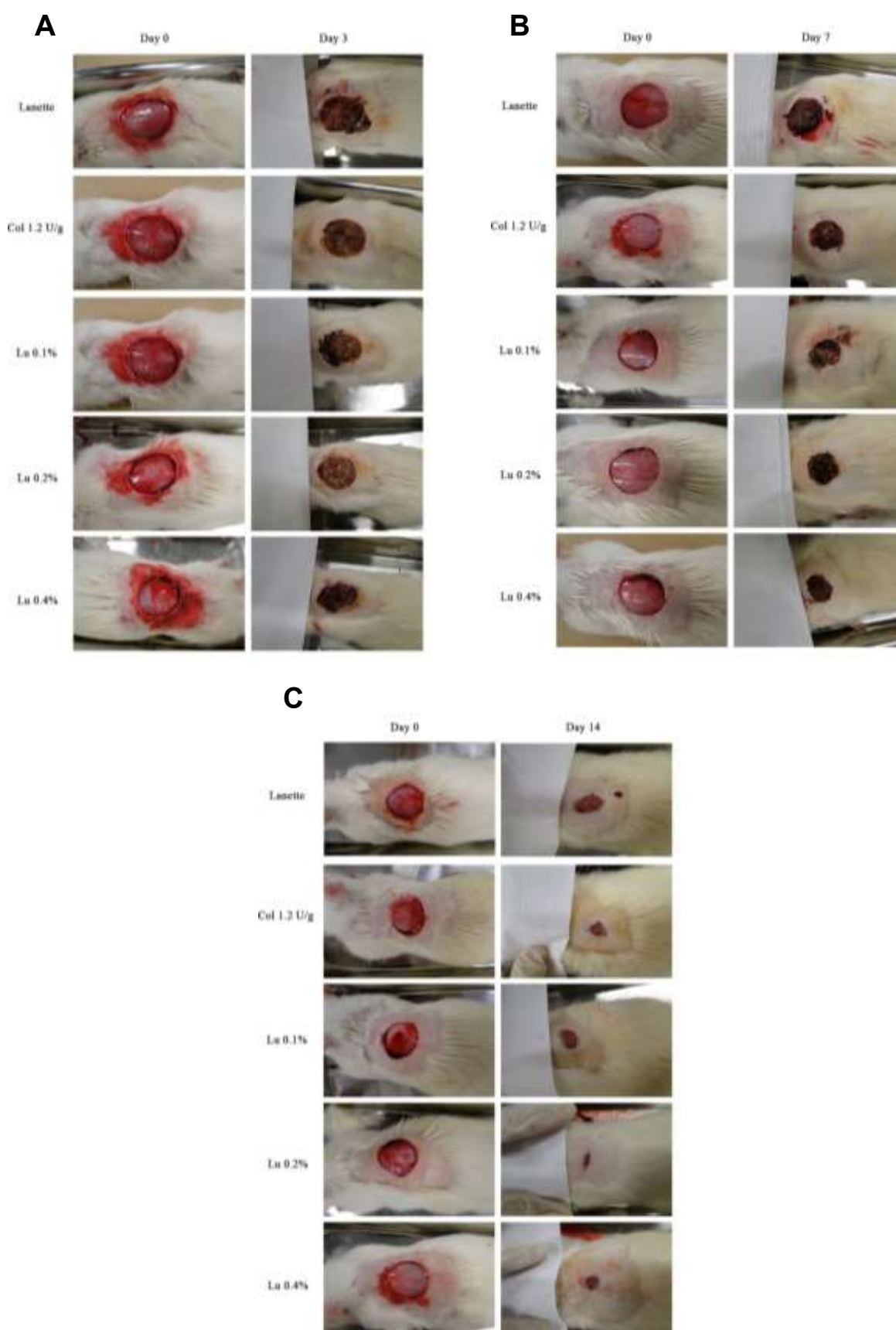
FW (forward), RV (reverse) and pb (base pairs). *National Center for Biotechnology Information, Nucleotide (NCBI - <https://www.ncbi.nlm.nih.gov>).

Table 2

Parameters	Groups	Days after wound induction		
		3 days	7 days	14 days
Exudation	Lanette	2 (2,3)	1 (0,3)	-
	Collagenase	1 (0,2)*	0 (0,1)	-
	Lupeol 0.1%	1 (1,2)	0 (0,1)	-
	Lupeol 0.2%	1 (0,3)*	0 (0,1)	-
	Lupeol 0.4%	1 (0,3)*	0 (0,1)	-
Edema	Lanette	2 (1,3)	-	-
	Collagenase	1 (0,2)	-	-
	Lupeol 0.1%	1 (1,3)	-	-
	Lupeol 0.2%	1 (0,2)	-	-
	Lupeol 0.4%	1 (0,3)	-	-
Hemorrhage	Lanette	1 (0,3)	1 (0,2)	-
	Collagenase	0.5 (0,1)	0 (0,1)	-
	Lupeol 0.1%	1 (0,2)	0.5 (0,2)	-
	Lupeol 0.2%	0 (0,2)	0 (0,1)	-
	Lupeol 0.4%	0 (0,1)	0 (0,2)	-
Presence of crust	Lanette	0 (0,2)	1.5 (0,3)	1 (1,3)
	Collagenase	1 (0,3)	2 (1,3)	1 (0,3)
	Lupeol 0.1%	0.5 (0,2)	2.5 (1,3)	0.5 (0,2)
	Lupeol 0.2%	1 (0,3)	3 (2,3)	0.5 (0,1)*
	Lupeol 0.4%	1.5 (0,3)	3 (2,3)*	0.5 (0,1)*
Granulation	Lanette	0 (0,1)	1 (0,2)	2 (1,3)
	Collagenase	1 (0,2)	2 (1,3)	1 (1,3)
	Lupeol 0.1%	1 (0,2)	2 (1,3)	1 (0,2)
	Lupeol 0.2%	1 (0,1)	2 (2,3)*	1 (0,2)
	Lupeol 0.4%	1 (0,2)	2.5 (1,3)*	1 (0,2)

FIGURES

Fig. 1



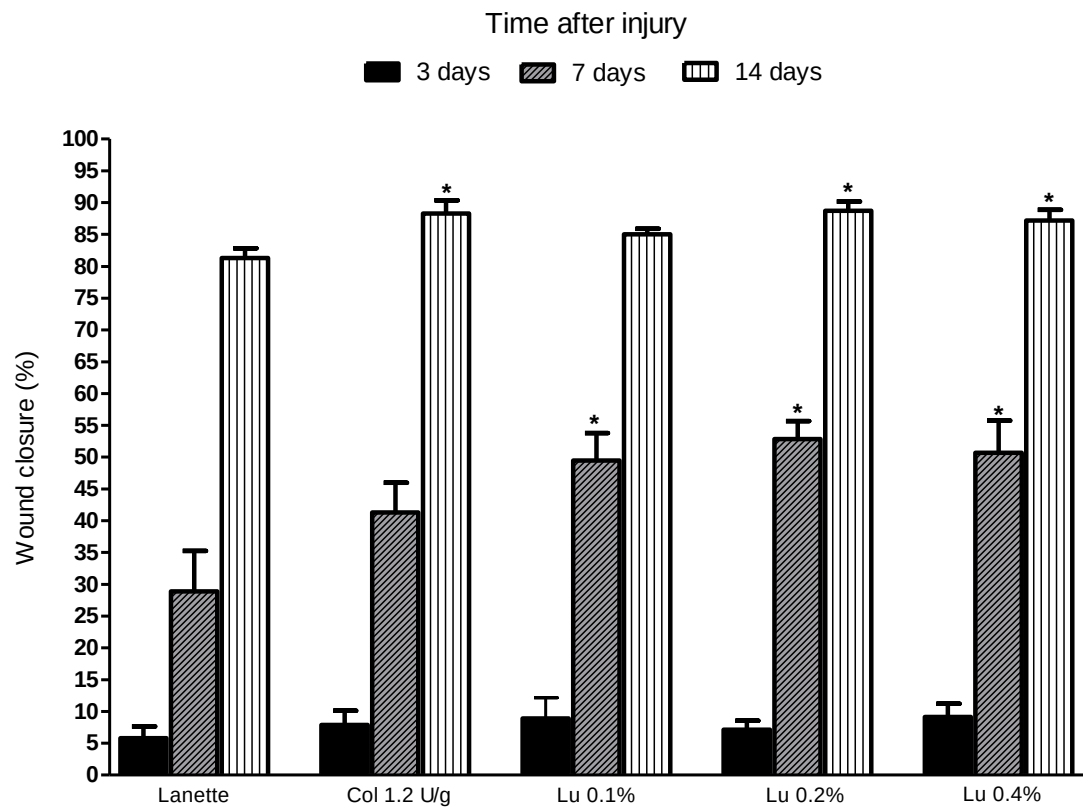
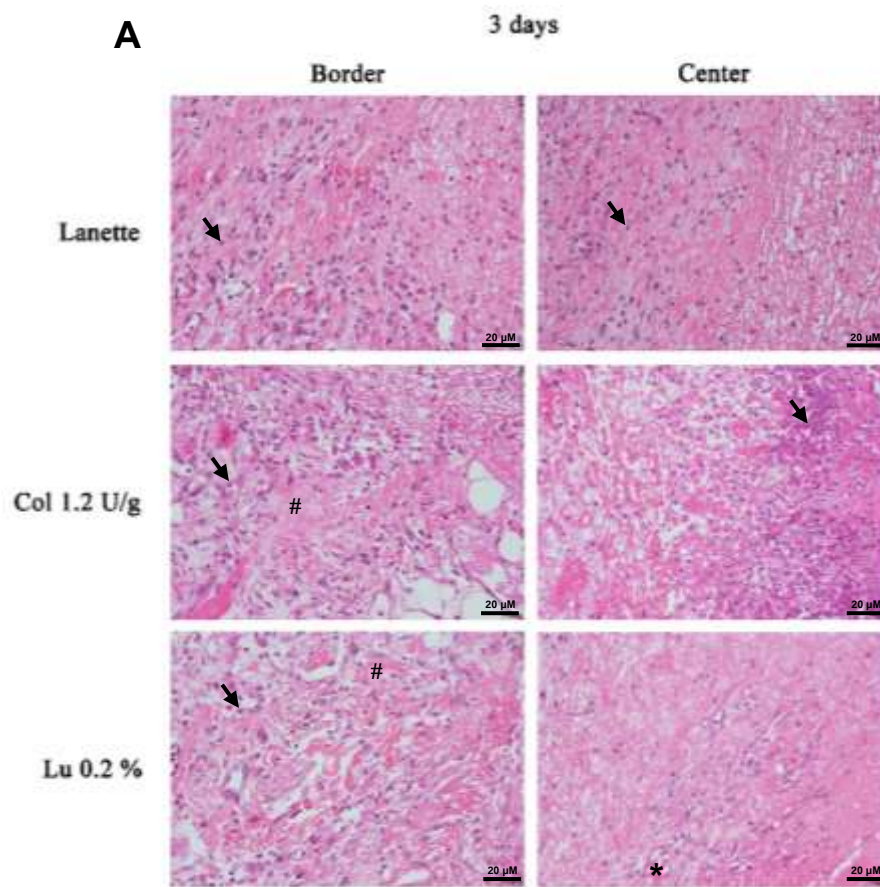
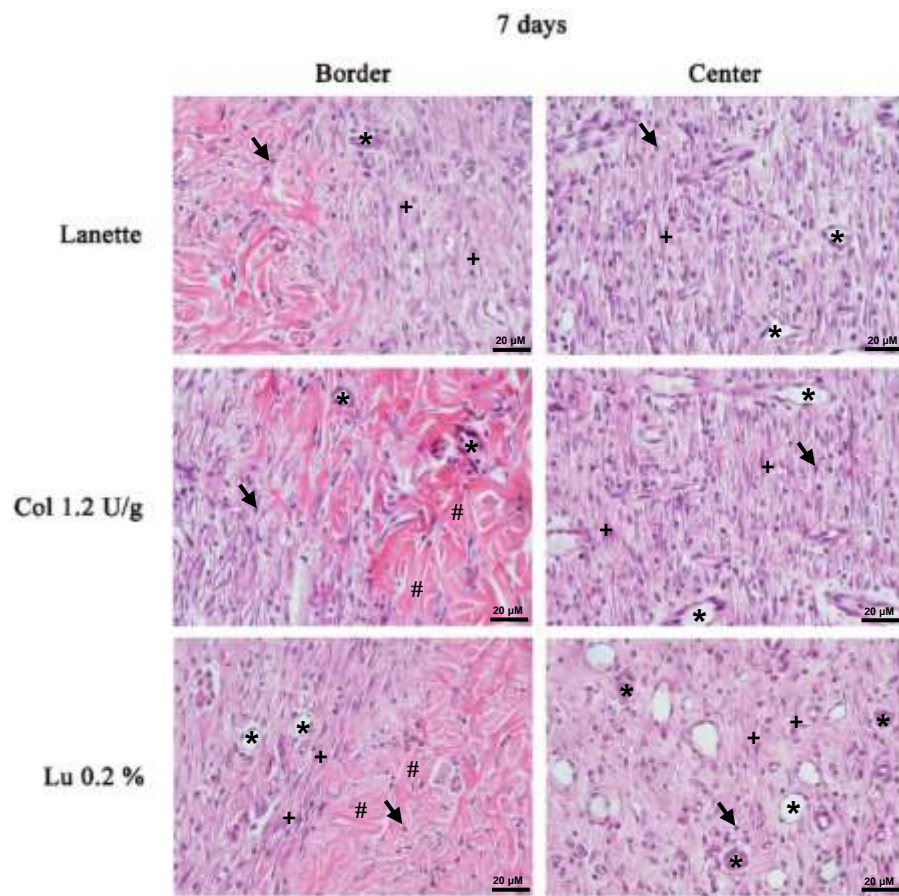
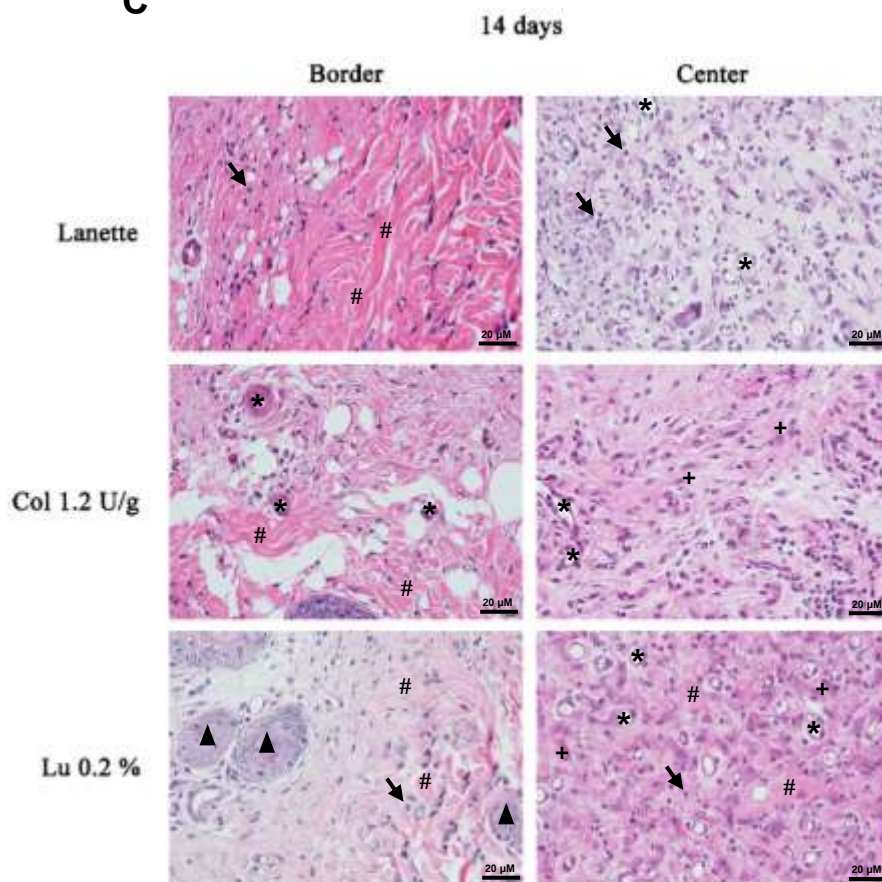
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Fig. 2

