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PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

EFEITO PRÓ-CICATRIZANTE DO TRITERPENO 3β,6β,16β-TRIHYDROXYLUP-20(29)-ENE (CLF-1) ISOLADO DE FOLHAS DE *Combretum leprosum* E ATIVIDADE ANTITUMORAL DE UMA LECTINA ISOLADA DA ESPONJA MARINHA *Haliclona caerulea*

LUIZ GONZAGA DO NASCIMENTO NETO

FORTALEZA

2016

UNIVERSIDADE FEDERAL DO CEARÁ
FACULDADE DE MEDICINA
DEPARTAMENTO DE PATOLOGIA E MEDICINA LEGAL
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Tese apresentada ao curso de Pós-Garduação em Biotecnologia – RENORBIO da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Doutor em Biotecnologia. Área de concentração: Biotecnologia em Saúde

Orientador: Prof. Dr. Edson Holanda Teixeira
Co-Orientador(a): Profa. Dra. Paula Alexandra Videira

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“Fantasiar com um motivo, contemplar com consideração, ser feliz suavemente, sofrer com nobreza e, depois, esvaziar a taça para que o amanhã a encha de novo”

Gibran Khalil Gibran

RESUMO

O número de casos de morbimortalidade em pacientes com feridas crônicas vem aumentando a cada ano. Nesse sentido, a cicatrização de feridas tem se tornado um dos principais problemas dermatológicos. Metabólitos secundários de plantas tem sido utilizados em vários estudos como moléculas promissoras no tratamento de várias patologias. Entretanto, são poucos os dados sobre seu efeito na cicatrização de feridas. *Combretum leprosum* Mart. é uma planta nativa da Caatinga e seu extrato etanólico é bastante utilizado na medicina popular para cicatrização de feridas, como sedativo ou tratamento de hemorragias. Um dos objetivos do presente trabalho foi avaliar o efeito do triterpeno 3β, 6β, 16β-trihydroxylup-20 (29)-ene (CLF-1) isolado do extrato etanólico de folhas de *Combretum leprosum* sobre lesões induzidas *in vitro* e *in vivo*. No ensaio *in vitro*, o CLF-1 (2,5 μg/mL) não apresentou toxicidade a fibroblastos dermais humanos (HDF). Além disso, o CLF-1 induziu a migração de fibroblastos para o fechamento da lesão artificial de maneira mais efetiva, comparado ao controle, em 24h. Além disso, o mecanismo molecular do efeito do CLF-1 foi estudado. Os resultados sugerem que a migração de fibroblastos pode ocorrer pelo aumento da expressão de TGF-β1 e redução nos níveis da citocina inflamatória TNF-α. No estudo *in vivo*, lesões experimentais foram induzidas na região dorsal de camundongos e o tratamento com o extrato etanólico de *C. leprosum* (EECL) e CLF-1 foi avaliado por um periodo de 12 dias. Os tratamentos com EECL e CLF-1 induziram uma reepitelização mais rápida e efetiva em comparação ao controle. O estudo histopatológico mostrou que EECL e CLF-1 apresentam perfis similares durante o processo regenerativo. Esses resultados sugerem que, provavelmente o componente ativo no EECL possa ser o CLF-1. Além dos elevados custos relacionados a cicatrização de feridas, patologias crônicas como o câncer são estimados como uma das principais causas de morte e um dos principais fatores dispendiosos para os sistemas de saúde. Os tratamentos convencionais como cirurgia, quimioterapia e radioterapia são efetivos contra tumores primários, contudo, não possuem o mesmo efeito nos estágios mais avançados da doença. Nesse sentido, muitas biomoléculas tem atraído interesse da comunidade científica devido ao seu potencial antitumoral. Esponjas marinhas são consideradas reservas biológicas de várias biomoléculas, sobretudo lectinas. Lectinas são proteínas que se ligam a carboidratos de maneira reversível sem alterar sua estrutura. De modo diferente como relação a lectinas de plantas, há poucos relatos na literatura descrevendo o mecanismo de ação de lectinas de esponjas sobre a indução de apoptose de células tumorais. O efeito antitumoral de uma lectina isolada da esponja marinha *Haliclona caerulea* (H3) sobre células do adenocarcinoma de mama humano MCF7 foi avaliado. H3 induziu a redução da viabilidade celular de MCF7 em mais de 50% (IC_{50}=100 μg/mL) em 6h, 24h e 48h. Contudo, sobre células normais, o tratamento com H3 induziu a redução em mais de 50%, apenas na maior dose testada (500 μg/mL). Além disso, H3 provoca arraste no ciclo celular na fase G1 e induz apoptose das células MCF7 em 24 e 48h. Ensaios utilizando *Lysotracker Red* e PCR em tempo-real sugerem que o efeito de H3 pode estar relacionado a um balanço dinâmico entre apoptose e autofagia, mediados pelo aumento da expressão de caspase-9 e LC3. Em conclusão, os resultados sugerem que o CLF-1 e H3 podem ser biomoléculas promissoras para o tratamento de feridas agudas e crônicas e enfermidades como câncer.

Palavras-chave: Produtos naturais, triterpeno, lectinas, cicatrização e câncer.

ABSTRACT

The number of cases of morbidity and mortality in patients with chronic wounds has increasing every year. In this sense, wound healing has become one the major dermatological problems. Secondary metabolites from plants have been used in several assays as promising molecules on various diseases attracted importance on treatment of various diseases. However, there are few data about it effect on wound healing. *Combretum leprosum* Mart. is a native species of the Caatinga and its ethanolic extract is commonly used as a healing agent, as a sedative and treatment of haemorrhages. A of the aim of the study was to evaluate the effect of triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene (CLF-1) isolated from ethanolic extract from the leaves of *Combretum leprosum* on skin wounds induced *in vitro* and *in vivo* lesions was avaluated. *In vitro* assay, the CLF-1 (2.5 μg/mL) not showed toxicity to human dermal fibroblasts (HDF). Moreover, the CLF-1 induced fibroblasts migration to the healing of the artificial injury with most effectiveness than control at 24h. In addition, the molecular mechanism of the CLF-1 treatment was studied. The results suggest that migration of fibroblasts occurs by upregulated expression of TGF-β1 and reduced levels of the inflammatory cytokine TNF-α. In the *in vivo* study, experimental skin lesions were created in the back of mice and treatment with the ethanolic extract from *C. leprosum* (EECL) and CLF-1 was evaluated for 12 days. The treatment with EECL and CLF-1 induced a faster and more effective epithelialization when compared to control. The histopathological assessment showed that EECL and CLF-1 has similar profiles during the regenerative process. This result suggest that probably the active component in the EECL may be CLF-1. Beyond of the spend about the wound healing chronic diseases as cancer are estimated as one of the main causes of dead and one of the main spending factors to the health systems. The conventional treatments, such as surgery, chemotherapy and radio are effective against primary tumors, hence not have the same effect in the later stages of the disease. In this sense, several biomolecules has attracted interest from the researchers because its antitumor potential. Marine sponjes are a biological reservoir of biomolecules, especially lectins. Lectins are proteins that bind reversibel way to carbohydrate epitops without modified them. Unlike plant lectins, there are few reports describing the mechanism of action of lectins from marine sponges to induce apoptosis in tumor cells. The effect of a lectin isolated from the marine sponge *Haliclona caerulea* (H3) on human breast cancer MCF-7 cell line was evaluated. H3 caused MCF7 cell reduction of viability in more than 50% (IC_{50}=100 μg/ml) at 6h, 24h and 48h. However, on normal cells, the treatment with H3 induced a reduction in over 50% only at the highest dose tested (500 μg/ml). Furthermore, H3 provoke arrest in the G1 cell cycle phase and induce MCF7 cells apoptosis in 24h and 48h. *Lysotracker Red* and real-time qPCR assays suggest that effect of H3 may be related to a dynamic balance between apoptosis and autophagic cell death mediated by increase of expression of caspase-9 and LC3. In conclusion, the results suggest that CLF-1 and H3 lectin may be promising biomolecules to treatment of acute and chronic wounds and rare diseases like cancers.

Keywords: Natural products, triterpene, lectins, wound healing and cancer.

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LISTA DE ABREVIATURAS E SIGLAS

7AAD	*7- Aminoactinomicyn D*
AJ	Ácido Arjunólico
ANOVA	Análise de variância
Asn	Asparagina
ATP	Trifosfato de Adenosina
AVC	Acidente Cérebrovascular
Bax	Proteína X associada a bcl-2
Bcl2	*B-cell lymphoma 2*
Bcl-xL	*B-cell lymphoma extra large*
BIOCEN	Biotério Central da Universidade Federal do Ceará
Caco-2	Adenocarcinoma colorretal
CD95	*Cluster of Differentiation 95*
CD-MPR	Receptor de manose-6-fosfato cácio dependente
CLF-1	*Combretum leprosum* fração 1
CI-MPR	Receptor de manose-6-fosfato cálcio independente
cm^2	Centímetros quadrados
Con A	Lectina de *Canavalia ensiformis*
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimetil sulfoxido
DNA	Ácido Desoxiribonucleico
DRC	Domínio de reconhecimento de carboidrato
EGF	Fator de Crescimento Epidermal
EECL	Extrato etanólico de *Combretum leprosum*
EPI	(-) Epicatecina
EROS	Espécies Reativas de Oxigênio
FA	Fator de Agregação
FADD	*Fas-associed death domain*
FGF 2	Fator de Crescimento de Fibroblastos tipo 2
g	grama
GAG	Glicosaminoglicano
GalNac	N-actilgalactosamina