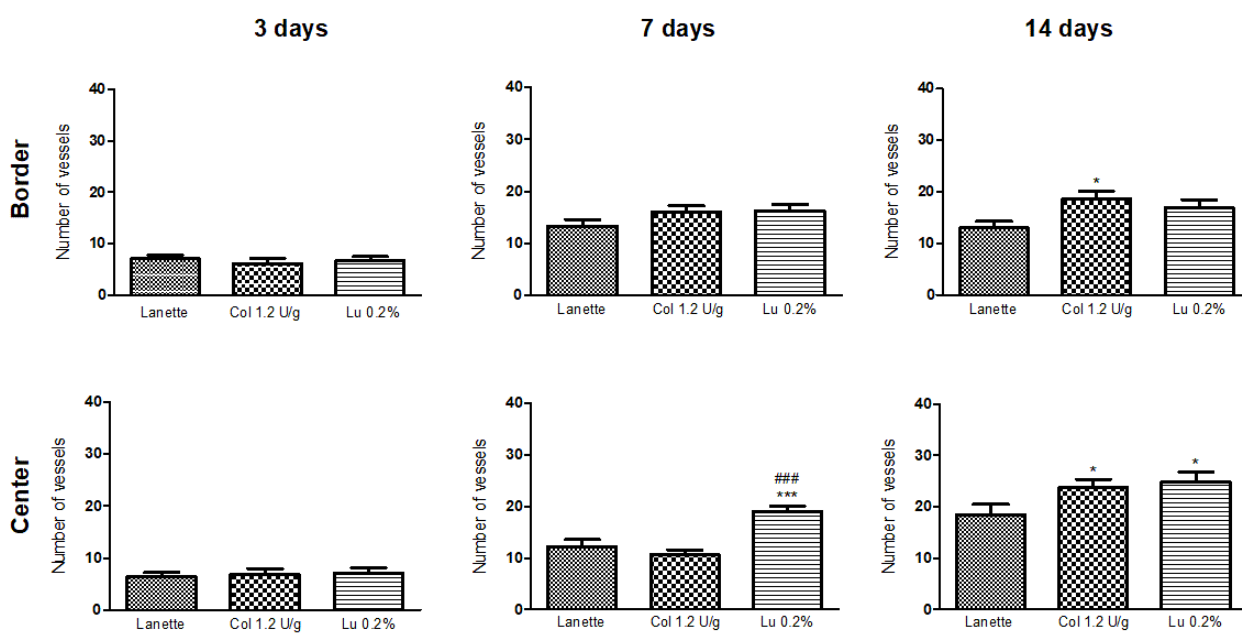


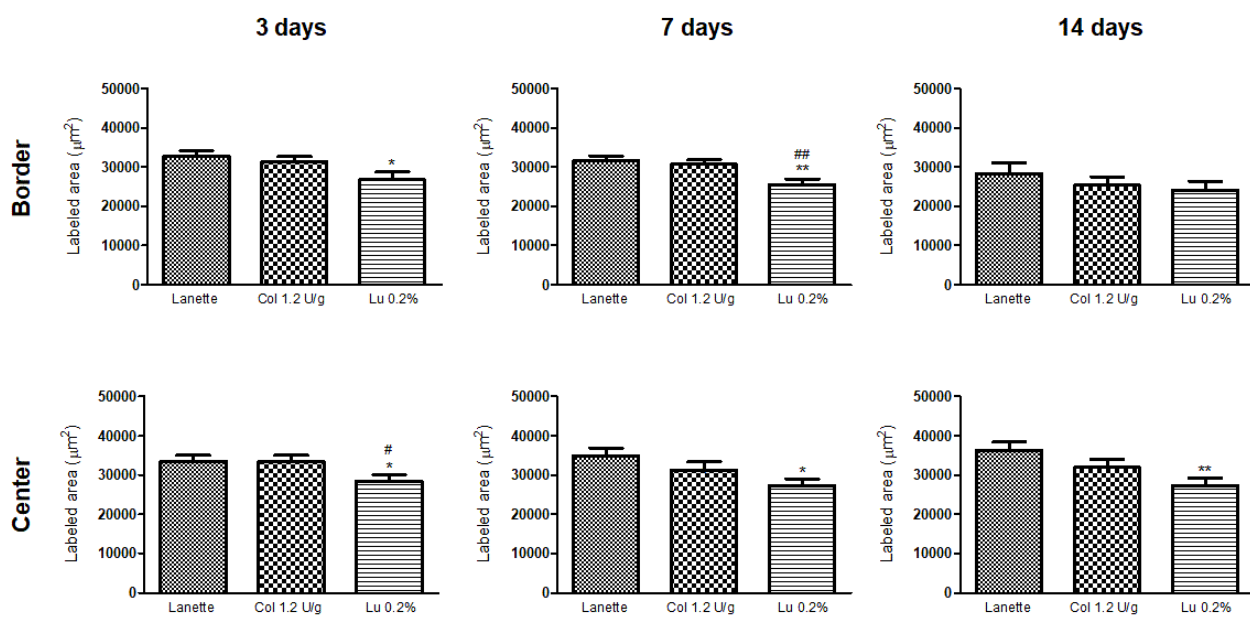
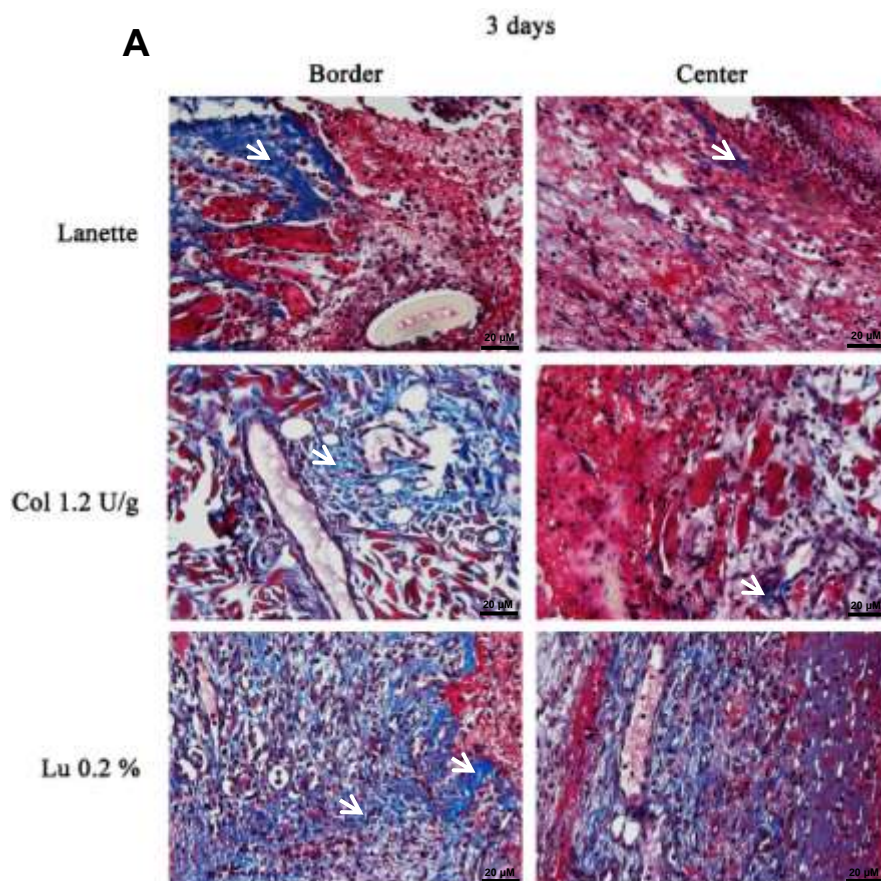
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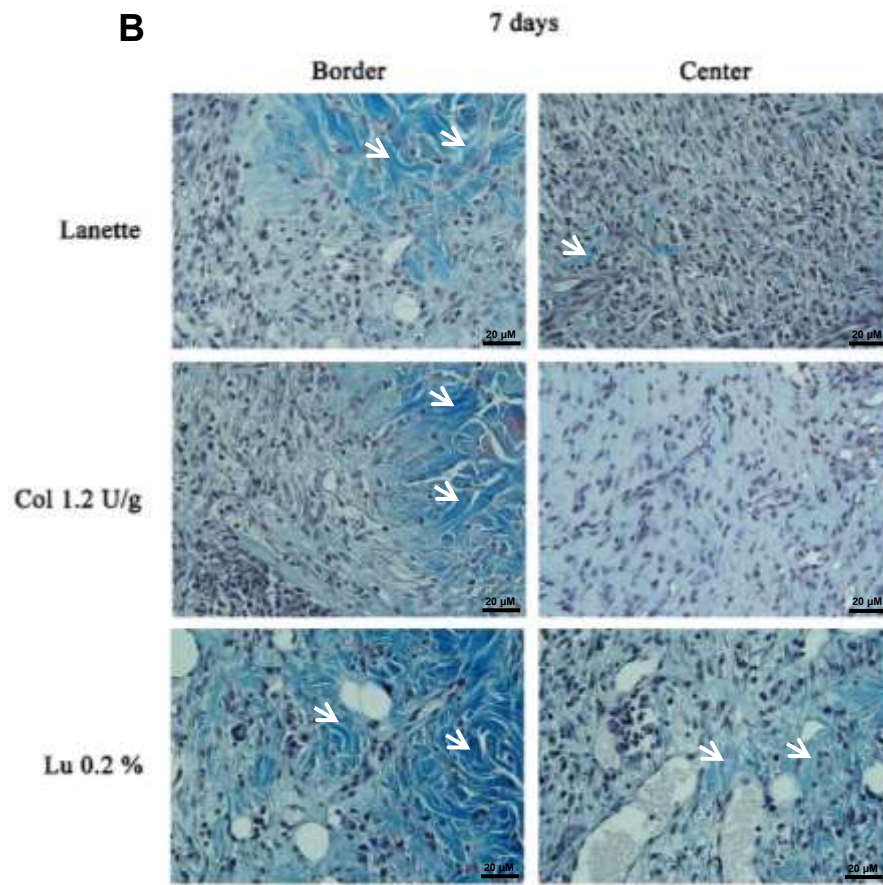
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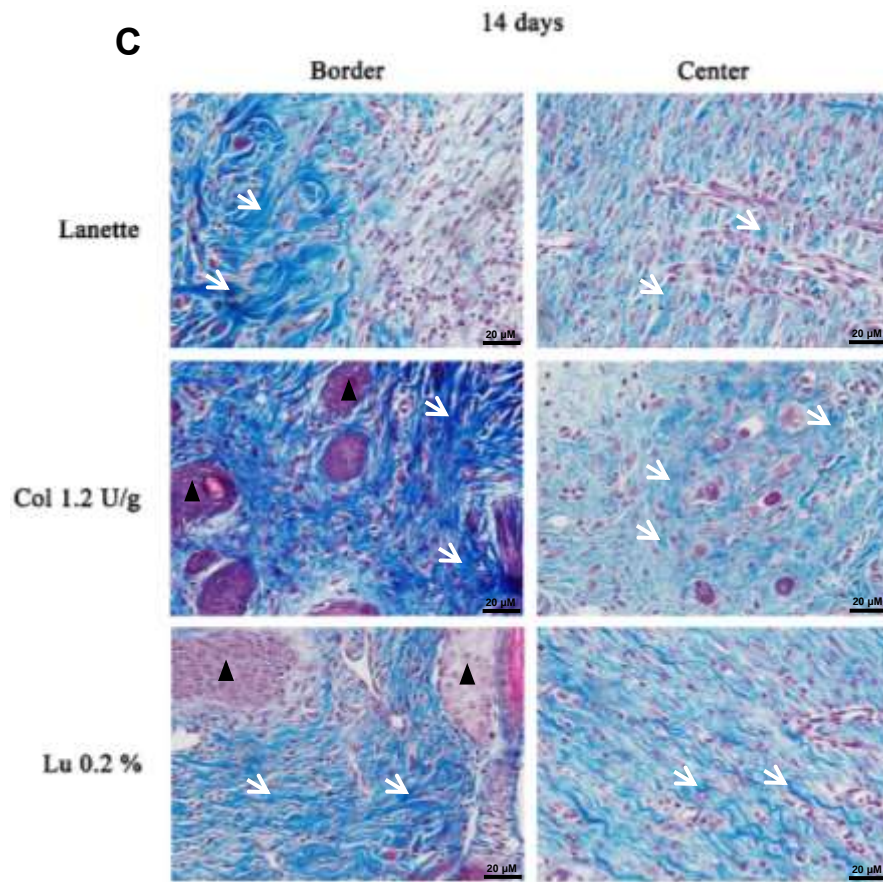


D



E**Fig. 3**





D

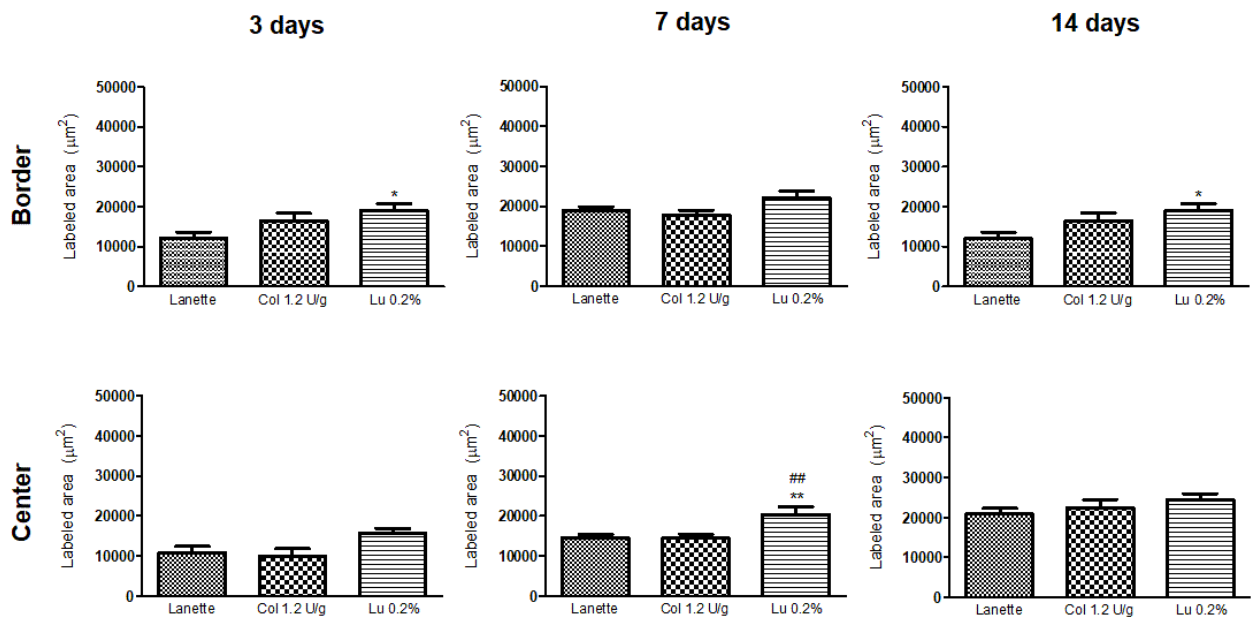
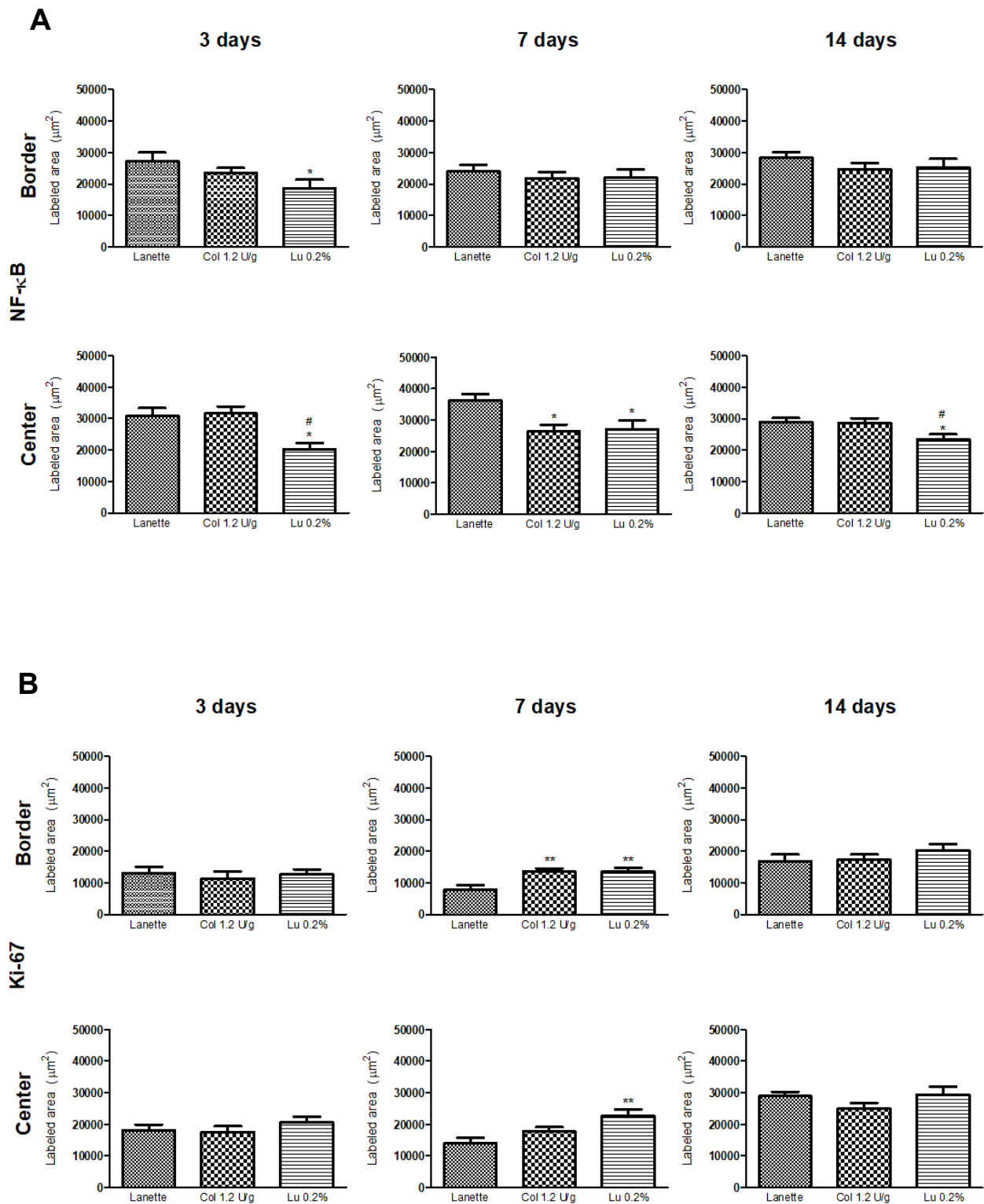


Fig. 4

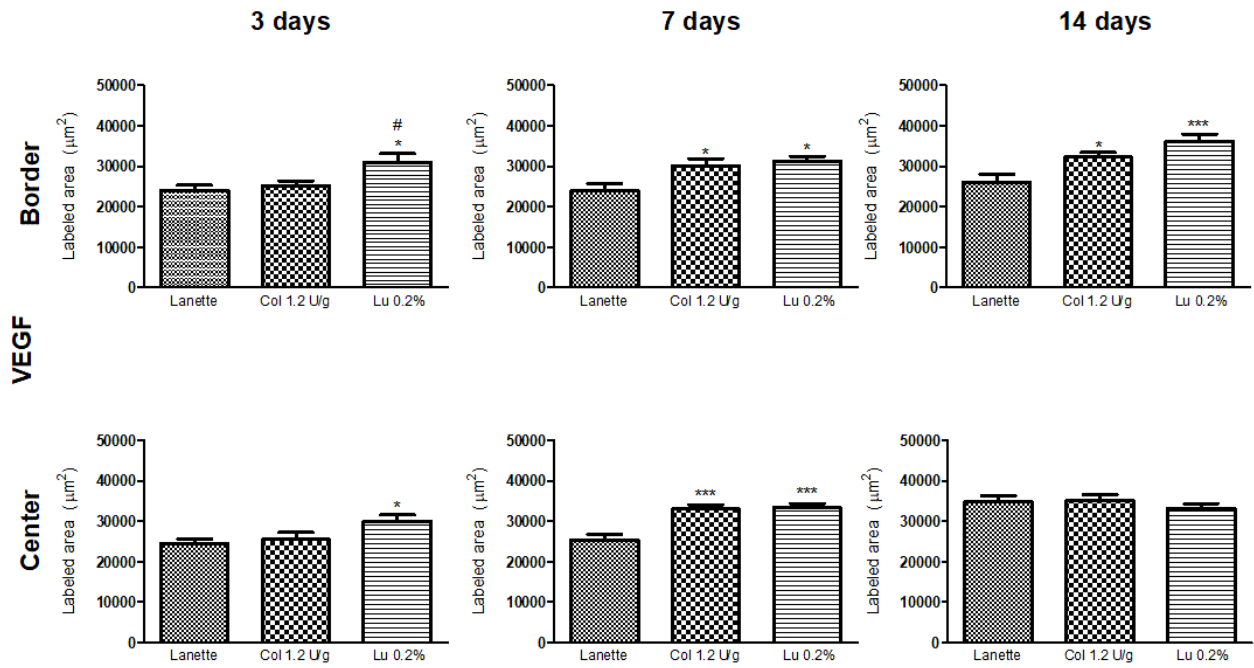
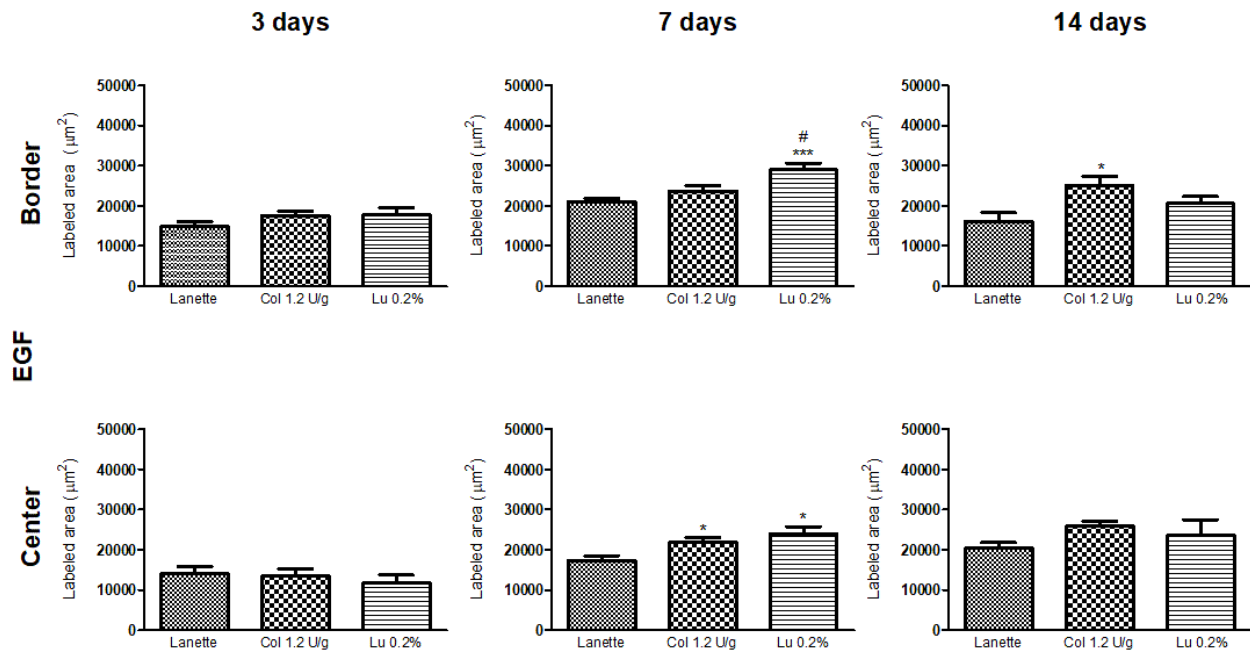
C**D**