H3	Halilectina 3 (Lectina de *Haliclona caerulea*)
HDF	*Human Dermal Fibroblast*
HEP G2	Carcinoma hepatocelular
IAP	*Inhibitor of Apoptosis Protein* (proteína inibidora de apoptose)
IL1	Interleucina 1
IL6	Interleucina 6
IL8	Interleucina 8
IL10	Interleucina 10
KGF	Fator de Crescimento de Queratinócitos
Kg	Quilograma
Km	Quilômetro
MEC	Matriz Extracelular
MBL	*Mannan Binding Lectin* (lectina ligante a manose)
MCF 7	Adenocarcinoma de mama humano
ml	mililitro
mM	miliMolar
MMP	Metaloproteinase de matriz
OD	Densidade ótica
OMS	Organização Mundial de Saúde
PDGF	Fator de Crescimento Derivado de Plaquetas
PF4	Fator plaquetário 4
PMN	Polimorfonuclear
POD	*Post Operatory Day*
RE	Retículo Endoplasmático
RIP	*Ribosome Inactivating Protein* (Proteína inativadora de ribossomo)
RMN	Ressonância Magnética Nuclear
Ser	Serina
Siglec	*Sialic acid binding Ig-type lectins* (Lectinas tipo imunoglobulinas ligantes de ácido siálico)
SMA	*Smooth Muscle Actin*
ST3GAL1	Beta-galactosídeo- alfa-2,3-sialiltransferase 1
sT	Antígeno sialil T
TF	Antígenos *Thomsen-Friedenreich*

TGF	Fator de Crescimento Transformante
Thr	Treonina
TIMP	Inibidor de Metaloproteinase Tissular
TNF	Fator de Necrose Tumoral
VEGF	Fator de Crescimento Vascular Endotelial

LISTA DE SÍMBOLOS

α	alfa
β	beta
Δ	delta
°C	Grau Celsios
±	mais ou menos
<	menor que
μ	micro
®	Marca registrada
™	*Trade mark*

SUMÁRIO

Capítulo I

INTRODUÇÃO E OBJETIVOS

I.1 INTRODUÇÃO

Feridas cutâneas têm se constituído um grave problema de saúde, acometendo a população de forma geral. Mesmo com uma abordagem interdisciplinar, com relação a cuidados e formas de tratamento, representam um grande desafio de gestão em países desenvolvidos e principalmente nos em desenvolvimento (SCHREML, 2010).

No Brasil, apenas 2% do orçamento da saúde é dedicado aos cuidados de pacientes acometidos por feridas agudas ou crônicas (Brasil, MS, 2008). No entanto, esse valor ainda é bastante reduzido face ao elevado número de mortalidades e morbidades causados em pacientes acometidos por feridas crônicas. Demograficamente, o número de pacientes que sofrem com feridas crônicas está alcançando proporções epidêmicas, aumentando a problemática em saúde pública e em termos econômicos (EMING; MARTIN; TOMIC-CANIC, 2014). Dados do Ministério da Saúde, mostram que mais de 145 mil pessoas foram vítimas fatais devido a feridas crônicas, apenas em 2011, representando uma das principais causas de morte no país (BRASIL, MS, 2011). Além disso, mais de um milhão de internações foram registrados pelo Sistema Único de Saúde (SUS) desde 2013 (BRASIL, MS,2015). Apesar dos cuidados médicos, uma larga proporção de pacientes com feridas crônicas sofrem permanentemente devido a morbidade que se traduz na amputação dos membros, prejudicando a qualidade de vida (CAMPOS *et al*., 2015).

Apesar de vários avanços tecnológicos, principalmente nas estratégias de tratamento no campo da cicatrização de feridas, muitos pacientes tem adotado a medicina tradicional como forma de tratamento, através do uso de remédios baseados em plantas medicinais. O reino Plantae oferece uma variedade de espécies ainda utilizadas como remédios para várias doenças em várias regiões do mundo como Ásia (DURAIPANDIYAN; AYYANAR; IGNACIMUTHU, 2006), África (BRUSOTTI *et al*., 2011; KHALID *et al*., 2012) e América do Sul (BOLZANI *et al*., 2012). A utilização de plantas medicinais com o objetivo de acelerar a cicatrização tem sido, frequentemente, realizada sem qualquer evidência científica de eficácia ou conhecimento sobre os mecanismos de ação dos compostos naturais que podem ser responsáveis pelo efeito regenerativo (GELLER *et al*., 2015). Assim, a pesquisa fitoquímica, baseada na etnofarmacologia, é considerada uma abordagem efetiva na descoberta de novas entidades químicas com potencial biotecnológico (BRUSOTTI *et al*., 2014).

Nesse contexto, a prospecção de moléculas úteis no tratamento das doenças humanas tem se constituído como um campo de imenso interesse para a ciência. Nos últimos anos, vários trabalhos vem destacando a atividade biológica de novos compostos (principalmente metabólitos secundários) pertencentes às classes de alcaloides, terpenóides e compostos fenólicos, reforçando a evidencia do papel crucial de produtos naturais como moléculas importantes para o desenvolvimento de fármacos modernos (LI;VEDERAS, 2009; BRUSOTTI *et al*., 2014). A produção de fármacos derivados de plantas, tem feito com que a medicina popular ou tradicional, torne-se uma moderna indústria capaz de caminhar na direção de uma significativa contribuição para os cuidados com a saúde.

No Brasil, por exemplo, existe uma demanda aumentada pela prospecção de drogas derivadas de plantas, principalmente devido a segura e clinicamente efetiva "medicina verde", que vem sendo constantemente observada por indústrias farmacêuticas residentes em diversos países, por ser melhor tolerada por pacientes, com custos mais baratos em relação a fármacos convencionais tornando-se mundialmente competitiva (PESSOA *et al*., 2006; SCHMIDT *et al*., 2009; RAHMAN; RAZAK; BAKRI, 2014).

Além dos problemas de morbimortalidade causados por feridas, outras doenças crônicas como as coronarianas, cerebrovasculares e câncer estão em constante ascensão com relação ao número de vítimas fatais. O câncer, por exemplo, é considerado uma doença heterogênea e complexa, constituindo-se como um dos principais problemas de saúde pública. Segundo a Organização Mundial de Saúde (OMS), apenas em 2012 foram registrados 14 milhões de novos casos e 8,2 milhões de mortes relacionadas. Com base no relatório mundial sobre o câncer, recentemente divulgado, a OMS solicitou que governos, autoridades e sociedade em geral, empreendam ações urgentes para evitar a ampliação do número de vítimas dessas patologias.

Nesse sentido, diversas terapias biológicas e tecnologias alternativas tem emergido para tratar efetivamente alguns tipos de câncer e/ou alvos específicos que são expressos por diferentes tipos de tumores (HANAHAN; WEINBERG, 2011). Dentre esses alvos, carboidratos assumem um papel crucial para a metastatização ou como antígenos associados a tumores (TACA) que servem como biomarcadores. Dessa forma, moléculas que possam reconhecer e discernir carboidratos com alterações em seu padrão na superfície celular podem ser importantes alvos terapêuticos (PADLER-KARAVANI, 2014). Com o avanço da biotecnologia, muitas substâncias isoladas de fontes naturais têm sido soluções promissoras contra o câncer. Entre esses compostos, lectinas detém elevado potencial biotecnológico no tratamento do câncer.

Lectinas são um grupo de proteínas que se ligam de forma reversível a carboidratos sem modificá-los estruturalmente (PANDA *et al*, 2014). Essas proteínas se caracterizam por serem amplamente encontradas na natureza desde micro-organismos até multicelulares superiores, exercendo funções fisiológicas importantes. Essa classe de proteínas, tem chamado atenção devido a seu vasto repertório de atividades biológicas, entre elas, a notável capacidade de destruir células tumorais usando mecanismos que implicam nos processos de apoptose por morte celular programada ou mesmo por autofagia (LIU *et al*., 2013).

A busca por novas moléculas com potencial biotecnológico, têm se expandido para habitats marinhos. Em contraste as várias biomoléculas isoladas do ambiente terrestre, produtos naturais oriundos dos mais variados tipos de organismos residentes nos mares vem ganhando destaque nas últimas décadas pelas atividades biológicas promissoras com relação ao tratamento de várias doenças, incluindo a atividade antineoplásica contra vários tipos de câncer (CHEUNG *et al*., 2015; BLUNT *et al*., 2016).

Nesse sentido, um novo enfoque tem sido direcionado a lectinas de origem marinha, onde suas novas aplicabilidades biológicas tem se tornado mais evidentes. Até o momento, mais de 300 espécies têm sido estudadas com relação a sua estrutura molecular, sequência de aminoácidos e especificidade a carboidratos (CHERNIKOV *et al*., 2013). Dentro desse grupo, lectinas de esponjas marinhas tem sido intensamente estudadas e têm revelado potenciais farmacológico e biotecnológico promissores.