Fig. 5

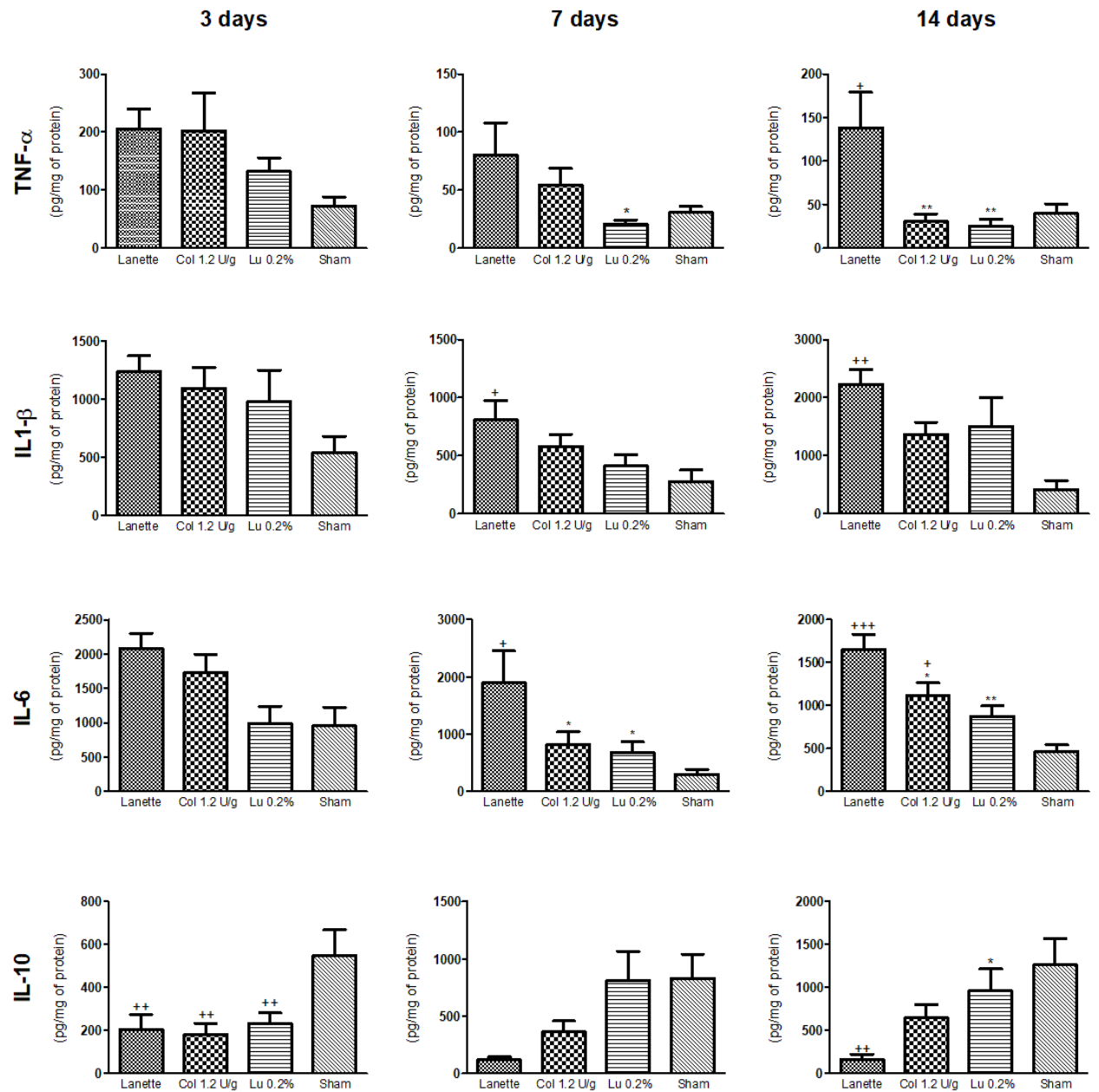
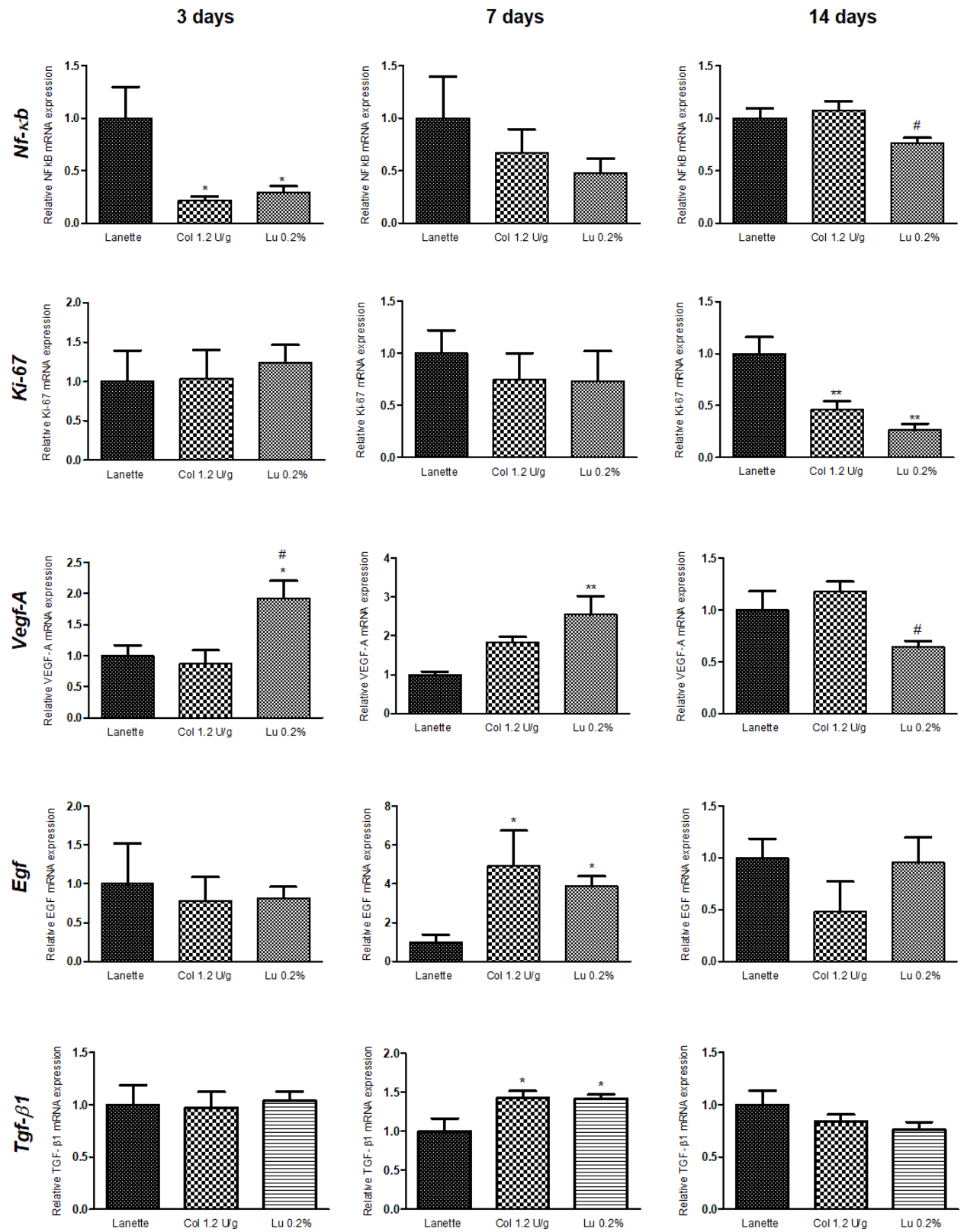
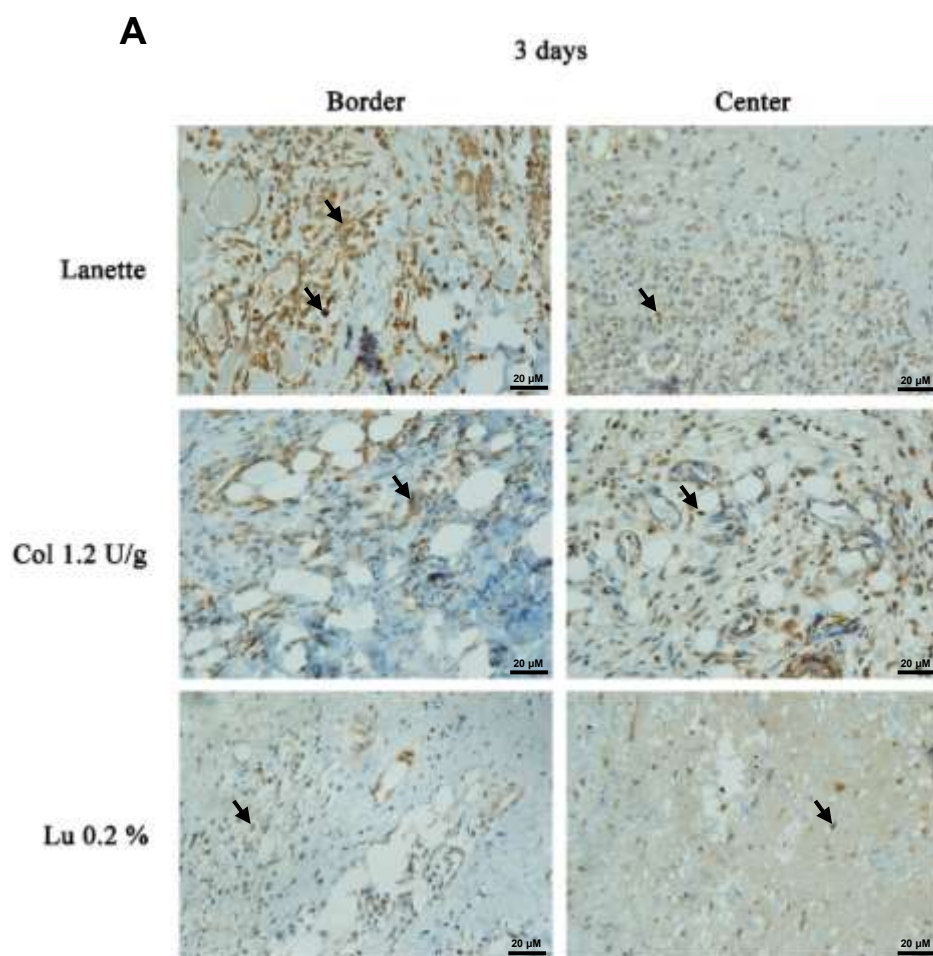
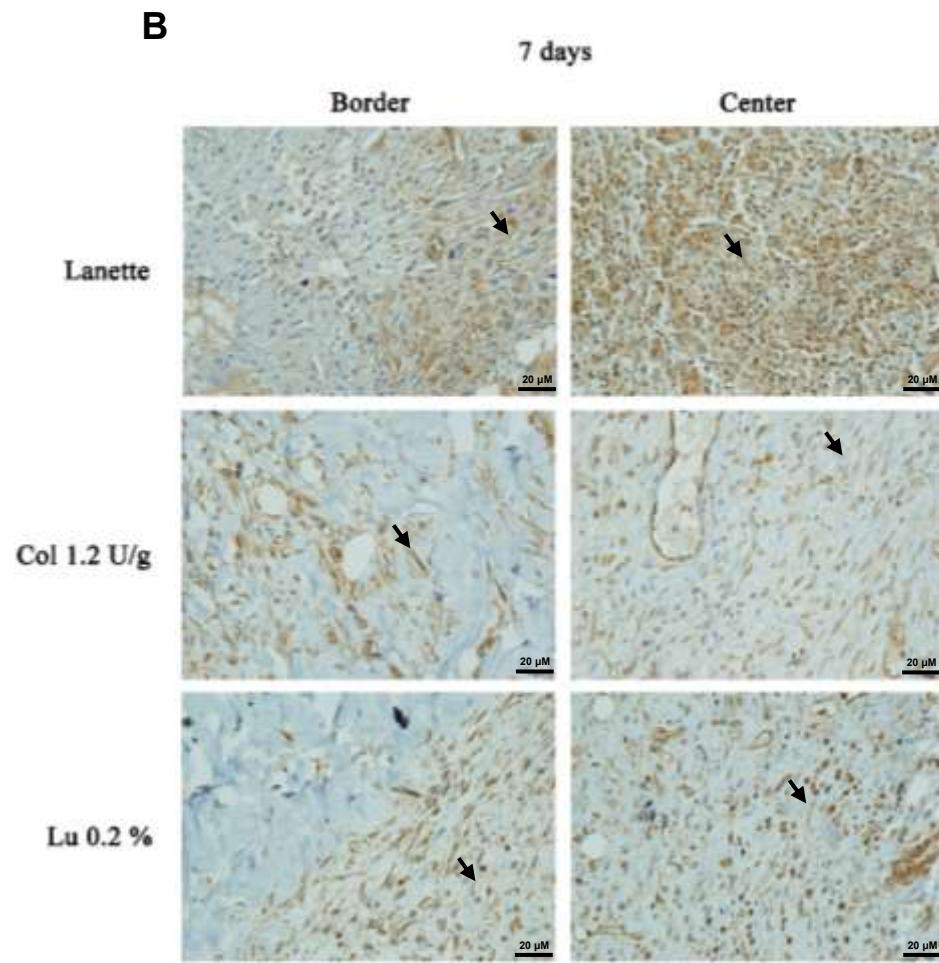


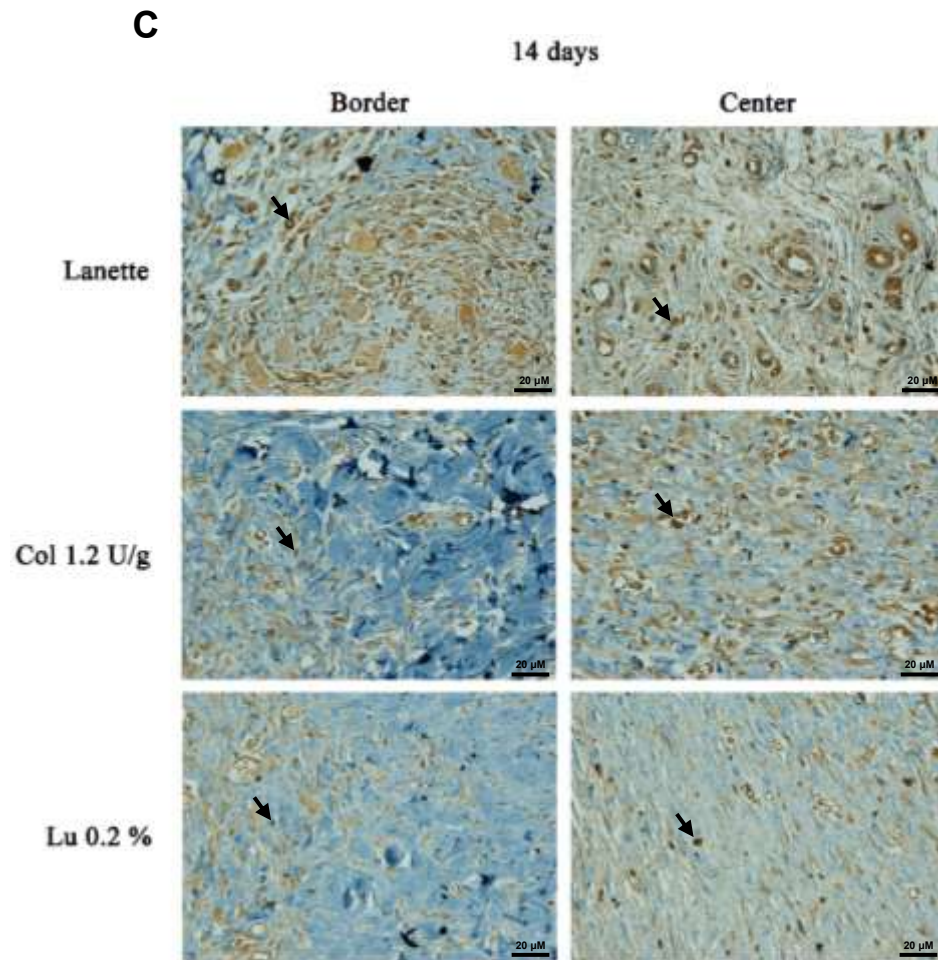
Fig. 6



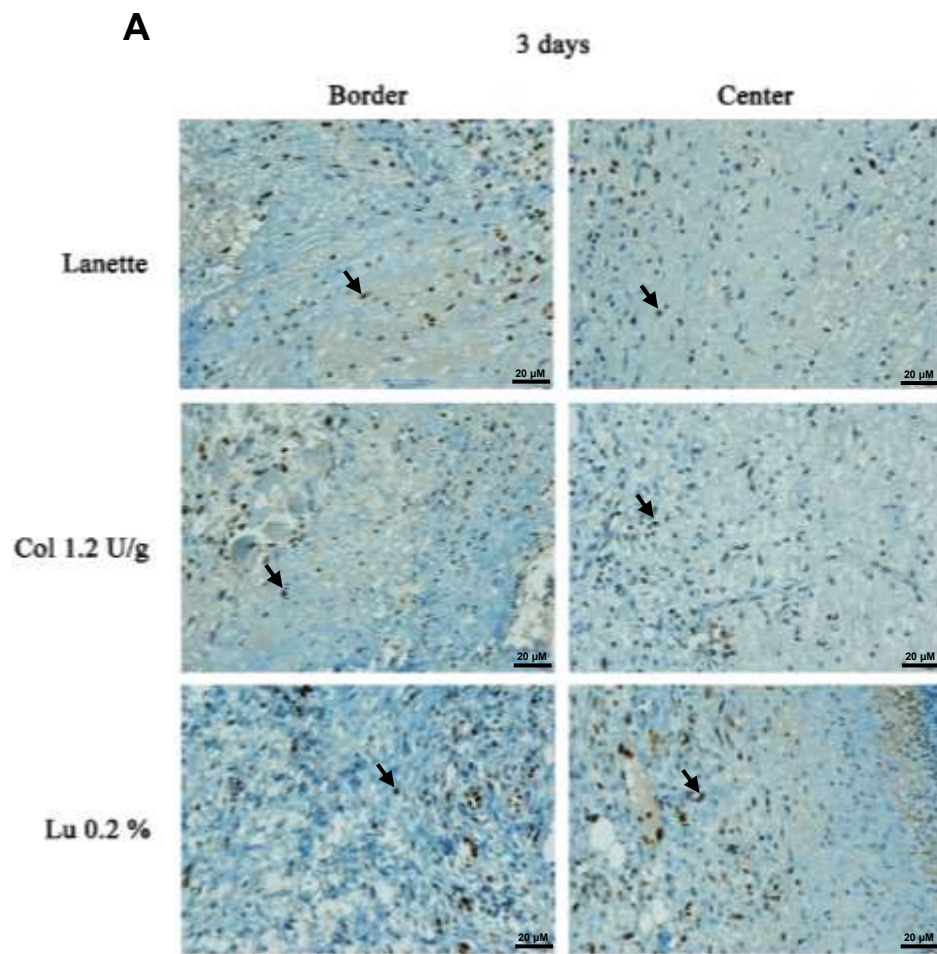
SUPPLEMENTARY MATERIALS

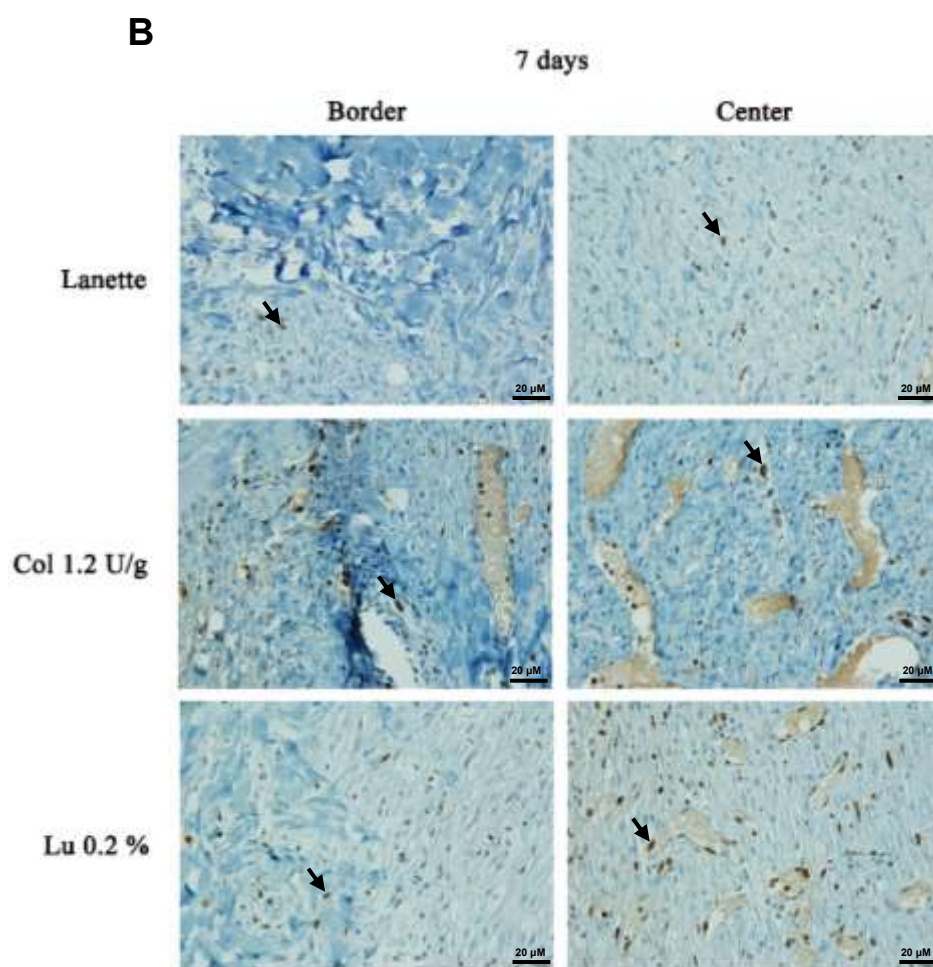


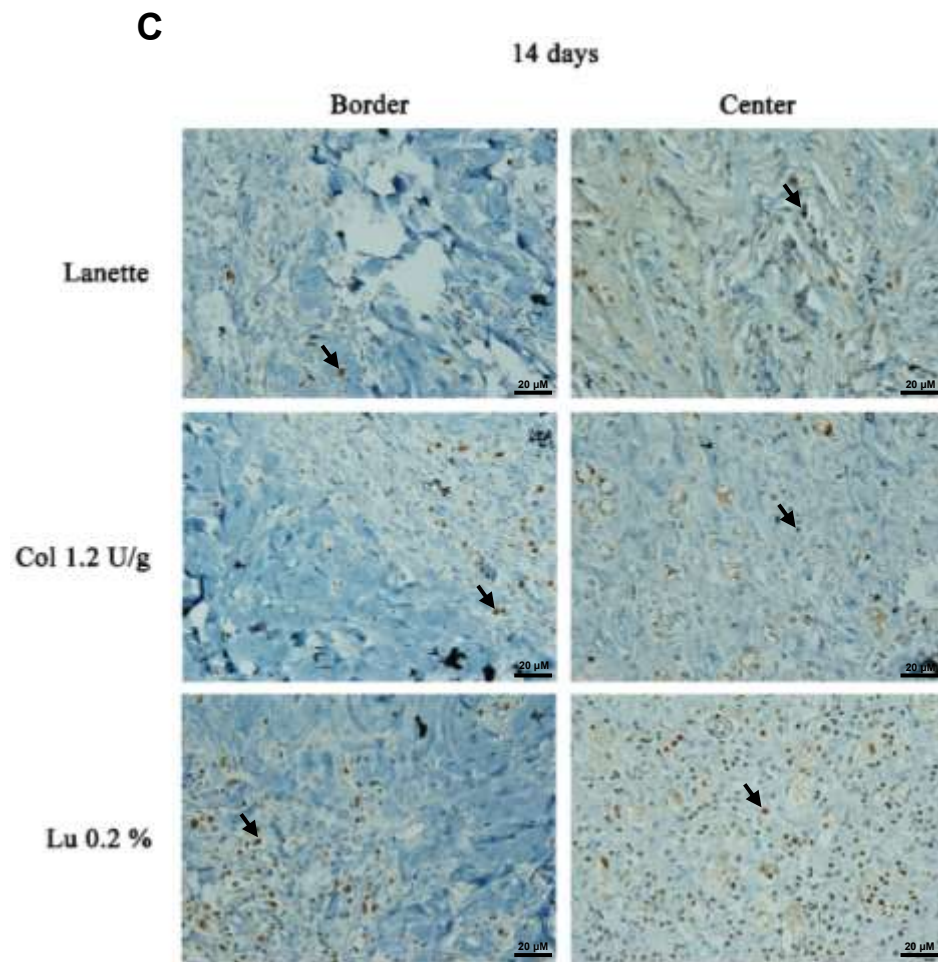




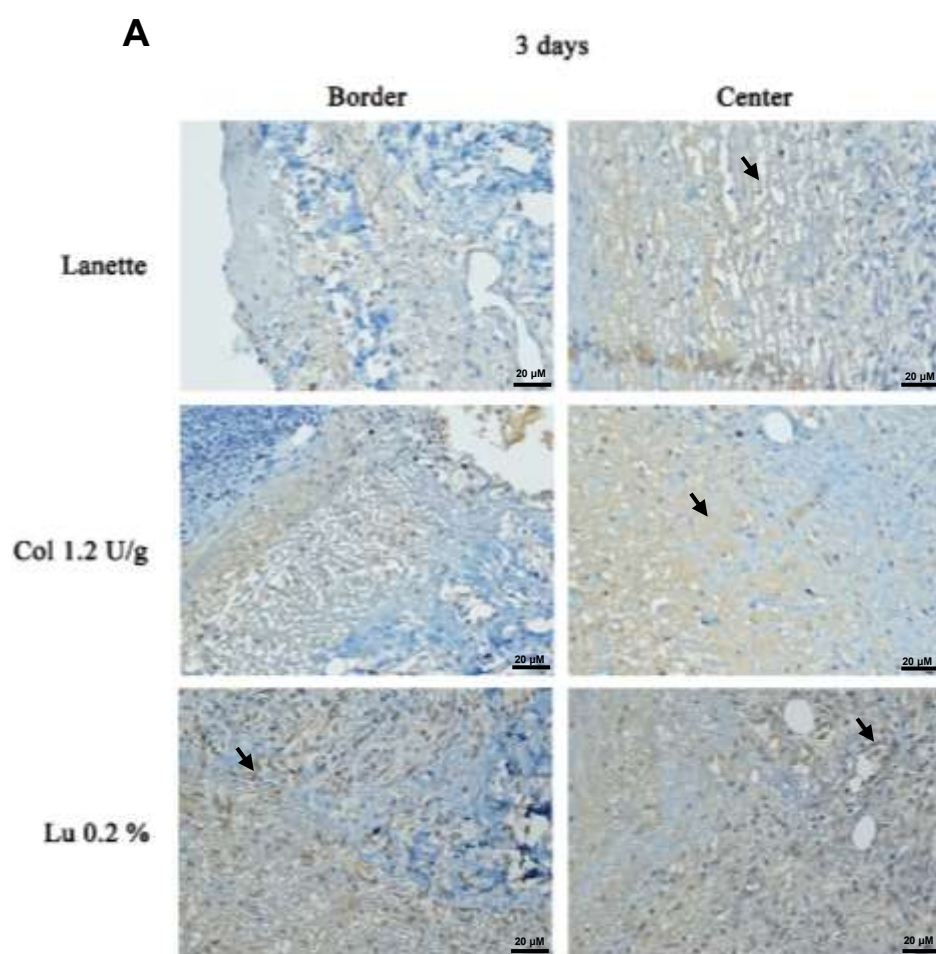
Supplementary Fig. S1: Photomicrography of the NF κ B immunostaining in the border and lesion center of rats topically treated with Lanette, Collagenase 1.2 U/g or lupeol 0.2% creams. A: 3 days after wound induction. B: 7 days after wound induction. C: 14 days after wound induction. Bar represents 20 μ m. Col: collagenase. Lu: lupeol. Black arrows indicate antibody staining against NF- κ B.

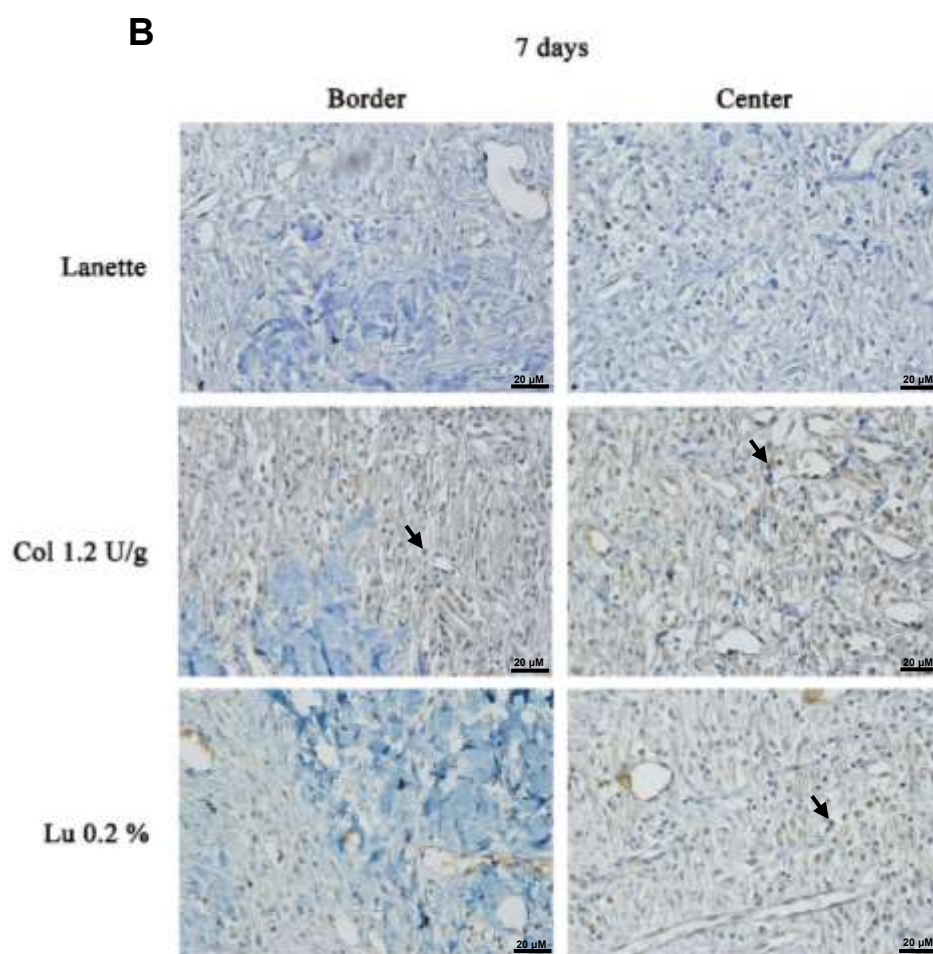


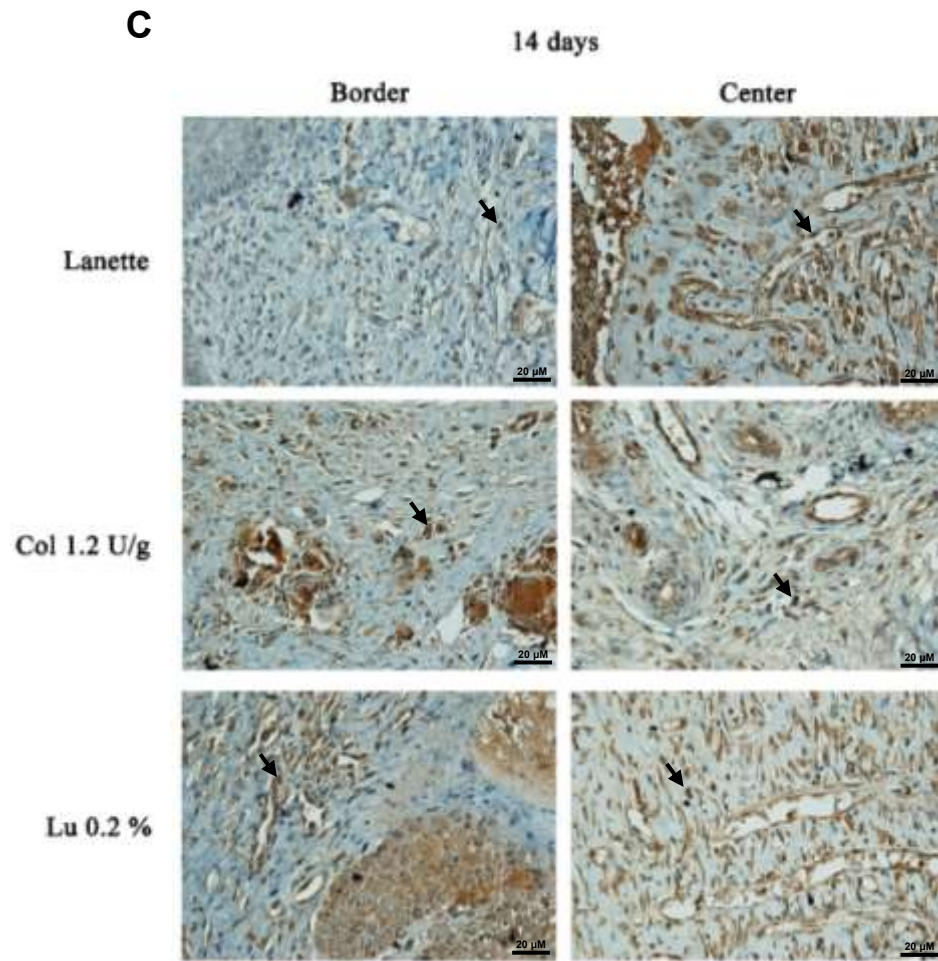




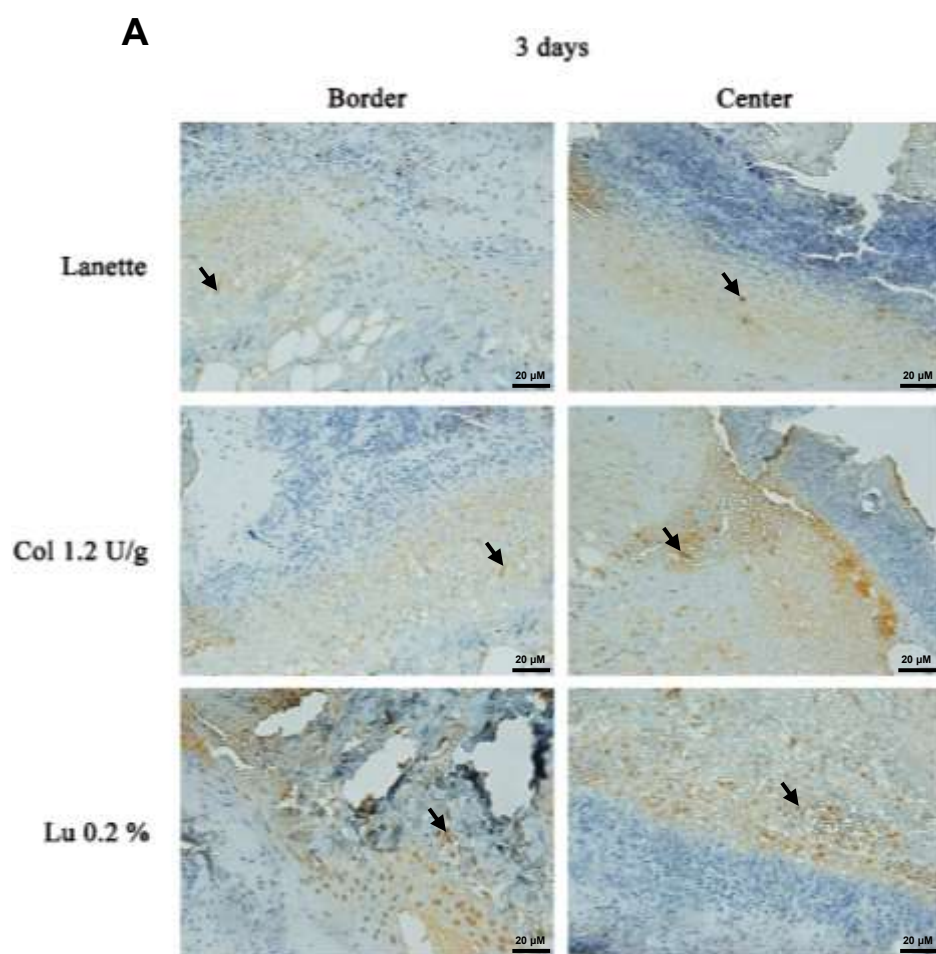
Supplementary Fig. S2: Photomicrography of the Ki-67 immunostaining in the border and lesion center of rats topically treated with Lanette, Collagenase 1.2 U/g or lupeol 0.2% creams. A: 3 days after wound induction. B: 7 days after wound induction. C: 14 days after wound induction. Bar represents 20μm. Col: collagenase. Lu: lupeol. Black arrows indicate antibody staining against Ki-67.

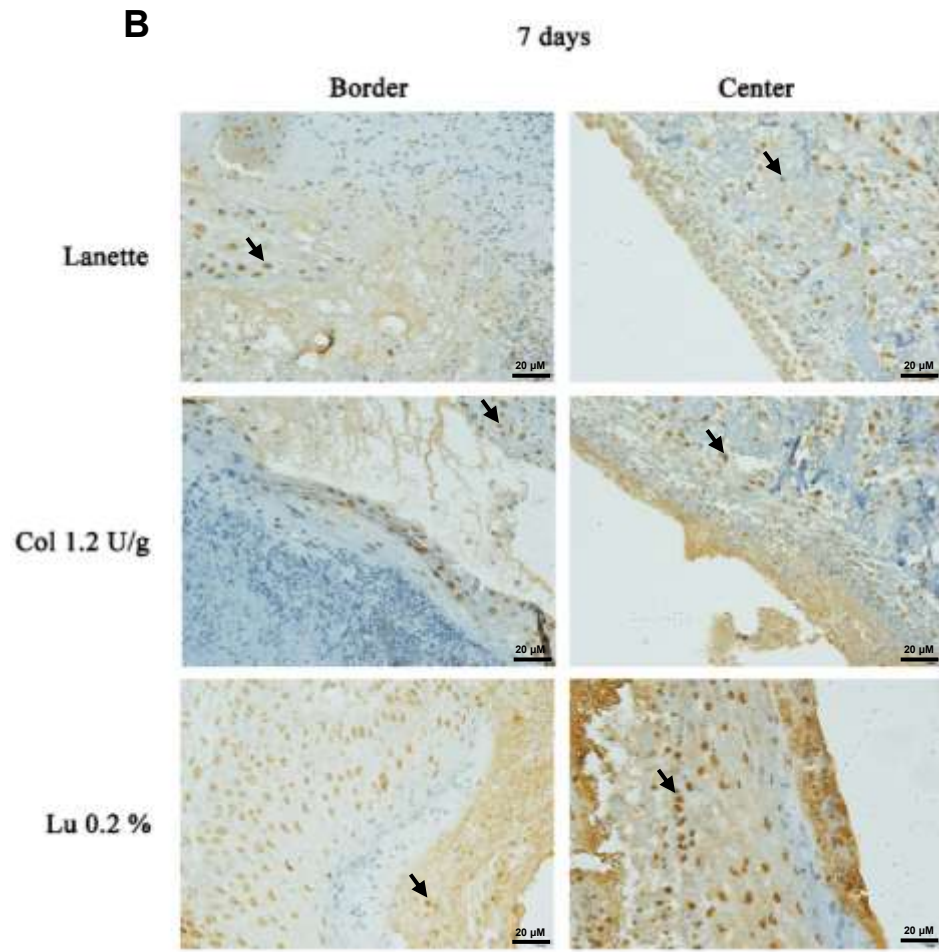


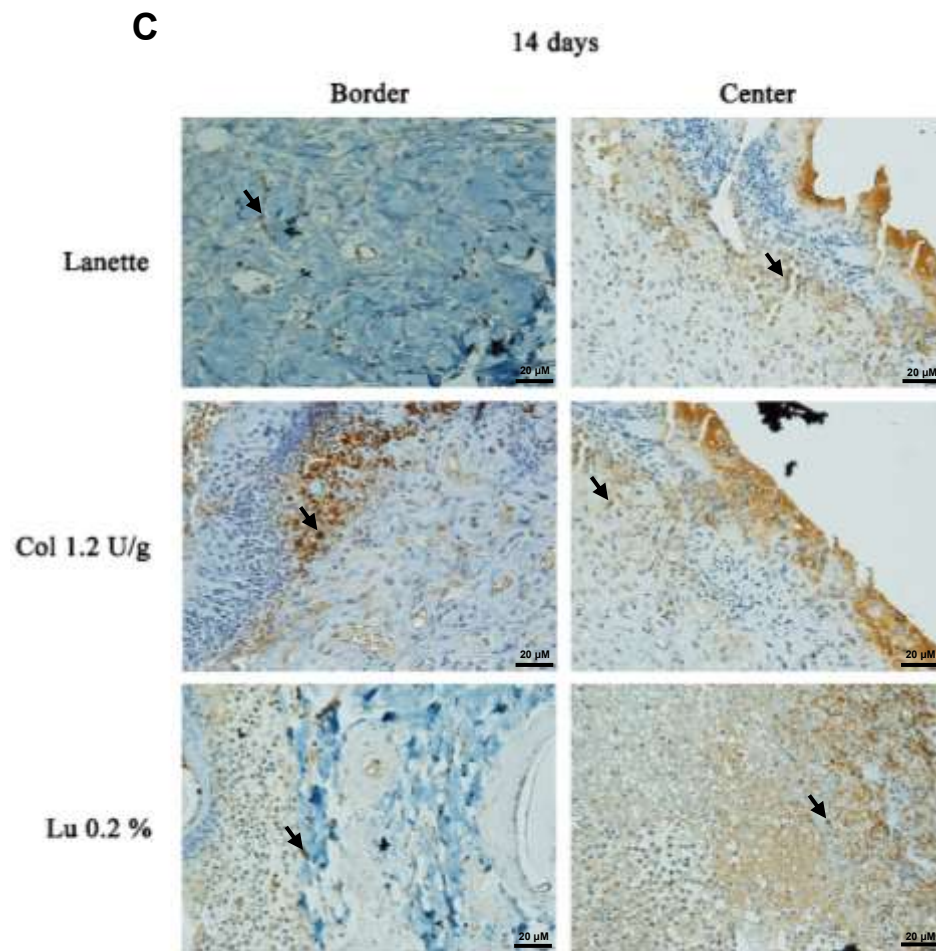




Supplementary Fig. S3: Photomicrography of the VEGF immunostaining in the border and lesion center of rats topically treated with Lanette, Collagenase 1.2 U/g or lupeol 0.2% creams. A: 3 days after wound induction. B: 7 days after wound induction. C: 14 days after wound induction. Bar represents 20μm. Col: collagenase. Lu: lupeol. Black arrows indicate antibody staining against VEGF.







Supplementary Fig. S4: Photomicrography of the EGF immunostaining in the border and lesion center of rats topically treated with Lanette, Collagenase 1.2 U/g or lupeol 0.2% creams. A: 3 days after wound induction. B: 7 days after wound induction. C: 14 days after wound induction. Bar represents 20μm. Col: collagenase. Lu: lupeol. Black arrows indicate antibody staining against EGF.

Ação do lupeol nas feridas cutâneas de ratos hiperglicêmicos

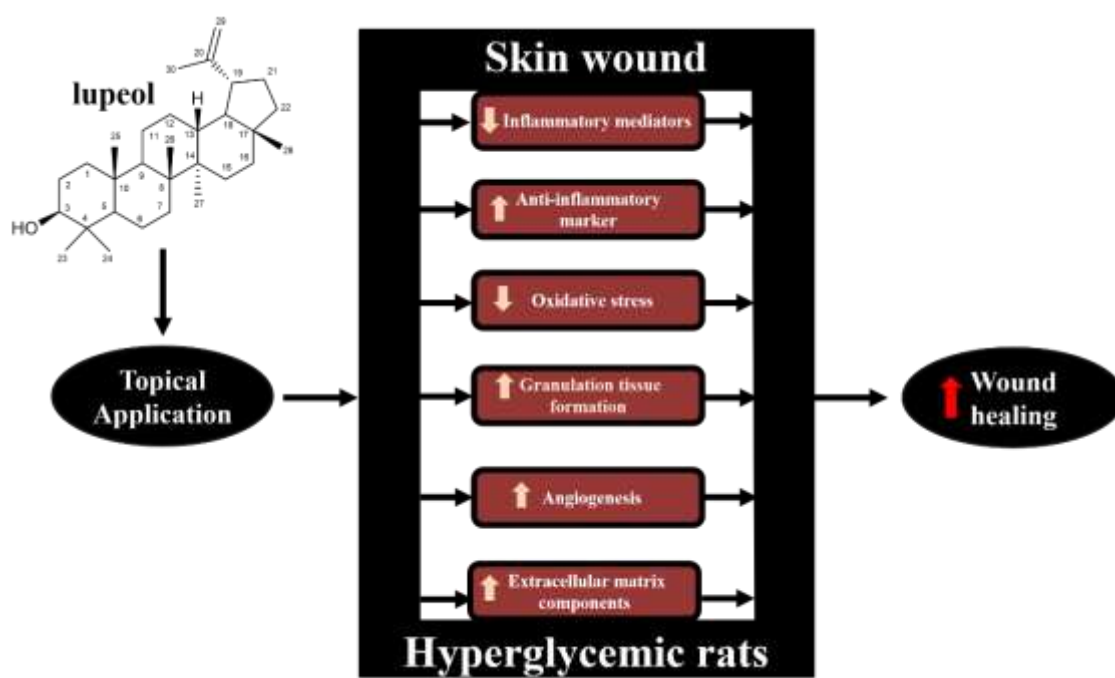
(Manuscrito referente aos resultados *in vivo* do lupeol escrito e formatado nos padrões da revista *Journal of Diabetes and its Complications*)



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Capítulo IV

Graphical abstract



ORIGINAL RESEARCH MANUSCRIPT

Lupeol enhances wound healing in streptozotocin-induced hyperglycemic rats with modulatory effects on inflammation, oxidative stress, angiogenesis and extracellular matrix components

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Abstract

Background: Impaired wound healing is a debilitating complication of diabetes that leads to significant morbidity, particularly foot ulcers. Natural products have shown effective in treating skin wounds. Lupeol is known to stimulate angiogenesis, fibroblasts proliferation and expressions of cytokines and growth factors involved in wound healing. *Methods:* The study is performed to evaluate the wound healing activity of lupeol in streptozotocin-induced hyperglycemic rats by macroscopical, histological, immunohistochemical, immunoenzymatic and molecular methods.