A magnitude da investigação de novas substâncias com efeitos pró-cicatrizante e antineoplásico está atrelada ao fato de que agentes clássicos mais utilizados para o tratamento dessas doenças de natureza crônica podem induzir efeitos adversos resultantes de uma inadequada seletividade de ação. Assim, se faz necessário explorar uma maior quantidade de alternativas terapêuticas, visando a busca por novos fármacos para a introdução de novas perspectivas relacionadas ao bem estar dos pacientes.

I.2 OBJETIVOS

I.2.1 Objetivo geral

Avaliar o efeito pró-cicatrizante do triterpeno 3β,6β,16β-trihydroxylup-20(29)-ene (CLF-1) isolado do extrato etanólico de folhas de *Combretum leprosum* e investigar o potencial antineoplásico de uma lectina isolada da esponja marinha *Haliclona caerulea* (H3) em células do adenocarcinoma de mama humano MCF 7.

I.2.2 Objetivos específicos

- Verificar se o triterpeno CLF-1 exerce efeito citotóxico sobre fibroblastos humanos;
- Avaliar o efeito do CLF-1 sobre a migração de fibroblastos *in vitro*;
- Estudar o mecanismo molecular da atividade pró-cicatrizante do CLF-1, investigando seu efeito sobre a expressão relativa de citocinas e fatores de crescimento envolvidos na cicatrização;
- Avaliar o potencial cicatrizante do CLF-1 sobre lesões cutâneas induzidas *in vivo*;
- Investigar o efeito de H3 sobre a viabilidade de células normais e tumorais;
- Investigar o efeito de H3 sobre as propriedades adesivas relacionadas a metástase de células MCF 7;
- Analisar a interferência de H3 sobre o ciclo celular de células MCF 7;
- Avaliar o efeito de H3 sobre a indução de apoptose/autofagia em células MCF 7;
- Determinar o efeito de H3 sobre a expressão relativa de genes pró e anti-apoptóticos.

Capítulo II

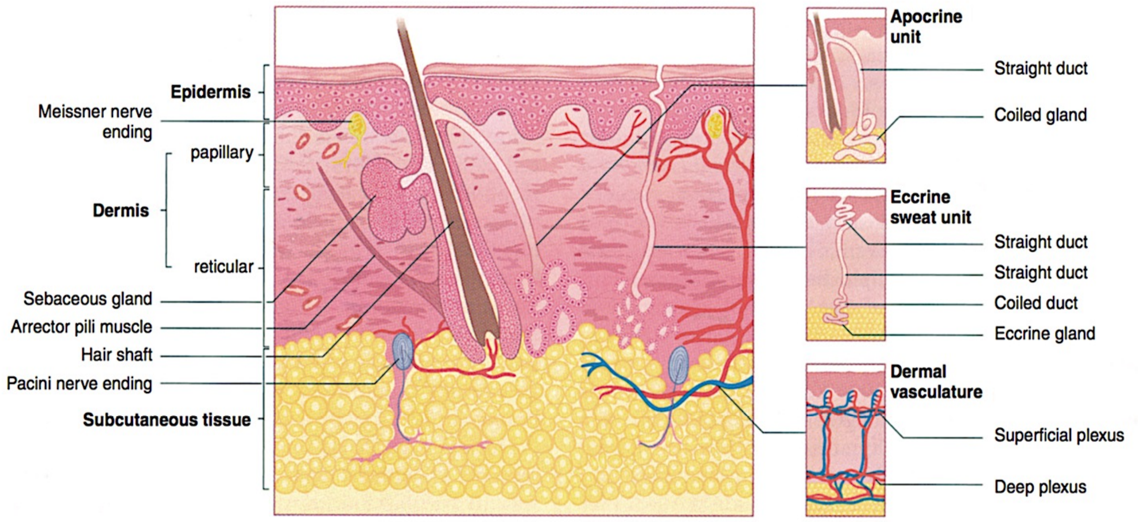
FUNDAMENTAÇÃO TEÓRICA

II.1 Pele: Anatomofisiologia

A pele é considerada o maior órgão do corpo humano, sendo capaz cobrir de maneira total a superfície externa do corpo, e é o principal sítio de interação com o ambiente externo (McLAFFERTY; HENDRY; FARLEY, 2012). Além disso, a pele serve como uma barreira protetora que é capaz de prevenir ou minimizar a exposição dos tecidos mais internos a eventos traumáticos, radiação ultravioleta, variações de temperatura (termoregulação), toxinas e contato direto com micro-organismos (CHEN; TSAO, 2013; VENUS, 2010). Outras funções importantes incluem a percepção sensorial, vigilância imunológica, a regulação do transito de moléculas e controle da perda de fluidos (McLEAN, 2003; SVOBODA; BÍLKOVÁ; MUTHNY, 2016).