Results: Percentage of wound closure and contraction was increased in the lupeol-treated group when compared to lanette group. Histopathological observation revealed decreased

inflammatory cell infiltration, and increased proliferation of fibroblasts, vascularization and deposition of collagen fibers after lupeol treatment. Immunohistochemical analyzes showed decreased intensity of NF- κ B, and increased intensity of FGF-2, TGF- β 1 and Collagen III. ELISA results revealed downregulated IL-6 levels and upregulated IL-10 levels in response to lupeol. The mRNA expression of *Hif-1 α* , *Sod-2*, *Ho-1* were significantly increased in response to lupeol as compared to lanette whereas *Nf- κ b* and *Vegf-A* levels were decreased in relation to insulin and lupeol treatment.

Conclusion: These findings indicate that lupeol possesses wound healing potential in hyperglycemic conditions and may be useful as a treatment for chronic wounds in diabetic patients.

Keywords

Hyperglycemia; streptozotocin; wound healing; triterpene; lupeol

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease, which is characterized by elevated levels of blood glucose leading, over time, into, heart, blood vessel, eye, kidney and, nerve damage and failure of skin wounds repair to repair damage.¹ According to the World Health Organization (WHO), this disease affects 171 million people worldwide, and this number may be projected to reach approximately 366 million by 2030.² The most common complication in patients with diabetes is an altered skin wound healing process, leading to complications such as Diabetic Foot Ulcers (DFUs), which has caused major worldwide morbidity due to various clinical and socioeconomic issues.³ Several research studies have demonstrated that wound healing is delayed by hyperglycemia during DM.⁴⁻⁶

In patients with diabetes, the mechanism of delayed wound healing has multifactorial causes, including a prolonged inflammatory stage and postponed proliferation and remodelling stages.⁷ It has been reported that nuclear factor kappa B (NF- κ B) regulates the gene expression of several cytokines such as interleukin-1beta (IL-1 β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α) and interleukin-10 (IL-10), inducible nitric oxide synthase (iNOS), chemotactic and matrix proteins, immunological responses and cell proliferation.^{8,9} The persistence of the inflammatory reaction is associated with oxidative stress, which is one of the most common complications for the delayed wound healing in diabetics.¹⁰ The increased production of free radicals and decreased antioxidant activities of enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), heme oxygenase-1 (HO-1) and heme

oxygenase-2 (HO-2) may aggravate the situation leading to a delay in diabetic wound healing. Growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor-beta1 (TGF- β 1) and vascular endothelial growth factor (VEGF), and several molecules including hypoxia-inducible factor-1alpha (HIF-1 α), are involved in healing process by stimulating and activating cell proliferation via activation of various reactions, such as angiogenesis, re-epithelialization, and differentiation and production of extracellular matrix.¹¹⁻¹² In the present study, we investigated the role of the mediators involved in the healing process in hyperglycemic rat wounds.

There have been several attempts to accelerate the wound healing in diabetics, but so far, the therapeutic options available in the market are still limited due to the high cost, prolonged duration of treatment and side effects. Therapeutics involving new molecules isolated from natural, safer products in an attractive alternative.³

Several studies have suggested that plant-derived secondary metabolites are capable of promoting beneficial effects on wound healing in different models.¹³⁻¹⁵ A group of secondary metabolites that have attracted much attention in recent years are triterpenes.¹⁶ The pentacyclic triterpenoids belong to a class of C30 isoprenoids present in all parts of the plant, such as leaves, stems, pollen, seeds and fruits.¹⁷ They assist in wound healing mainly due to their inhibitory effects on the production and activity of inflammatory mediators and growth factors, resulting in contraction of the wound and increased rate of epithelization.¹⁸

Lupeol is a triterpene found in many medicinal plants, such as *Bowdichia virgiliodes* (Fabaceae). Previous studies have reported that lupeol has several bioactivities, including anti-diabetic, anti-inflammatory and antioxidant effects.¹⁹⁻²⁰ In particular, lupeol demonstrated an effect on cell proliferation *in vitro* through various mechanisms of action, including induction of differentiation²¹, activation of mitogen-activated protein kinases p38 (MAPK)²² and phosphoinositide-3-kinase-protein kinase (Pi3k/Akt).²³ These findings appear to be involved in the healing effects of lupeol. Harish et al. (2008)²⁴ showed that topical treatment with lupeol gel promoted cutaneous wound closure in normoglycemic rats by inducing the formation of granulation tissue, inhibiting the infiltration of macrophages and increasing the re-epithelialization. However, the efficacy of lupeol on hyperglycemia-induced impaired wound healing has not been investigated and the mechanisms underlying these effects remain unknown. In the present study, we investigated whether lupeol enhances wound healing in streptozotocin-induced hyperglycemic rats via reduction of the inflammatory process, and increase of markers involved in oxidative stress, angiogenesis, formation of granulation tissue and extracellular matrix remodeling.

2. Materials and methods

2.1. Chemicals and reagents

Streptozotocin (STZ) was obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA). Diagnostic kits for ELISA assay, such as TNF- α , IL-1 β , IL-6 and IL-10 were bought from R&D Systems, Minneapolis, MN, USA. Primary antibodies specific to NF- κ B, TGF- β 1, FGF-2, Collagen 3, β -actin, and anti-rabbit and/or anti-mouse secondary antibodies conjugated to horseradish peroxidase were attained from Santa Cruz Biotechnology (Santa Cruz, CA). All the chemicals used in the experiments were of an analytical grade.

2.2. Plant material, extraction and isolation of lupeol

Bowdichia virgilioides Kunth. (stem bark) was collected in December 2014 in the surroundings of Santa Rita, State of Paraíba, Brazil, a coastal area around the Atlantic Forest. A Voucher specimen (*Agra et Góis* 6243) was deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), and in the reference collection of the Laboratory of Pharmaceutical Technology from Federal University of Paraíba, João Pessoa, Brazil. Three kg of air-dried ground stem bark of *Bowdichia virgilioides* were exhaustively extracted with 95% alcohol solution. The extracted solution was filtered and the solvents were subjected to the evaporation method under reduced pressure with rotary evaporation at 40°C to obtain the final ethanolic extract (EtOHE, 250g). The EtOHE was partitioned using solvents in increasing polarity (hexane, chloroform and methanol). The hexane residue (49g) was subjected to repeated washings with acetone under stirring followed by filtration. The solid obtained was recrystallized from chloroform and hexane, resulting in white crystals which were performed by analyzing ^1H and ^{13}C NMR spectral data and identified as lupeol substance (3g).

2.3. Animals

Healthy male Wistar rats (*Rattus norvegicus*) of body weight around 250g $\pm$ 2g were procured from Central Animal House, UNESP, Botucatu and used for the study. The rats were housed individually in polyethylene cages in an experimental animal room with a 12 h light/dark cycle and maintained in standard conditions in animal house at a room temperature of 23 $\pm$ 2 °C and humidity at 55% $\pm$ 15%. They were fed with commercial rat fed a standard diet and water *ad libitum*. Prior 7 days to the start of the experiment, the rats were acclimatized to the animal house environment. All the experimental procedures were approved by the Ethics Committee on Animal Use (CEUA/IBB/UNESP) and the guidelines are followed strictly

throughout the experimental period in accordance with the experimental protocols (Protocol 610/2014) previously approved.

2.4. *Acute dermal irritation test*

Lupeol-based cream was used to evaluate skin irritation at concentration of 0.2% w/w lupeol as per OECD 402 directive. Healthy male *Wistar* rats weighing between 200-250g were divided into two groups: 1 group treated with lupeol cream at 0.2% w/w and another group treated with lanette cream (vehicle). After total removal of hairs in the dorsal region of the rats, treatments were performed over 14 days. The animals were observed twice daily for 14 days for any skin changes indicative of adverse reactions, such as irritation, edema, pruritus and erythema, and some signs of toxicity, such as tremors, convulsions, salivation, diarrhea, sleep and general pattern of behavior.

2.5. *Induction of experimental hyperglycemia*

Single intraperitoneal injection of streptozotocin (STZ) freshly prepared in citrate buffer (0.1 M, pH 4.5), at a concentration of 55 mg/kg body weight, was used to induce experimental hyperglycemia in overnight fasted rats. After two days of induction, animals with blood glucose level $\geq 250 \text{ mg dL}^{-1}$ were defined as hyperglycemic and kept under observation for another 7 days. Then, the hyperglycemic rats that maintained high glycemic levels were considered for the *in vivo* experiments. Furthermore, a weekly blood glucose test, body weight detection, and water and feed intakes assessment were used to ensure stable hyperglycemia status. This procedure followed the methodology described by Romana-Souza et al. (2009).²⁵

2.6. *Excision wound model and experimental design*

The hyperglycemic rats that maintained high glycemic levels were considered for the wounding induction. All rats were anesthetized with ketamine hydrochloride (0.08mg/100g) and xylazine (0.04mg/100g) by intraperitoneal (IP) injection. Then, all the animals received a single dose of ketoprofen (100 mg/kg, SC) as an ethical conduct to minimize postoperative discomforts. Next, after the shaving the hair on the back of each rat, the skin was sterilized by 70% alcohol to remove any type of contamination and a full-thickness excisional wound was created in the posterior dorsal region that extended through the panniculus carnosus of each animal using a 2cm diameter punch. To assess the cutaneous wound healing of lupeol-based cream, the rats were randomly divided into four groups (n=8), as shown below:

Group I - Topically treated with lanette cream (vehicle)

Group II - Topically treated with insulin-based cream 0.5 U/g (positive control)

Group III- Topically treated with 0.2% w/w lupeol cream (substance test)

Group IV- Sham group

Formulations was applied topically every day, once a day during a period. A schematic drawing of the experimental protocol is shown in figure 1. The rats were placed in their respective cages with one rat per cage.

2.7. Collection of blood samples

After 14 days of experimentation, all the animals were anaesthetized, and the 5 ml of blood samples were collected into ethylene diamine tetra-acetic acid (EDTA) embedded tubes, centrifuged and the serum was aspirated carefully for various biochemical assays, such as AST, ALT, γ -GT, Alkaline phosphatase, Creatinine and Urea.

2.8. Determination of macroscopic parameters

The wound area was measured daily through photographs with a professional camera and marked on a transparent sheet and the surface area of the wound was measured. After scanning, the wound area was measured using specific software Adobe Photoshop C5 - version 5 (Adobe Systems Inc., San Jose, California). The percentage of wound area was calculated using the following formula (Shenoy et al., 2011): % cicatricial = {initial lesion area - area of lesion on the day}/initial lesion area} *100. The clinical signs of the lesions, such as exudation, presence of crust and granulation tissue, were monitored by macroscopic examination and graded on a four-point scale: 0 - absent (0%), 1 - light (30%), 2 - moderate (30-70%) and 3 - intense (> 70%) based on the method from de Oliveira et al., (2014)²⁶, with modifications.

2.9. Histopathological examination

The tissue samples from the skin were fixed with alcohol, formalin and acetic acid and processed in paraffin. Skin tissues were sectioned (5 μ m). The inflammatory cell infiltration, proliferation of fibroblasts and number of blood vessels were assessed by hematoxylin and eosin (HE), and the deposition of collagen fibers by Masson's trichrome staining. For all analyzes, two distinct regions were photographed: border and the center of the wound. Ten photomicrographs of each sample were analyzed under a 40x magnifications according to the method as previously described (Gushiken et al., 2017)²⁷, being 5 for the border and 5 of the central lesion regions. All these parameters were quantified by the marked area count, totaling

an area of 100.000 μm^2 /slice. The photomicrographs were obtained with the software CellSens Standard (Olympus, USA) and the measurements were made using AVSoftBioView software.

2.10. Immunohistochemistry analysis

The immunohistochemical studies were performed with deparaffinized, rehydrated sections and fixed on silanized slides and submitted to antigen recovery by pressure (20 psi/125 °C). After washing the sections were blocked with bovine serum albumin (BSA), and then incubated with primary antibody against NF- κ B (Abcam, Cambridge, UK, 1:100 μL), FGF-2 (Abcam, Cambridge, UK, 1:500 μL), TGF- β 1 (Abcam, Cambridge, UK, 1:300 μL) and Collagen III (Abcam, Cambridge, UK, 1:100 μL) overnight at 4°C. The slices were incubated with appropriate biotinylated secondary antibodies. Then, the sections were stained with 3'-Diaminobenzidine (DAB) and counterstained with hematoxylin. Finally, ten photomicrographs at 40x magnifications of each slice were analyzed with software CellSens Standard (Olympus, USA), being 5 for the border and 5 of the central lesion regions (Gushiken et al., 2017)²⁷. The immunolabeled area was quantified totalizing 100.000 μm^2 /slice. The quantification was made with the software AVSoftBioView.

2.11. Enzyme-linked immunosorbent assay (ELISA)

The skin samples were homogenized in cold PBS supplemented with protease inhibitor cocktail (Sigma–Aldrich), centrifuged at 10000 rpm for 20 min at 4°C, and then the supernatants were used to measure the cytokines levels of TNF- α , IL-1 β , IL-6 and IL-10, as per the manufacturer's instructions from commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). Determination of total proteins were assessed by the biuret assay so that the data of the parameters analyzed are expressed relative to the amount of protein in the sample in milligrams (Krohn, 2002)²⁸.

2.12. RNA extraction and RT-qPCR

The total RNA of the skin samples (*R. norvegicus*) was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommendations and with adaptations, according to Nóbrega et al., (2010)²⁹. To avoid genomic DNA contamination, the RNA samples were treated with DNase I, RNase-free kit (Invitrogen, Carlsbad, CA, USA) prior to cDNA synthesis. The cDNA synthesis was performed with random hexamers using Superscript® II (Invitrogen, Carlsbad, CA, USA) with random hexamers according to standard protocols. The quantitative PCR reaction was carried out using designed and specific forward and reverse primers for *R. norvegicus* (table 1) to evaluate the expression of the genes for *Nf-*

κb, *Ki-67*, *Egf*, *Vegf-A*, *Ho-1*, *Ho-2*, *Gpx-1*, *Sod-2*, *Hif-1α*, *Nos-2*, *Angpt-4* and *Col3a1* among the different treatments used. For qPCR, Cts values were determined using SYBR Green kit (Invitrogen, Carlsbad, CA, USA). All qPCR reactions (10 µl) used 900 nM for each primer and 700 ng of total RNA. Each reaction was performed in duplicate in a StepOne system (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions, and relative gene expression profiles were calculated, according to the $\Delta\Delta C_t$ method as previously described (Vischer et al., 2003)³⁰. And for each gene of interest, the mRNA levels (Cts) were normalized by the reference gene, *β-actin* (table 1), and expressed with values relative to the Cts mean (point from which the system starts the quantification of the genetic material from the exponential phase threshold) of each group (ddCt - Ct normalized by means of the respective *β-actin* groups).

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.1; GraphPad software Inc. San Diego CA, California, USA). All the parametric data were expressed as mean $\pm$ standard error of the mean and the comparison between groups was performed by ANOVA followed by Newmann-Keuls test. The nonparametric data were expressed as median (maximum and minimum) and performed by the Kruskal-Wallis test, followed by the Dunn test. The values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Acute dermal irritation

Topical application of lupeol treatment at 0.2% w/w did not show any adverse reactions on rats skin, i.e. e., irritation, edema, pruritus and erythema, as compared to the control group. There were also no observed any signs of toxicity and behavioral changes.

3.2. Hyperglycemia status

All rats used in the present study showed the characteristic signs of hyperglycemia from the second day after the administration of STZ. Blood glucose levels increased significantly in all groups after 48 hours of streptozotocin administration and remained elevated throughout the experiment (Table 2). Other signs such as weight loss, polyphagia and polydipsia were also observed. Serum levels of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Gamma-GT, Alkaline Phosphatase and Urea were measured after 14 days of experimentation and showed significant changes in relation to sham group (supplementary material).

3.3. Wound closure

The evaluation of the lesion area revealed that the topical application of lupeol-based cream notably decreased wound size (Fig. 2A), with a significant increase in the percentage of lesion retraction in relation to lanette (Fig. 2B). The mean per cent lesion retraction was markedly higher in the lupeol-treated group from day 11 and this difference remained until day 15 at termination. The insulin-treated group showed a significant increase in the percentage of wound contraction on days 13 and 15 compared to the control group. Clinical parameters, such as coagulation, presence of crust and granulation tissue were analyzed in all rats. The macroscopic observations after 14 days of treatment showed that some lesions still presented little clot and granulation tissue. Lanette treatment still had a significant amount of crust adhered to the lesion, compared to lupeol-treated group, which did not present this crust, showing only the scar of the injured region (Table 3).

3.4. HE staining

Representative images of HE-stained wound sections are presented in Fig. 3A and allowed the counting of blood vessels, inflammatory cells infiltration and proliferation of fibroblasts in the border and the lesion center (Fig. 3B). It was not possible to observe any significant changes in the border using the evaluated parameters (Fig. 3B). However, we observed a significant reduction in inflammatory cell infiltration in the lupeol- and insulin-treated groups but not in the lanette group (Fig. 3B). In contrast, lupeol treatment was able to significantly increase fibroblast proliferation compared to lanette and insulin-treated groups (Fig. 3B). Both lupeol and insulin treatment significantly increased the number of blood vessels in the center of the lesions in relation to lanette (Fig. 3B).

3.5. Masson's trichrome staining

Analysis of total collagen fibers using Masson's trichrome method showed labeled area (μm^2) in the border and center regions of the lesion as shown in Fig. 4A. The deposition, orientation and organization of collagen fibers were considerably clearer in lupeol-treated wounds than in the other groups (Fig. 4C). Lupeol treatment caused a significant increase in the area labeled by collagen fibers in the border and the center of the lesion compared to lanette (Fig. 4C).

3.6. Effect of lupeol on immunostaining of NF- κ B, TGF- β 1, FGF-2, and Collagen III

Representative images of the immunohistochemistry of NF- κ B, TGF- β 1, FGF-2 and Collagen III in experimental wounds are shown in Figs. 5A, C, E, G. Lupeol treatment

significantly reduced the NF- κ B immunolabeling in the lesion border and center in relation to lanette treatment. Immunolabeling to localize TGF- β 1, lupeol treatment showed a significant increase of the antibody in the wound center in relation to lanette- and insulin-treated groups, as shown in Fig. 5D. Regarding FGF-2, there was significant increase in immunostaining of the both border and lesion center treated with lupeol and insulin cream compared to lanette treatment (Fig. 5F). Lupeol also significantly increased the Collagen III-immunolabelled area in the central region of the lesion as compared to lanette group (Fig. 5H).

3.7. Effect of lupeol on pro-and anti-inflammatory markers

mRNA expression of pro-inflammatory mediators, demonstrated a significant increase in the *Nf- κ b* expression in the insulin-treated group as compared to lupeol treatment and no significant change in the mRNA expression of *Nos-2*, as showed in Fig. 7A and 7G, respectively. On the other hand, the results obtained through ELISA showed that lupeol treatment caused a significant increase in TNF- α levels when compared to the sham group (Fig. 6A). In contrast, there was a significant reduction of IL-6 levels in the insulin and lupeol-treated groups in relation to lanette-treated group (Fig. 6C). IL-1 β levels were significantly increased only in the lanette-treated group as compared to sham group (Fig. 6B). However, IL-10 levels remained significantly elevated in the insulin-treated and lupeol groups relative to the lanette group (Fig. 6D). These results provide evidence for lupeol having anti-inflammatory potential by reducing *Nf- κ b* and IL-6, and increasing IL-10 levels.

3.8. Effect of lupeol on molecular markers of angiogenesis and cell proliferation

The mRNA expression of *Vegf-A*, *Hif-1 α* and *Angiopoietin-4* as markers of the angiogenic process in wound healing was evaluated. Lupeol treatment caused a reduction in *Vegf-A* expression compared to insulin treatment (Fig. 7D). On the other hand, lupeol effectively stimulated a significant increase in *Hif-1 α* expression compared to the lanette and insulin-treated groups, as shown in Fig. 7E. No significant change in *Angiopoietin-4* expression could be observed (Fig. 7F). There were also no significant changes in expression of *Ki-67*, *Egf* or *Col3a1*, markers involved in the proliferative wound healing phase (Fig 7B, 7C and 7M).

3.9. Effect of lupeol on molecular markers of oxidative stress

Analyzes of markers such as *Ho-1*, *Ho-2*, *Gpx-1* and *Sod-2*, related to oxidative stress through real-time gene expression were performed. The results showed that the lupeol and insulin-treated groups significantly increased the expression of *Sod-2* and *Ho-1* compared to

vehicle (Fig. 7H and 7J). There was no significant change in the expression of *Gpx-1* and *Ho-2* among the treated groups, as shown in Fig. 7I and 7L.