O sistema tegumentar é formado pela pele e suas estruturas derivadas, como ilustrado na figura II.1.1. Nesse sentido, a estrutura da pele é caracterizada por compor três camadas básicas: camada epitelial (epiderme), camada dermal (derme) e a hipoderme que compreende o tecido adiposo subcutâneo (KANITAKIS, 2002; GARTNER; HIATT, 2013).

Figura II.1.1: Secção cruzada da pele e tecido adiposo subcutâneo.



Fonte: Kolarsick, P. A. J.; Kolarsick, M. A.; Goodwin, C. Anatomy and Physiology of the Skin. **J. Dermatol. Nurses´Assoc**, 3, 203-213, 2011.

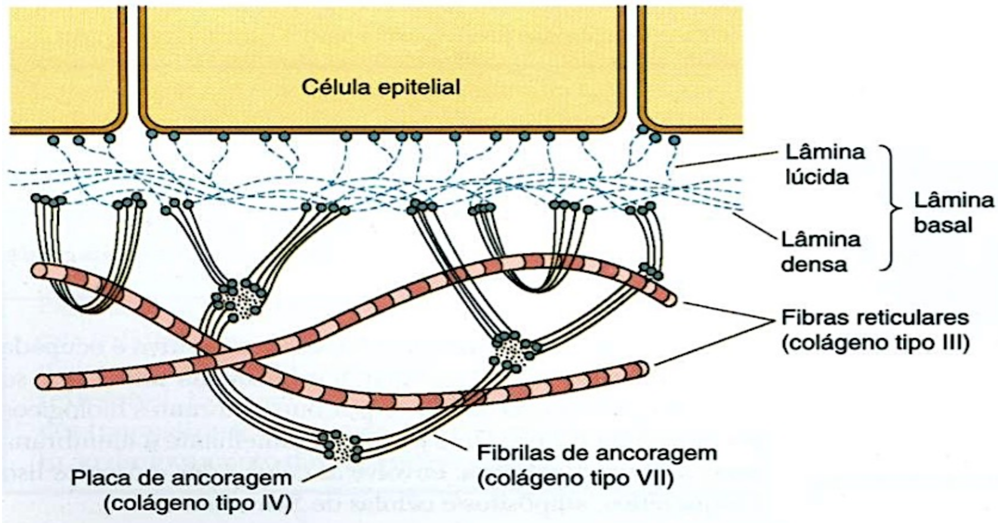
A camada epitelial é a mais externa sendo composta principalmente por dois tipos de células: queratinócitos e células dentríticas. Cerca de 80% das células que compõem a epiderme são queratinócitos que sintetizam queratina, uma proteína com função protetora, resultante do processo de queratinização (CHU, 2008).

Queratinócitos também são capazes de sintetizar peptídeos antimicrobianos (AMPs) que inibem a colonização de patógenos na superfície da pele (CHEN; TSAO, 2013). Além de queratinócitos, a epiderme possui outros tipos celulares, tais como melanócitos, células de Langerhans e células de Merkel. Melanócitos e células de Langerhans são importantes componentes relacionados à imunidade da pele, fagocitando e apresentando antígenos para linfócitos T transitórios (McLEAN, 2003). Células de Merkel são responsáveis por exercer comunicação com terminações nervosas aferentes e estão relacionadas à transdução sensorial na pele (MOLL, 1994; WOO; LUMPKIN; PATAPOUTIAN, 2015).

Apesar de ser uma camada delgada, a epiderme comumente é dividida em quatro camadas que são diferenciadas de acordo com a morfologia dos queratinócitos (KOLARSICK; KOLARSICK; GOODWIN, 2011). A camada de células basais, mais próximas a derme, compreendem o estrato germinativo, a camada de células escamosas o estrato espinhoso, a camada de células granulares o estrato granuloso e a camada mais externa composta por células amorfas ou corneócitos compreendem o estrato córneo (BLAYDON; KELSELL, 2014; JAMES et al. 2006).

No sentido longitudinal, logo abaixo da camada epidermal encontra-se uma fina camada especializada da matriz extracelular (MEC), a membrana basal (Figura II.1.2).

Figura II.1.2: Representação esquemática da membrana basal ancorada a epiderme.



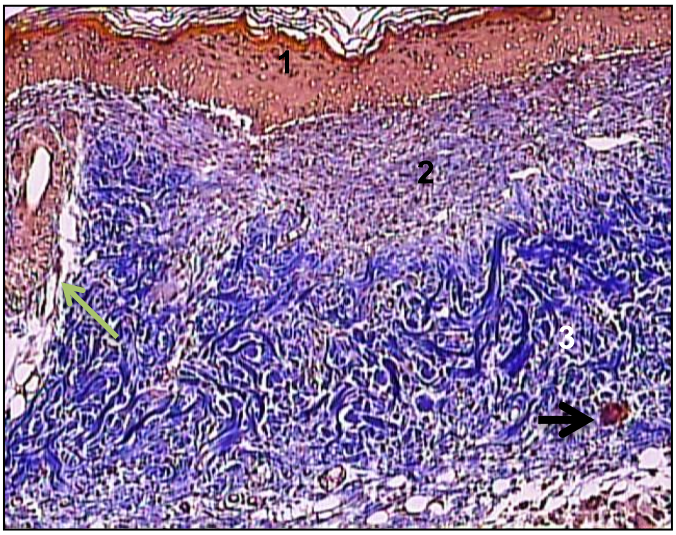
Fonte: Adaptado de GARTNER, L.P.; HIATT, J.L.: **Concise Histogy**, 1st ed., Philadelphia, Saunders, 2011.

A membrana basal é uma estrutura complexa constituída por parte dos componentes presentes na substância fundamental da derme, como colágeno tipo

IV, laminina, proteoglicanos e glicosaminoglicanos (GAGs) como nidogênio, entactina, heparan sulfato e condroitina (MIKESH et al., 2013; YURCHENCO; SCHITTNY, 1990). Além disso, outras proteínas tais como o colágeno tipo VII serve como fibrila de ancoragem juntando a lâmina reticular a lâmina densa na membrana basal promovendo, nesse sentido, a junção entre epiderme e derme (HASHMI; MARINKOVICH, 2011).

Por conseguinte, abaixo da membrana basal encontra-se a camada dermal que consiste em duas regiões, a derme papilar (mais externa) e a derme reticular (mais interna) (*Vide figura II.1.1*). A derme é fundamentalmente um sistema integrado de fibras, filamentos e tecido conjuntivo amorfo que contém anexos cutâneos, vasos e nervos intrincados, fibroblastos e células residentes como mastócitos e macrófagos (GARTNER; HIATT, 2011). A interação entre derme e epiderme mantém as propriedades morfofuncionais de ambos os tecidos (Figura II.1.3). Essa interação é importante para vários processos celulares, incluindo o reparo e remodelação após lesão tecidual e consequente cicatrização.

Figura II.1.3: Fotomicrografia representativa das camadas de pele de camundongo. 1) epiderme; 2) derme reticular; 3) derme papilar (notar: fibras colágenas mais densas – em azul); seta preta: vaso vestigial; seta verde: anexo cutâneo. Coloração: tricrômico de Masson. Magnificância: 10x em microscópio de luz.



Fonte: Próprio autor.

A derme é composta, principalmente, por uma proteína estrutural fibrilar conhecida como colágeno. Essa composição, inclui colágenos dos tipos I e III como os principais constituintes (KOLARSICK; KOLARSICK; GOODWIN, 2011). A região papilar é composta de fibras colágenas finas, enquanto a reticular é dotada de fibrilas mais espessas, conhecidas como fibras colágenas densas (MIKESH *et al*., 2013). Apesar de apresentar fibras colágenas com características diferentes, essas mesmas fibras estão intrincadas em uma unidade fundamental para o tecido conjuntivo ou camada dermal, que é a MEC. Além disso, a derme é também constituída por fibras elásticas que diferem estrutural e quimicamente de fibras colágenas e consistem de dois componentes: proteínas filamentosas e elastina. A associação entre fibras colágenas e elásticas é responsável pela força de tensão e tração da pele, protegendo o organismo e conferindo resistência mecânica a fricções externas (GARTNER; HIATT, 2011).

Fibroblastos são as células mais abundantes no tecido conjuntivo (GOLPOUR *et al*., 2014). São responsáveis por sintetizar aminoácidos essenciais para composição das fibras colágenas como hidroxiprolina e hidroxilisina (JAMES *et al*., 2006; KOLARSICK; KOLARSICK; GOODWIN, 2011). Além disso, participam do processo de fusão de fibras elásticas a MEC na presença de GAGs e de outros processos fisiopatológicos, onde através da secreção de citocinas exercem funções cruciais em diferentes aspectos para homeostasia da derme incluindo o processo cicatricial (GOEL *et al*., 2013; LEE *et al*., 2013).