4. Discussion

We have demonstrated a role for lupeol, a pentacyclic triterpene, in wound healing in streptozotocin-induced hyperglycemic rats. The results showed markedly decreased expression/levels of NF- κ B and IL-6, and increased of IL-10 in the lupeol-treated group compared to the control group. Lupeol also increased the expression of FGF-2, TGF- β 1, *Hif-1 α* , *Ho-1* and *Sod-2*. In addition, we have histologically observed a better formation of granulation tissue with marked proliferation of fibroblasts, increased vascularization and deposition of collagen fibers in lupeol treatment. The results suggest a better and accelerated wound healing in hyperglycemic rats treated with lupeol.

Impaired wound healing represents one of the major diabetic complications in clinical practice. In general, hyperglycemia causes changes in the functioning of endothelial cells and, consequently, vascular dysfunction in the wounds, which can result in severe infections and in many cases leading to amputations.³¹ It is well known that changes in several endogenous factors contribute to the delayed wound healing in diabetics, including low production of growth factors production,³² mediators involved in the angiogenic response,^{33,34} macrophage function,³³ collagen synthesis, epidermal barrier function, keratinocyte migration and fibroblast proliferation.³² Animal models have documented the healing process and excisional wound models have become effective methods of preclinical testing in diabetic rats studies (Scherer et al., 2008).³⁵

Measurement of blood glucose levels is one of the most effective diagnostic methods in monitoring of diabetes. Thus, glycemic evaluation was chosen as the main parameter of disease progression in this study. A single injection of STZ was able to significantly increase the glycemia of fasted rats. This is because STZ is a diabetogenic agent, inducing necrosis by damaging pancreatic β -cell DNA³⁶ with effective decrease insulin production, which eventually generates a diabetic phenotype. In the present study, all animals after STZ injection maintained blood glucose levels above 250 mg/dL, even after any of the topical treatments, suggesting they did not interfere with the blood glucose levels of the animals throughout the experimental period. Previous studies have also reported similar observations, with effective wound healing activity in rats without decreasing glycemia of these animals.^{37,38} In our study, characteristic signs were noted from the third day of STZ administration. We observed a decrease in body

weight gain, polyphagia, polydipsia and polyuria in all animals, which according to the literature are the main clinical manifestations of diabetes.^{39,40}

Studies conducted through clinical trials indicated that wound healing in patient with diabetes occurs in three phases: the inflammatory phase due to high release of pro-inflammatory mediators and impairment of the immune system; the proliferative phase through the proliferation of fibroblasts, collagen fibers deposition, formation of new blood vessels; and the remodeling phase involving restoration and repair of injured tissue.^{41,42} For a successful therapy, it would require a drug that accelerates the wound healing with potential involvement in all phases of the process, with low cost and less side effects. The findings of the present study showed that lupeol was effective in promoting skin wound healing in hyperglycemic rats, by reducing the lesion size from day 11, accompanied by wound contraction. Our results are in accordance with Harish et al., (2008)²⁴ who showed that lupeol-based gel enhanced skin wound healing in normoglycemic rats through excision, incision and dead space wound models.

It is known that diabetic wounds show exacerbated production of inflammatory cells at wound sites, which may limit wound closure efficacy.⁴³ Our data showed a reduction of inflammatory cells in diabetic rats wound site treated with lupeol-based cream, specifically in central region of the lesion. Chronic inflammation is also one of the main factors contributing to the delayed wound healing. Previous studies have shown that hyperglycemic wounds are capable of causing a decrease in the expression of anti-inflammatory cytokines, such as IL-10 and TGF- β , and an increase in the expression of proinflammatory cytokines, such as TNF- α , IL-1 β and IL-6 as well as inflammatory cells.^{44,45} In the present study, lupeol treatment favorably regulated these mediators by reducing pro-inflammatory cytokines such as IL-6 and by upregulating the levels of IL-10, an anti-inflammatory cytokine.

In addition to cytokines, studies report the involvement of NF- κ B with chronic inflammation in diabetic wounds. Once activated, NF- κ B translocates from the cytoplasm to the nucleus of the cell, where it binds to DNA and promotes a transcription of several proinflammatory mediators, including NOS-2 or iNOS gene expression.⁴⁶ It is known that overproduction of iNOS during hyperglycemia is correlated with prolonged inflammation within the dermis and delays wound healing.⁴⁷ The results of the present study showed that topical application of lupeol reduced the expression of NF- κ B, but not iNOS expression and enhanced wound healing by preventing prolonged inflammation. Our data are similar to the recent study published by Pereira-Beserra et al., (2018),²³ who showed that lupeol successively promoted wound healing in human epidermal keratinocytes via anti-inflammatory mechanisms

through inhibition of pro-inflammatory mediators such as NF- κ B. Thus, the study indicates an anti-inflammatory effect of lupeol in hyperglycemic wounds

A persistent inflammatory state through high infiltration of inflammatory cells into the wound site also produces oxidative stress by generating several reactive oxygen species (ROS) and reactive nitrogen species, capable of producing various cytotoxic effects on cells.^{48,49} SOD and CAT are the natural cellular antioxidants that can play a crucial role in oxygen defense metabolism by converting superoxide ions to water and molecular oxygen.⁵⁰ Another essential enzyme expressed under conditions of elevated oxidative stress is HO-1.⁵¹ Studies report that, in addition to its potent antioxidant effect, HO-1 also presents pro-angiogenic and anti-inflammatory properties.^{52,53} The results obtained evidenced a significant increase in the gene expression of both enzymes, *Sod-2* and *Ho-1*, in lupeol- and insulin-treated groups, suggesting an antioxidant effect by neutralizing the action of the free radicals generated in the wound.

Wound healing quality depends on the granulation tissue formation that is directly affected in diabetics. Increase in granulation tissue formation requires an increase in cell proliferation of fibroblasts and endothelial cells, and increased collagen synthesis.^{54,55} It is well documented that collagen is one of the major and most important components of granulation tissue and its synthesis occurs in fibroblasts, which is dependent on TGF- β 1, a crucial factor for proper wound healing.⁵⁶ The role of lupeol on the proliferative phase was validated through histopathological studies, where it was possible to observe an increase in the proliferation of fibroblasts in the central region of the wounds, an increase in the formation of collagen fibers in both the borders and the center of the lesion, and elevated immunostaining of collagen type III in wound center.

Fibroblasts play a predominant role during the wound contraction process.⁵⁷ Wound contraction is a fundamental phenomenon in the complete wound closure, and is associated with differentiated myofibroblasts, specialized cells present in granulation tissue.⁵⁸ FGFs (fibroblast growth factor) stimulate the proliferation and migration of various cells involved in wound healing. They also stimulate collagen synthesis, epithelialization and neovascularization.⁵⁹ FGF-2 is one of the most important growth factors of the proliferative phase, produced mainly by keratinocytes and stimulates proliferation and migration of fibroblasts, new blood vessels formation and ECM production.⁴² Our results showed a strong expression of FGF-2 in border and center of hyperglycemic lesions treated with lupeol.

Angiogenesis is important for the maintenance of tissue health and quicker wound healing. Impairment in new blood vessels formation delays the healing process and induces severe ulcerations. Moreover, cutaneous blood flow and neovasculogenesis are completely

impaired in diabetes, which in turn affects the healing process of wounds. Improved angiogenesis was demonstrated in our findings through histopathological analyses in the central region of the lesions after treatment with lupeol cream. Numerous molecules, growth factors and cytokines are involved in regulating the angiogenesis, such as VEGF, FGF-2 and HIF-1 α .⁶⁰ HIF-1 α has a crucial role in, since it is necessary for expression of multiple angiogenic growth factors, cell motility, and recruitment of endothelial progenitor cells.⁶¹ In our study, increased expression of TGF- β 1 and *Hif-1 α* in the lupeol-treated group possibly explains the formation of new vessels observed in histopathological analyzes.

In the present study, as expected, the application of the insulin-based cream produced some beneficial effects compared to the control. Our data are consistent with Lima et al. (2012)⁶² who showed an improvement in diabetic wound healing by insulin-based cream. Insulin treatment caused an increase in the percentage of wound contraction from the 13th day, while the lupeol had an effect from the 11th. Although most effects on molecule expression showed similarities to lupeol-treated group, insulin treatment differed by not increasing the levels of TGF- β 1 and *Hif-1 α* , crucial mediators in the angiogenic process.

Overall, the study demonstrated through an excisional wound model in streptozotocin-induced hyperglycemic rats, that topical application of lupeol accelerates cutaneous wound healing by promoting anti-inflammatory and antioxidant effects, neovascularization, and granulation tissue formation. Thus, lupeol, a pentacyclic triterpene, isolated from the bark of stem of *B. virgilioides*, might be considered as a therapeutic agent for wounds in patients with diabetes.

5. Conclusion

Wound treatment using lupeol-based cream effectively enhanced the healing process in hyperglycemic rats through the anti-inflammatory effect of NF- κ B signalling pathways, as suggested by a reduction of inflammation-associated mediators, such as IL-6 (proinflammatory cytokine), and elevated IL-10 levels (anti-inflammatory cytokine). Histopathological findings also revealed a decrease in the inflammatory process, and faster neovasclogenesis and proliferation of fibroblasts in the lupeol-treated group. The involvement of *Hif-1 α* , FGF-2 and TGF- β 1, crucial to angiogenesis process as well as fibroblast infiltration, proliferation, and migration in wound sites efficiently mediated the injury and returned the wound to its original state. In addition to this, lupeol also minimized the oxidative stress and improved antioxidant status of through increased mRNA expression of *Ho-1* and *Sod-2*. This study provided us with

good scientific evidence that the application of lupeol cream is promising for treating wounds in hyperglycemic rats.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version.

Abbreviations

Akt: protein kinase B

ALT: Alanine aminotransferase

ANOVA: one-way analysis of variance

AST: aspartate aminotransferase

CAT: Catalase

cDNA: complementary DNA

Cq: quantification cycle

Ct: cycle threshold

DAB: 3'-diaminobenzidine

ddCT: overall average of cycle threshold

EGF: epidermal growth factor

ELISA: enzyme-linked immunosorbent assay

EtOHE: ethanolic extract

FGF-2: fibroblast growth factor-2

FW: forward

GPx: glutathione peroxidase

HE: hematoxylin and eosin
HIF-1 α : hypoxia-inducible factor-1alpha
HO-1: heme oxygenase-1
HO-2: heme oxygenase-2
IL-10: interleukin 10
IL-1 β : interleukin-1beta
IL-6: interleukin 6
iNOS: inducible nitric oxide synthase
IP: intraperitoneal
Ki-67: antigen Ki-67
MAPK: mitogen-activated protein kinases
mRNA: messenger RNA
NCBI: National Center for Biotechnology Information
NF- κ B: factor nuclear kappa B
NOS-2: nitric oxide synthase-2
PCR: polymerase chain reaction
Pi3k: phosphoinositide-3-kinase-protein kinase
RT-qPCR: quantitative reverse transcription PCR
RV: reverse
SC: subcutaneous
SEM: standard error of the mean
SOD: superoxide dismutase
STZ: streptozotocin
TGF- β 1: transforming growth factor-beta 1
TNF- α : tumor necrosis factor alpha
VEGF: vascular endothelial growth factor

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Table legends

Table 1: Sequence of primers used in RT-qPCR.

Table 2: Blood glucose levels in different groups before and after administration (i.p) of streptozotocin (STZ). *** $p < 0.001$ vs. sham group, using ANOVA followed by the Newman-Keuls test.

Table 3: Clinical parameters of hyperglycemic cutaneous wound after topical treatment with Lanette, Insulin 0.5 U/g and lupeol 0.2 % (n=8) in rats. Data are expressed as median (minimum and maximum) and analyzed by the Kruskal-Wallis test, followed by the Dunn test. * $p < 0.05$ vs.

Figure legends

Fig. 1: Overview of experimental protocol for wound healing in streptozotocin-induced hyperglycemic rats.

Fig. 2: Effect of lupeol on skin wound healing in streptozotocin-induced hyperglycemic rats. Representative images of lesion area (initial and from the last day of experimentation) of animals treated with Lanette, Insulin 0.5 U/g and lupeol 0.2% (**A**) and wound closure (%) on days 3, 5, 7, 9, 11, 13 and 15 post-wounding (**B**). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ represents lupeol vs. Lanette group. ++ $p < 0.01$ and +++ $p < 0.001$ represents insulin vs. Lanette group, using ANOVA followed by the Newman-Keuls test.

Fig. 3: HE stained skin tissue sections on day 14 post wound induction in streptozotocin-induced hyperglycemic rats (**A**). Inflammatory cells infiltration, Proliferation of fibroblasts and Number of blood vessels (**B**) in HE staining of the border and central region of rats hyperglycemic wounds treated with Lanette, Insulin 0.5 U/g or lupeol 0.2% for 14 days. * $p < 0.05$ and *** $p < 0.001$ vs. Lanette group. # $p < 0.05$ vs. Insulin group, using ANOVA followed by the Newman-Keuls test. Bar represents 20 μ m. (black arrows) indicate the presence of inflammatory cells, (#) collagen fibers, (*) blood vessels, (+) fibroblasts and (▲) sebaceous glands.

Fig. 4: Masson's trichrome stained skin tissue sections on day 14 post wound induction in streptozotocin-induced hyperglycemic rats (**A**). Labeling area of total collagen fibers (**B**) (μm^2) in the border and central region of rats hyperglycemic wounds treated with Lanette, Insulin 0.5 U/g or lupeol 0.2% for 14 days. * $p < 0.05$ and ** $p < 0.01$ vs. Lanette group, using ANOVA

followed by the Newman-Keuls test. Bar represents 20 μ m. (black arrows) indicate the presence of total collagen fibers.

Fig. 5: Photomicrography of the immunostaining and immunolabeled area (μ m²) for NF- κ B (**A-B**), TGF- β 1 (**C-D**), FGF-2 (**E-F**) and Collagen III (**G-H**) in the border and central region of rats hyperglycemic wounds treated with Lanette, Insulin 0.5 U/g or lupeol 0.2% for 14 days. * p <0.05, ** p <0.01 and *** p <0.001 vs. Lanette group. # p <0.05 vs. Insulin group, using ANOVA followed by the Newman-Keuls test. Bar represents 20 μ m. (black arrows) indicate antibody staining against NF- κ B, TGF- β 1, FGF-2 and Collagen III.

Fig. 6: Quantification of TNF- α (**A**), IL-1 β (**B**), IL-6 (**C**) and IL-10 (**D**) levels (pg/mg protein) in rats hyperglycemic wounds treated with Lanette, Insulin 0.5 U/g or lupeol 0.2% for 14 days. * p <0.05, ** p <0.01 and *** p <0.001 vs. Lanette group. + p <0.05 and ++ p <0.01 vs. sham group, using ANOVA followed by Newman-Keuls test.

Fig. 7: Gene expression (RT-qPCR) of *Nf- κ b* (**A**), *Ki-67* (**B**), *Egf* (**C**), *Vegf-A* (**D**), *Hif-1 α* (**E**), *Angiopoietin-4* (**F**), *Nos-2* (**G**), *Sod-2* (**H**), *Gpx-1* (**I**), *Ho-1* (**J**), *Ho-2* (**L**) and *Col3a1* (**M**) in rats hyperglycemic wounds treated with Lanette, Insulin 0.5 U/g or lupeol 0.2% for 14 days. * p <0.05 and ** p <0.01 vs. Lanette group. # p <0.05 vs. insulin group, using ANOVA followed by Newman-Keuls test.

TABLES

Table 1

Gene	Primers sequence 5'-3'	Product Size	Melting Temperature	Access number*
<i>β-actin</i>	FW: CCCTGGCTCCTAGCACCAT RV: GATAGAGCCACCAATCCACACA	80 pb	60°C	NM_031144.3
<i>Nf-κb</i>	FW: CCTCATCTTTCCCTCAGAGCC RV: CGCACTTGTAACGGAAACGC	98 pb	60°C	NM_199267.2
<i>Ki-67</i>	FW: GGGTTTCCAGACACCAGACC RV: CCAGGAAGACCAGTTAGAACC	100 pb	60°C	NM_001271366.1
<i>Egf</i>	FW: CTCAGGCCTCTGACTCCGAA RV: ATGCCGACGAGTCTGAGTTG	93 pb	60°C	NM_012842.1
<i>Vegf-A</i>	FW: TGC GGATCAAACCTCACCAA RV: GGCTCACAGTGATTTTCTGGC	115 pb	60°C	NM_001110333.2
<i>Ho-1</i>	FW: GTCCCAGGATTTGTCCGAGG RV: GGAGGCCATCACCAAGCTTAAA	133 pb	60°C	NM_012580.2
<i>Ho-2</i>	FW: CCGGGCAGAAAATACCCAGT RV: ATCAGTGCTTCCCTCCGGTG	192 pb	60°C	NM_J05405.1
<i>Gpx-1</i>	FW: CATTGAGAATGTCGCGTCCC RV: TTGCCATTCTCCTGATGTCCG	141 pb	60°C	NM_030826.4
<i>Sod-2</i>	FW: GTGGAGAACCCAAAGGAGAGT RV: GGTCTGATTAGAGCAGGCG	177 pb	60°C	<u>NM_017051.2</u>
<i>Hif1-α</i>	FW: ATCCATTTTCAGCTCAGGACAC RV: GGTAGGTTTCTGTAAGTGGGTCTG	182 pb	60°C	NM_AF057308.1
<i>Nos-2</i>	FW: GCCTAGTCAACTACAAGCCCC RV: AGAAACTTCCAGGGGCAAGC	87 pb	60°C	NM_012611.3
<i>Angpt-4</i>	FW: AACTGTTCCAGAAGGTAGCCC RV: TCAAGAGGTCAATCTGGCTCTG	90 pb	60°C	NM_199115.2
<i>Col3α1</i>	FW: GGGATCCAATGAGGGAGAAT RV: CCTTGCCTGTTTGATATT	128 pb	60°C	NM_032085.1

FW (forward), RV (reverse) and pb (base pairs). *National Center for Biotechnology Information, Nucleotide (NCBI - <https://www.ncbi.nlm.nih.gov>).

Table 2

Groups	Day -9 (STZ injection)	Day -7 (post STZ)	Day 0 (Before wounding)	Day 8 (post wounding)	Day 15 (Euthanasia)
Lanette	104.04 ± 3.93	498.12 ± 14.02***	482.53 ± 11.37***	543.09 ± 8.85***	530.61 ± 21.37***
Insulin 0.5 U/g	108.12 ± 4.21	501.09 ± 23.87***	524.84 ± 19.99***	501.67 ± 15.4***	511.36 ± 11.87***
Lupeol 0.2%	101.43 ± 5.80	476.89 ± 16.29***	498.89 ± 14.82***	554.03 ± 25.8***	543.08 ± 27.32***
Sham	104.45 ± 4.73	112.04 ± 6.91	109.26 ± 7.02	99.85 ± 8.853	113.93 ± 9.24

Table 3

Parameters	Groups	Days after wound induction
		14 days
Coagulation	Lanette	0 (0,2)
	Insulin 0.5 U/g	0 (0,1)
	Lupeol 0.2%	0 (0,1)
Presence of crust	Lanette	1 (0,2)
	Insulin 0.5 U/g	0 (0,1)
	Lupeol 0.2%	0 (0,1)*
Granulation tissue	Lanette	1 (0,2)
	Insulin 0.5 U/g	0 (0,1)
	Lupeol 0.2%	0 (0,2)

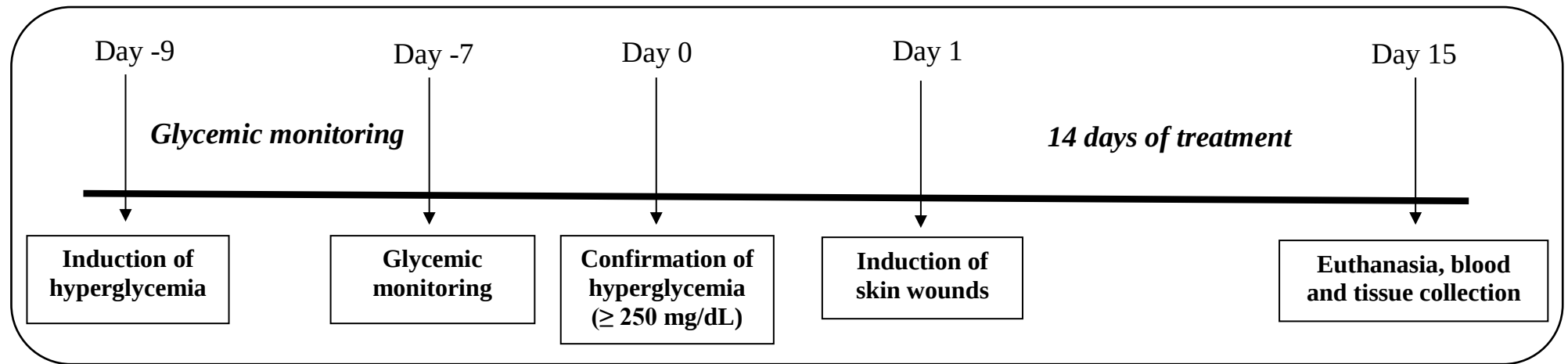
FIGURES**Figure 1**

Figure 2

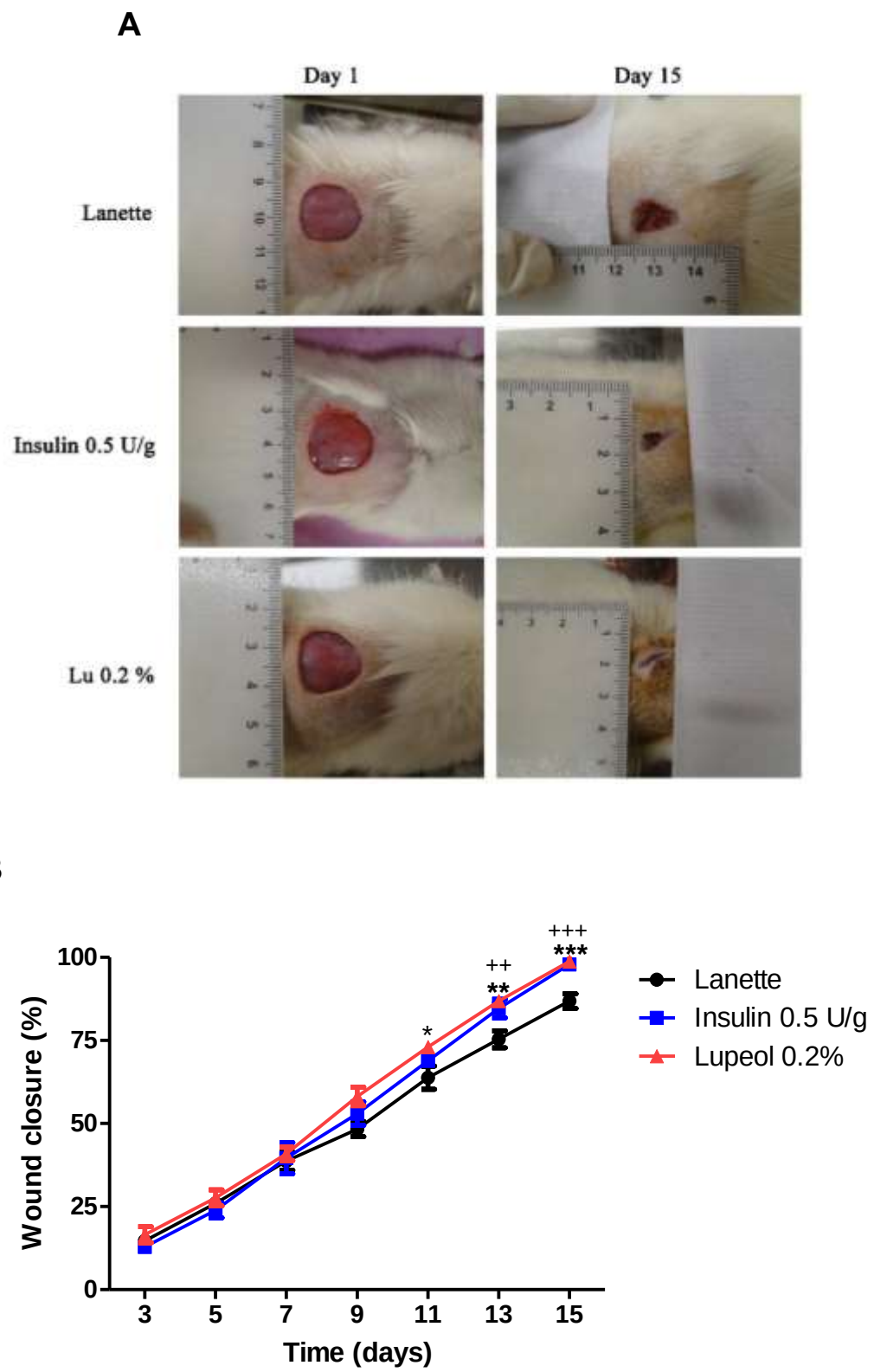


Figura 3

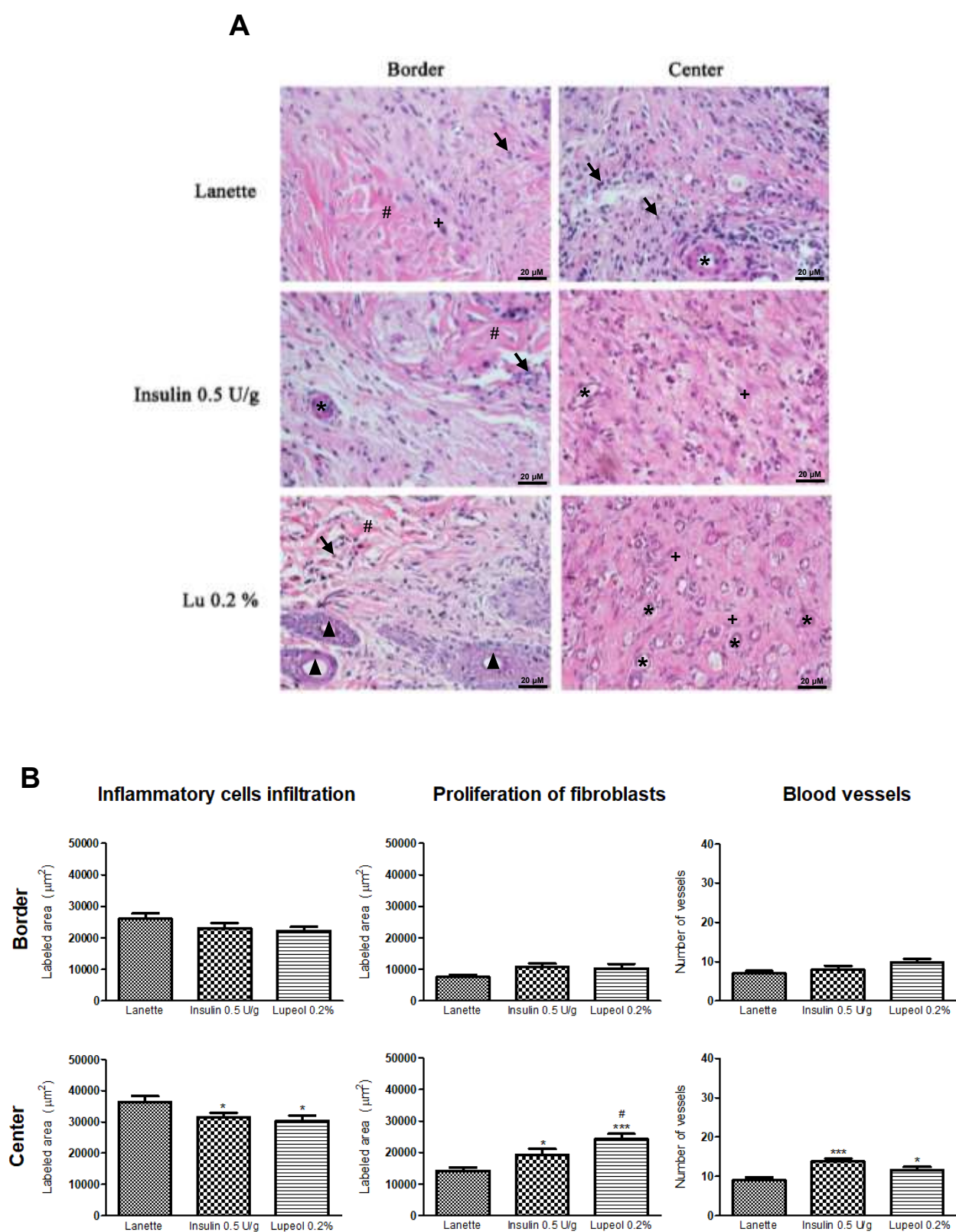


Figura 4

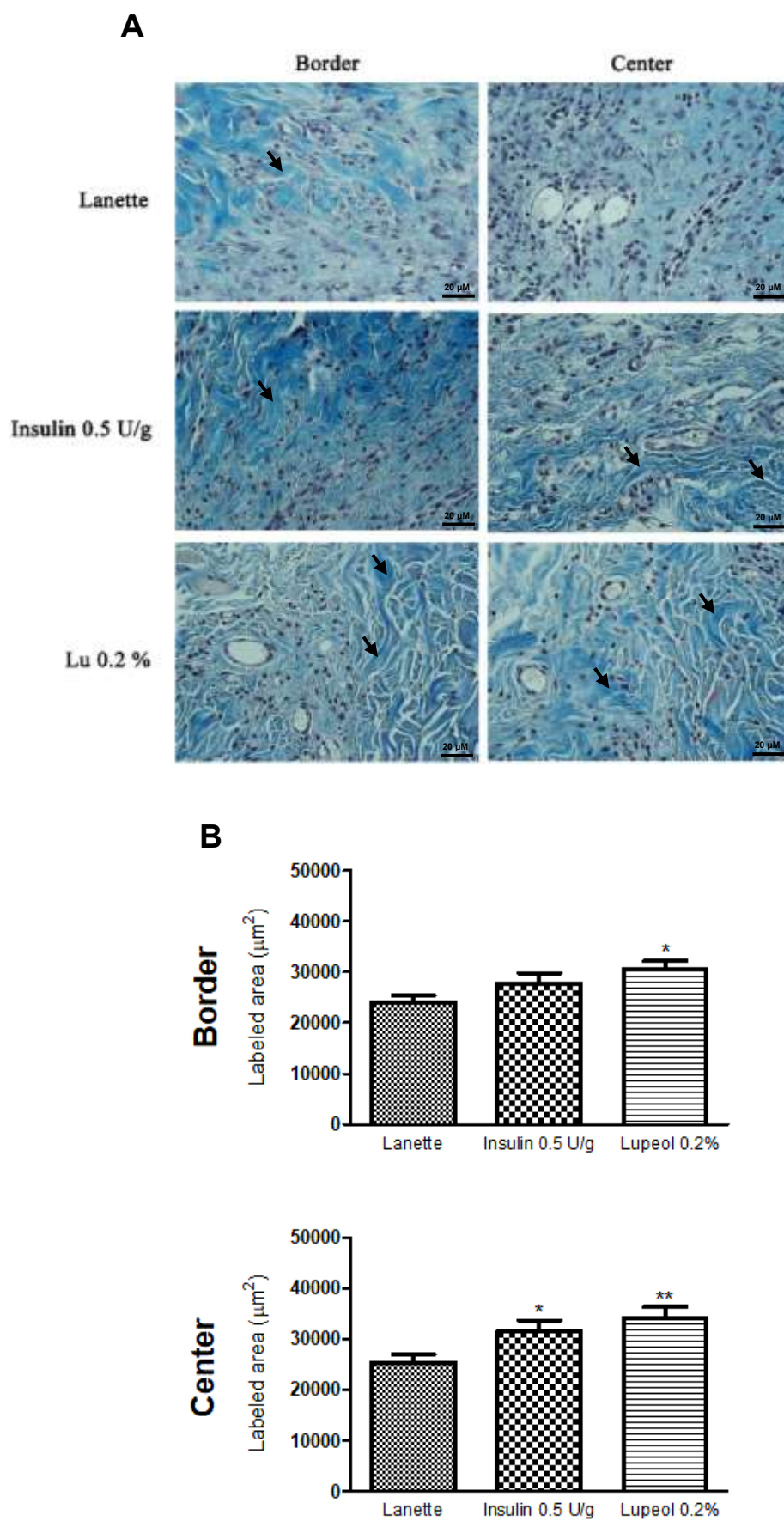
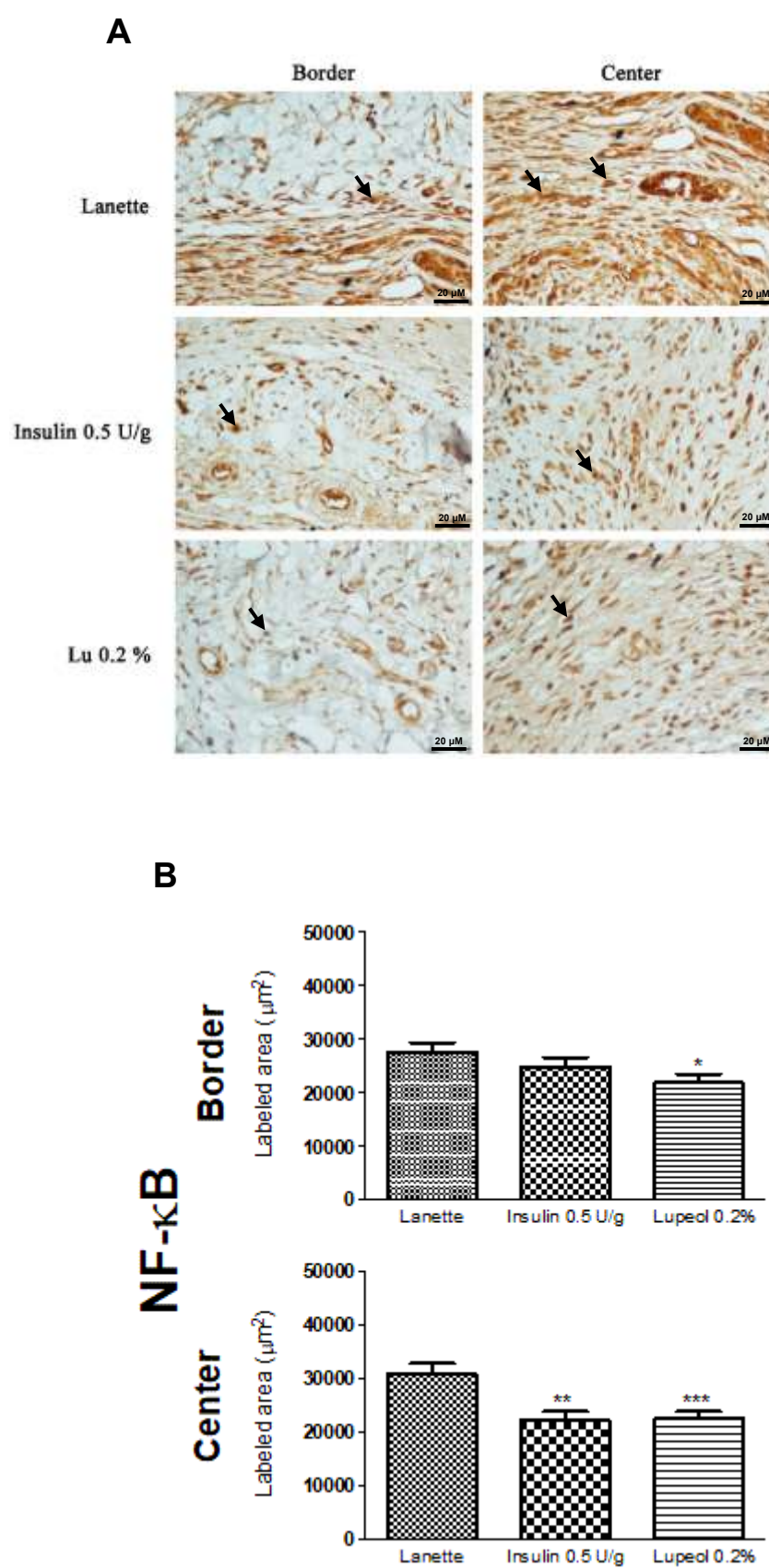
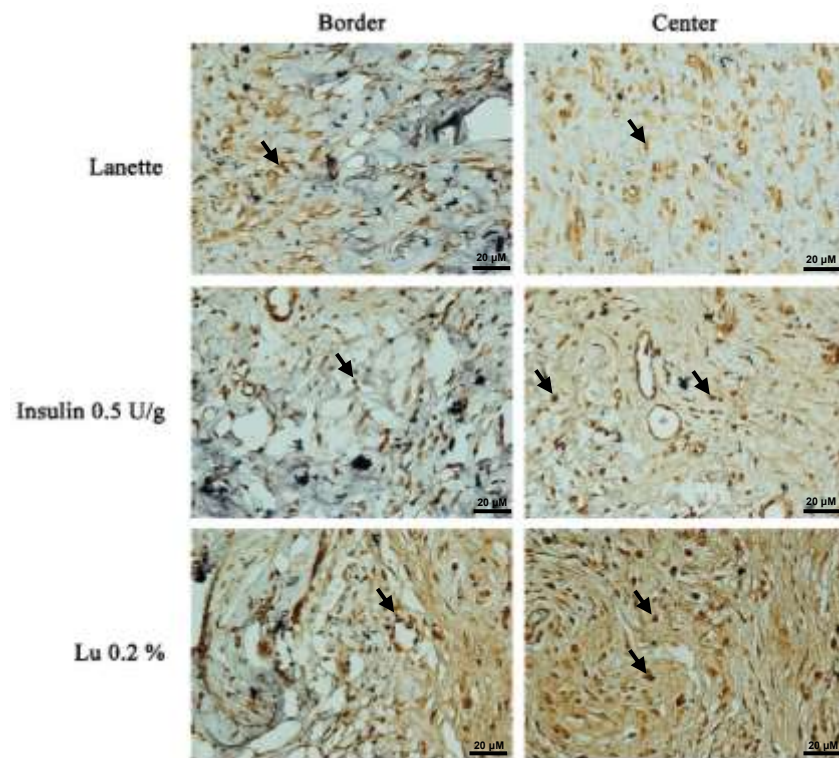


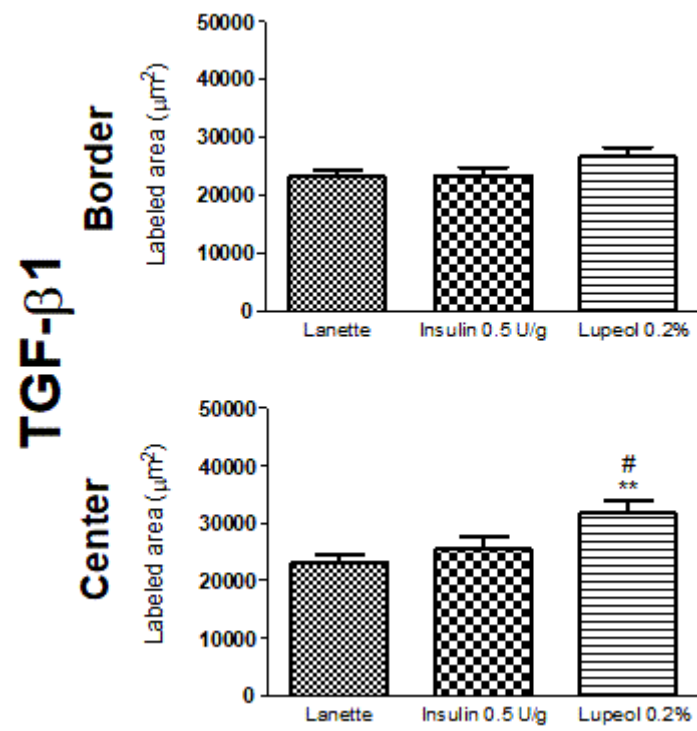
Figure 5

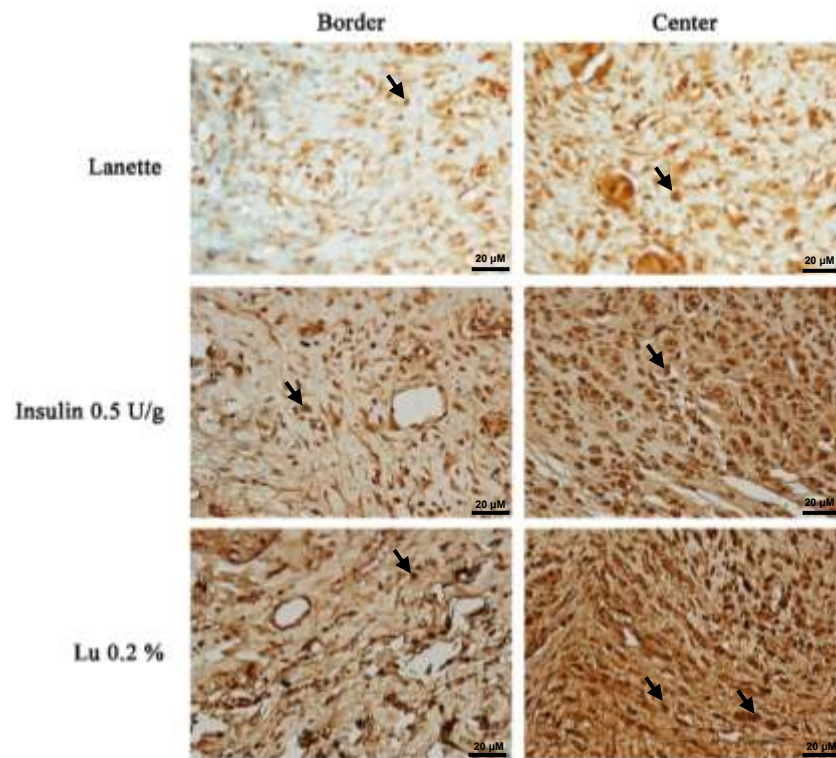
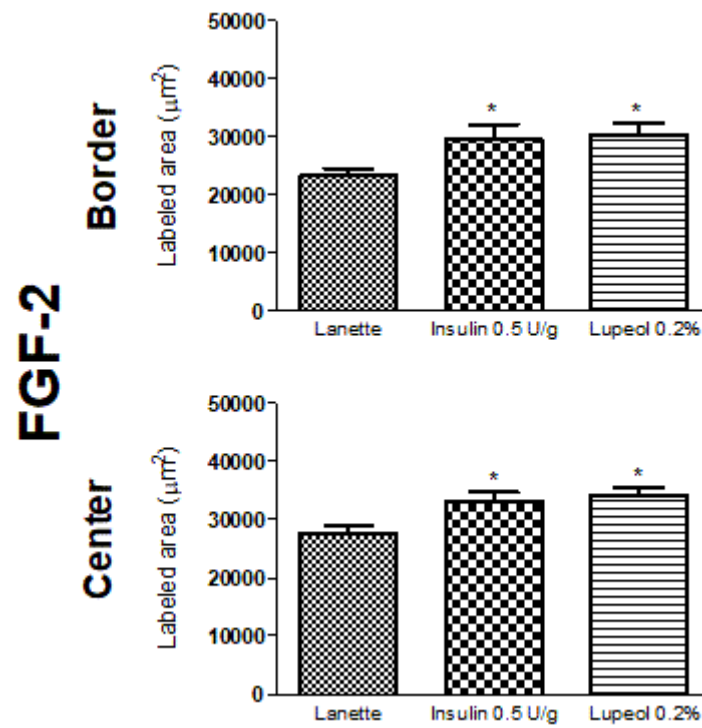


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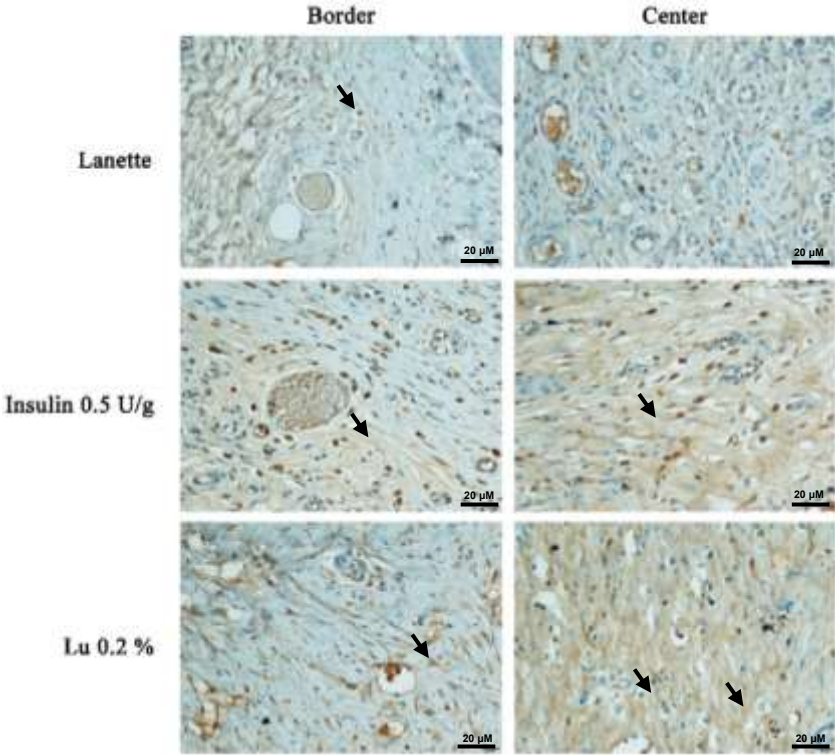


D



E**F**

G



H

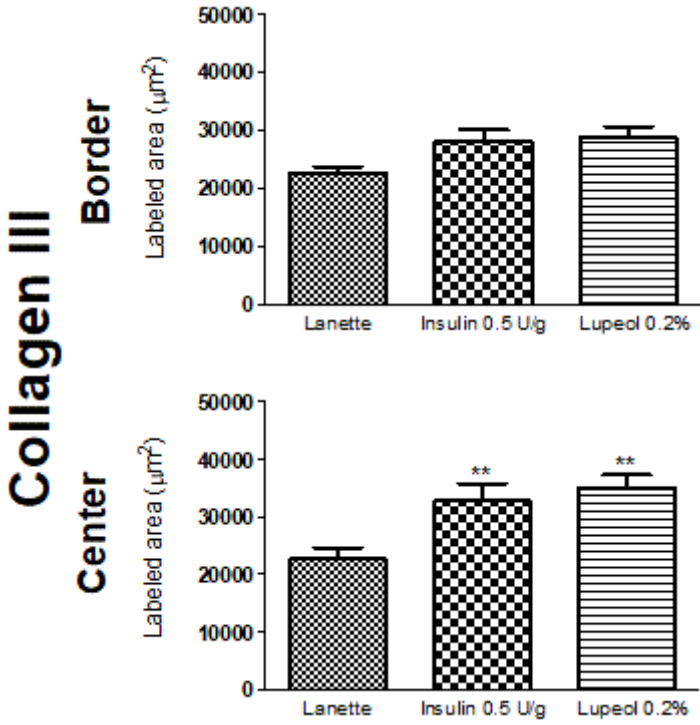


Figure 6

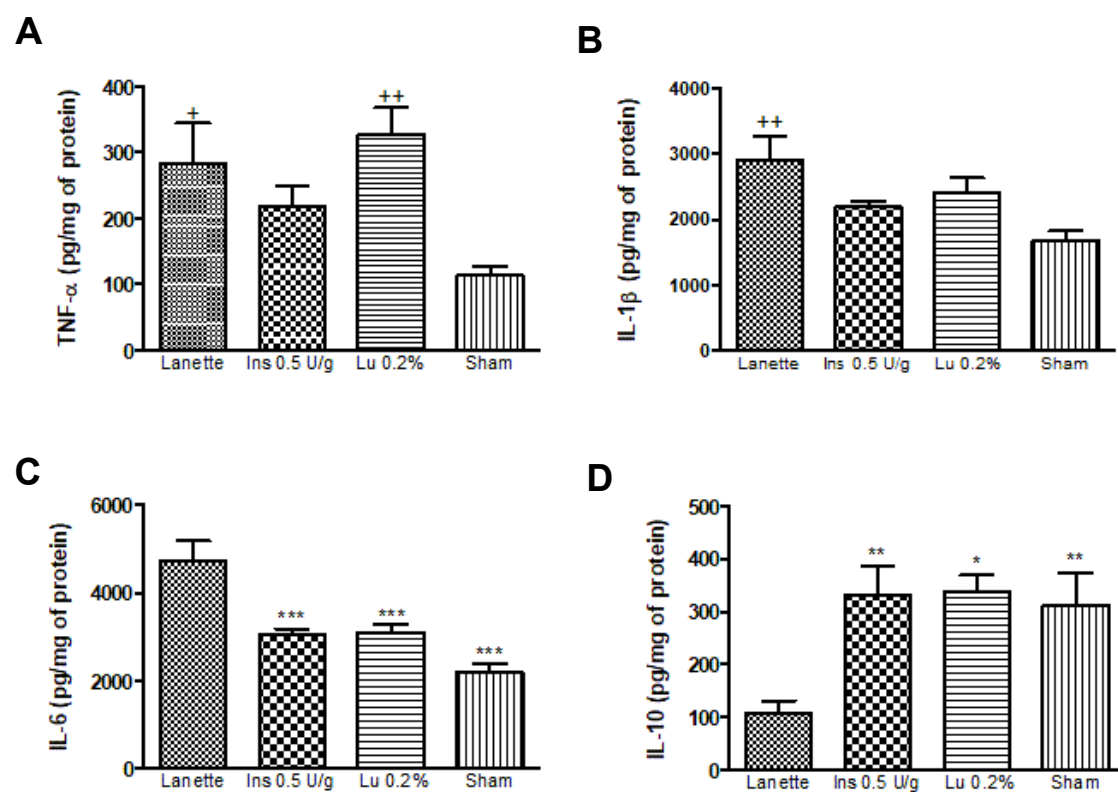
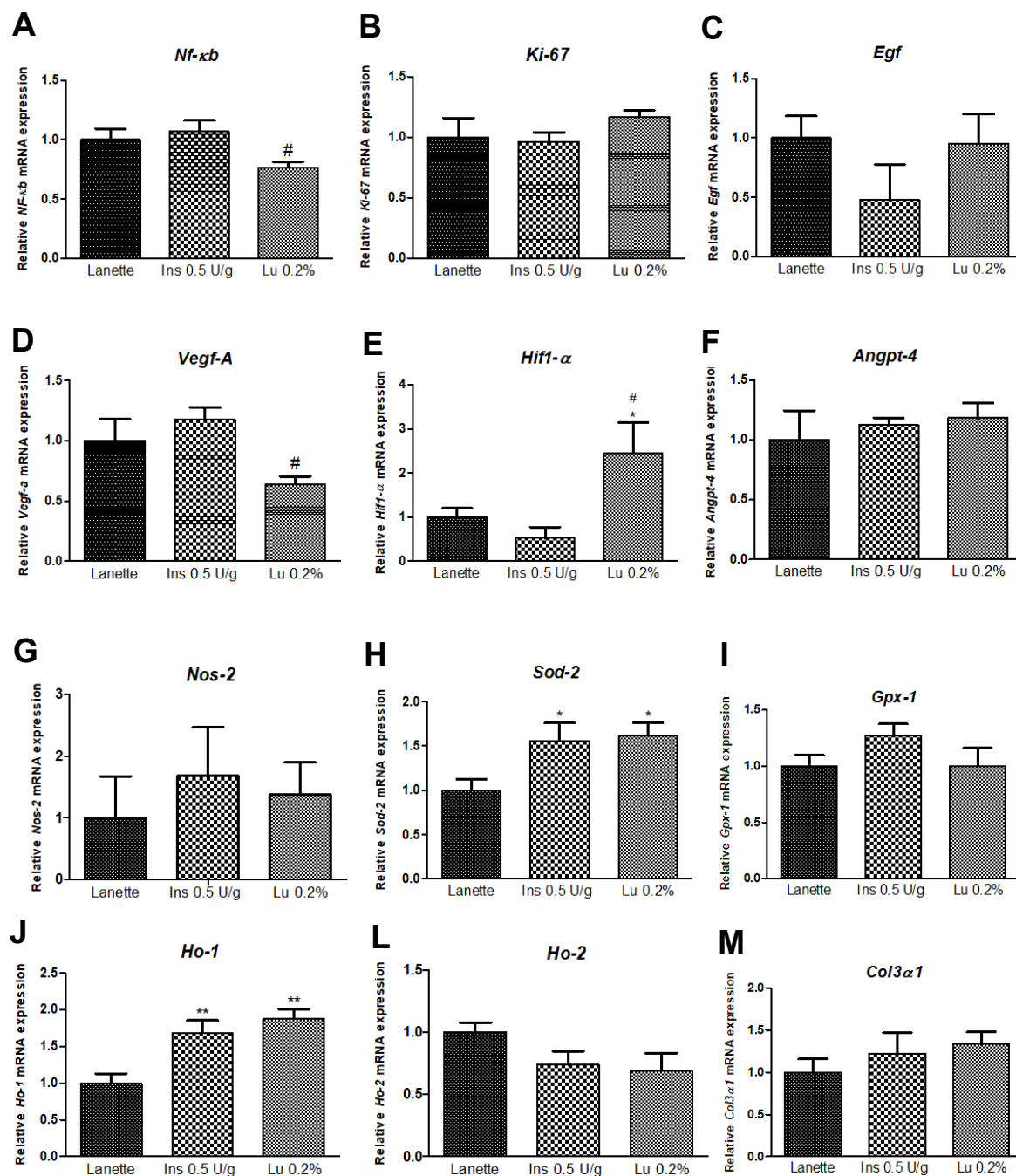


Figure 7



SUPPLEMENTARY MATERIALS

Body weight (g)	Lanette	Insulin 0,5 U/g	Lupeol 0,2%	Sham
Initial weight	276,6 ± 10,56	258,9 ± 6,479	285,6 ± 13,27	279 ± 7,125
Final weight	242,7 ± 10,60***	244,6 ± 5,961***	253,7 ± 12,91***	294,9 ± 44,27

Supplementary Table 1: Body weight evaluation of rats treated with Lanette, Insulin 0.5 U/g or 0.2% lupeol creams for 14 days of experimentation (n=8). *** $p < 0.001$ vs. sham group, using ANOVA followed by the Newman-Keuls test.

Biochemical parameters (mg/dL)	Lanette	Insulin 0,5 U/g	Lupeol 0,2%	Sham
AST	286,30 ± 35,54*	275,90 ± 33,23*	317,60 ± 48,19*	151,0 ± 17,76
ALT	1153 ± 261,61***	717,30 ± 103,90*	738,60 ± 184,50*	88,50 ± 17,52
γ-GT	13,71 ± 3,41*	11,13 ± 2,47*	10,86 ± 3,08*	1,42 ± 0,42
Alkaline phosphatase	761,42 ± 75,90***	807,61 ± 55,74***	687,64 ± 137,10***	78,86 ± 7,52
Creatinine	0,39 ± 0,03	0,39 ± 0,01	0,45 ± 0,01	0,40 ± 0,02
Urea	74,91 ± 8,76***	69,83 ± 3,46***	74,76 ± 4,41***	37,63 ± 0,91

Supplementary Table 2: Biochemical parameters of rats treated with Lanette, Insulin 0.5 U/g or lupeol 0.2% for 14 days of experimentation (n=8). * $p < 0.05$ and *** $p < 0.001$ vs. sham group, using ANOVA followed by the Newman-Keuls test.

Groups	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13
	(post wounding)	(post wounding)	(post wounding)	(post wounding)	(post wounding)	(post wounding)
	Water intake (mL)	Water intake (mL)	Water intake (mL)	Water intake (mL)	Water intake (mL)	Water intake (mL)
Lanette	280,0 ± 33,33***	301,3 ± 40,84***	331,9 ± 46,69***	444,3 ± 29,26***	442,3 ± 24,90***	477,0 ± 21,53***
Insulin 0.5 U/g	396,30 ± 26,10***	413,1 ± 13,29***	423,8 ± 10,25***	521,3 ± 12,02***	486,9 ± 14,27***	527,8 ± 14,07***
Lupeol 0.2%	413,11 ± 26,59***	425,0 ± 16,31***	400,6 ± 35,70***	509,3 ± 17,02***	458,6 ± 25,53***	458,8 ± 66,52***
Sham	114,30 ± 9,02	64,29 ± 3,68	82,14 ± 9,24	113,6 ± 9,55	80,00 ± 6,075	106,6 ± 8,70

Supplementary Table 3: Water intake (mL) in hyperglycemic rats treated with Lanette, Insulin 0.5 U/g or lupeol 0.2% during 14 days of experimentation (n=8). *** $p < 0.001$ vs. sham group, using ANOVA followed by the Newman-Keuls test.

Groups	Day 3	Day 7	Day 10	Day 13
	(post wounding)	(post wounding)	(post wounding)	(post wounding)
	Food intake (g)	Food intake (g)	Food intake (g)	Food intake (g)
Lanette	46,25 ± 5,756***	135,8 ± 9,874**	185,6 ± 8,594**	150,3 ± 13,97**
Insulin 0.5 U/g	61,75 ± 61,75***	144,1 ± 11,37**	173,1 ± 13,36**	177,6 ± 10,41*
Lupeol 0.2%	69,00 ± 6,58***	149,5 ± 10,72*	171,3 ± 14,30**	142,6 ± 24,48**
Sham	144,6 ± 8,92	215,4 ± 22,48	249,0 ± 14,74	251,9 ± 26,12

Supplementary Table 4: Food intake (g) in hyperglycemic rats treated with Lanette, Insulin 0.5 U/g or lupeol 0.2% during 14 days of experimentation (n=8). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. sham group, using ANOVA followed by the Newman-Keuls test.

Conclusão geral

Capítulo V

CONCLUSÃO GERAL

A análise dos resultados geral permite concluir que:

- Lupeol tem efeito cicatrizante de feridas *in vitro* em baixas concentrações, através de estímulos da migração de queratinócitos e aumento da contração de fibroblastos em uma matriz gel de colágeno;
- Este efeito *in vitro*, ocorre possivelmente a ativação das vias PI3k/Akt e MAPK/p38;
- Lupeol aumenta a expressão de moléculas citoprotetoras da barreira epidérmica, como Tie-2 e MMP-2;
- Os resultados das análises *in vivo*, mostram efeito cicatrizante do lupeol nas concentrações de 0.2% e 0.4% em feridas de ratos normoglicêmicos tratados após 7 e 14 dias da indução da lesão;
- A concentração de 0.2% do creme à base de lupeol mostrou cicatrização acelerada em feridas de ratos hiperglicêmicos tratados durante 14 dias;
- Este potencial efeito observados em ambos os modelos, envolve a regulação do processo inflamatório, através da redução de moléculas pró-inflamatórias, como NF- κ B, TNF- α e IL-6, e aumento da expressão de IL-10, citocina anti-inflamatória;
- Aumenta a expressão de mediadores envolvidos na formação de tecido de granulação, angiogênese e reepitelização, como Ki-67, VEGF, HIF-1 α , EGF, FGF-2 e TGF- β 1;
- Os achados histopatológicos em ambos os modelos *in vivo*, revelaram uma diminuição no processo inflamatório, neovasculogênese e aumento na deposição de fibras colágenas totais após tratamento com lupeol;
- Lupeol também minimizou o estresse oxidativo e melhorou o status antioxidante através da expressão gênica aumentada de HO-1 e SOD-2 em feridas de ratos hiperglicêmicos;
- Nenhum sinal de toxicidade avaliado pelo teste de irritação dérmica foi observado, garantindo a segurança da utilização do creme à base do lupeol.

Nossos achados, de uma forma geral, indicam o potencial de cicatrização de feridas cutâneas do lupeol, um triterpeno isolado de *Bowdichia virgilioides*, e fornece boas evidências científicas de que a aplicação de creme à base lupeol, é promissor no tratamento de feridas normais e em condições hiperglicêmicas.



Figura 1: Mecanismos de regulação na cicatrização *in vitro* e *in vivo* (normoglicêmicos e hiperglicêmicos)

Anexo A – Certificado de aprovação do estudo pela Comissão de Ética no Uso de Animais

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu



Certificado

Certificamos que o Protocolo nº 610-CEUA, sobre "Emprego do creme à base do *triterpeno lupeol* para aceleração do processo de cicatrização de feridas cutâneas em ratos diabéticos", sob a responsabilidade de **Cláudia Helena Pellizzon**, está de acordo com a legislação vigente (Lei 11.794/2008 e Decreto 6.899/2009), com as resoluções normativas aplicáveis à luz dos Princípios Éticos na Experimentação Animal elaborados pela Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL/COBEA), e foi aprovado "Ad referendum" da **COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)**, nesta data.

Botucatu, 22 de maio de 2014.


Prof. Dr. **Wellerson Rodrigo Scarano**
Presidente da CEUA

Anexo B – Estágio de Pesquisa no Exterior (CNPq)



Fernando Pereira Beserra
Rua Benedito Mathias Blumer, 35, Jardim Paraíso
18610310 - Botucatu -
SP - Brasil

Diretoria de Ciências Agrárias, Biológicas e da Saúde
Programa Especial de Biotecnologia e Recursos Genéticos - COBRG

Ofício SN/CGEFO/CNPq

Brasília, 17 de dezembro de 2015

Processo número: 202513/2015-7

Modalidade: Doutorado Sanduiche no Exterior - SWE

Chamada: Doutorado Sanduiche - SWE

Inst.: The University of Sydney/Austrália

Em aditamento a mensagem eletrônica na qual lhe foi comunicada a concessão de uma bolsa de estudos no exterior, vimos informar-lhe os benefícios, prazos e condições da referida bolsa, em conformidade com as normas em vigor no CNPq.

Modalidade: Doutorado Sanduiche no Exterior - SWE

Vigência: 01/05/2016 até 30/10/2016

Mês de remessa da documentação para análise quanto à prorrogação (se pertinente): Agosto de 2016

Benefícios:

Mensalidade: AUD 1.650,00 (valor-base: AUD 1.650,00)

Adicional de Localidade para Cidade de alto custo: AUD 507,00

Auxílio Instalação: AUD 1.650,00

Seguro-Saúde: AUD 660,00

Taxa de Bancada (conforme aprovado na proposta)

Auxílio Deslocamento: USD 3.121,00

CONDIÇÕES

No prazo de 30 (trinta) dias contados a partir do início das atividades, o bolsista deverá enviar as cópias digitalizadas do **comprovante de embarque**; do **comprovante de matrícula ou carta da instituição de destino no exterior atestando o início das atividades**; e **comprovante da contratação do seguro-saúde**, por meio do Formulário "Envio de Documentação Complementar de Bolsa no Exterior", disponível na página do CNPq, Plataforma Carlos Chagas.

Caso os documentos não sejam enviados no prazo previsto, a bolsa será suspensa pelo CNPq.

Ministério da Ciência e Tecnologia e
Inovação MCTI

Conselho Nacional de Desenvolvimento Científico e
Tecnológico - CNPq
Coordenação de Execução de Bolsas no Exterior
SHIS Quadra 01 Conjunto B - Bloco B, Térreo
Edifício Santos Dumont
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Handwritten signature

ANEXO C - Estágio de Pesquisa no Exterior (FAPESP)



TO WHOM IT MAY CONCERN

We hereby declare that FUNDAÇÃO DE AMPARO À PESQUISA DO ESTADO DE SÃO PAULO - FAPESP, CNPJ 43.828.151/0001-45, has approved a financial support to FERNANDO PEREIRA BESERRA in order to develop his research studies abroad, which will be as below:

1. 5 months and 2 days of International Fellowship in the amount of 7,600.00 euros, from June 01st, 2018 to November 02nd, 2018.
2. Health Insurance in the amount of R\$ 2,356.00.
3. Transportation Expenses totalizing 2,200.00 american dollars.
4. International Installation Aid, in the amount of 1,500.00 euros.
5. International Technical Reserve, in the amount of 1,140.00 euros.

The fellowship granted by FAPESP does not configure any kind of labor relationship and does not impose payment of wages.

This financial support provides resources to FAPESP RIA - Doctorate fellowship student to participate in research activities at Medical University of Gdansk/MUG, in Poland.

FAPESP Process No.2018/06042-0.

São Paulo, May 07th, 2018.


 Andréia Alves Neves
 Department Manager
 Support and Contract Agreement Department

Andréia Alves Neves
 Gestor Tec. Adm. Chale
 SAC