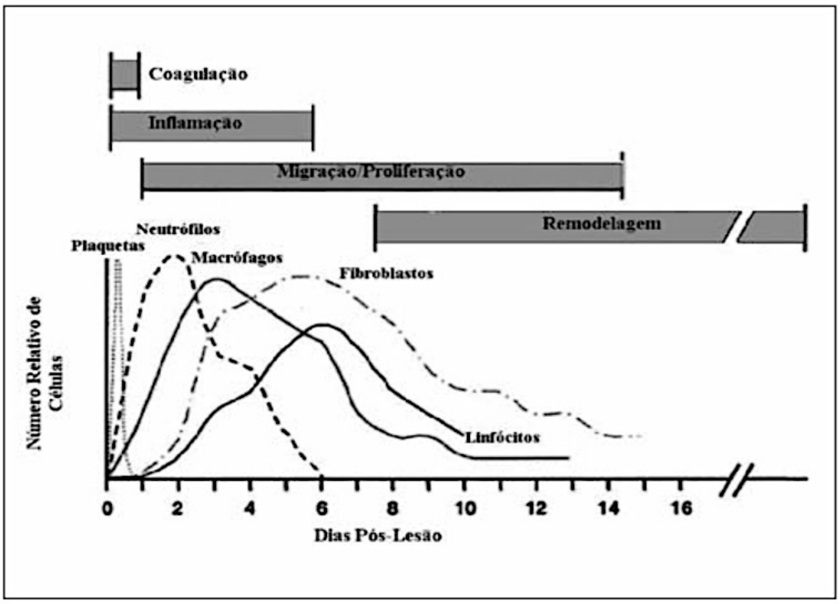
II.2 Cicatrização: aspectos gerais

Feridas cutâneas compreendem uma quebra na continuidade da camada epidermal e são caracterizadas pelo rompimento da estrutura e função dos tecidos subjacentes (GREAVES *et al*., 2013; KAHN; BEERS; LENTZ, 2011). As feridas podem ser classificadas como agudas ou crônicas, dependendo de sua tendência para o processo de cicatrização (PYUN *et al*., 2015). Feridas crônicas como úlceras arteriais, úlceras de pressão e úlceras diabéticas são definidas como lesões teciduais que não cicatrizam em estágios ordenados, ou seja, podem atingir um tempo superior a 12 semanas para regeneração completa (MOHANDAS *et al*., 2015; PHAECHAMUD *et al*., 2015). Contudo, o tempo de cicatrização em ambos os tipos de ferida é dependente de vários fatores de influência local e/ou sistêmicos. Como fatores de influência local podemos citar: a oxigenação e a presença de infecções. Fatores de influência sistêmica compreendem idade, estresse, hormônios sexuais, diabetes e obesidade, medicações, consumo de álcool, tabagismo e dieta (GUO;DI PIETRO, 2010).

A perda da integridade da pele através da indução da lesão tecidual ou ainda por alguma enfermidade pode levar a estágio de morbidez e até possível mortalidade (SINGER; CLARK, 1999). Nesse sentido, a integridade da pele deve ser restaurada para restabelecer seus mecanismos de homeostase, prevenindo infecções e minimizando a perda de fluidos (GURTNER *et al*., 2008).

A cicatrização é descrita como um processo complexo onde múltiplas vias, de eventos fisiopatológicos e bioquímicos que ocorrem em paralelo e de forma inter-relacionada, são ativadas e sincronizadas para induzir o reparo tecidual (GREAVES *et al*., 2013; GURTNER *et al*., 2008). Tradicionalmente, a cicatrização de feridas agudas está dividida em 4 fases sobrepostas conhecidas como hemostasia (coagulação), inflamação, proliferação celular e remodelação (MAAROF *et al*., 2016; MARTIN, 1997) (Figura II.2.1).

Figura II.2.1: Etapas do processo cicatricial relacionados aos tipos celulares ao longo do tempo.



Fonte: Adaptado de PARK, J.E.; BARBUL, A. Understanding the role of imune regulation in wound healing. **Am. J. Surg**., v. 187, S. 11-6, 2004.

Imediatamente após a lesão, o processo cicatricial inicia com hemostasia e processo inflamatório. A vasoconstrição e formação do tampão de fibrina ou plaquetário levam a interrupção da hemorragia (TELLER; WHITE, 2009). A primeira resposta do processo hemostático é a vasoconstrição. Vários mediadores químicos como endotelinas, catecolaminas (epinefrinas) e prostaglandinas são liberados das células adjacentes àquelas que sofreram danos após a lesão tecidual (BROWN, 2015). Paralelamente, a coagulação e a ativação plaquetária contribuem com estímulos adicionais para a vasoconstrição, mediadores como serotonina e tromboxano A2 (KALISH *et al*., 2013).

A cascata de coagulação consiste de duas vias convergentes: extrínseca e intrínseca. Nesse sentido, as vias são ativadas por diferentes fatores nas quais ambas levam a ativação do fator X e Xa com a finalidade de produzir trombina (SCHMAIER, 2008). A trombina executa duas funções importantes durante a formação do tampão de fibrina: catalisar a conversão de fibrinogênio em fibrina e iniciar a ativação plaquetária (BLIDEN *et al*., 2016).

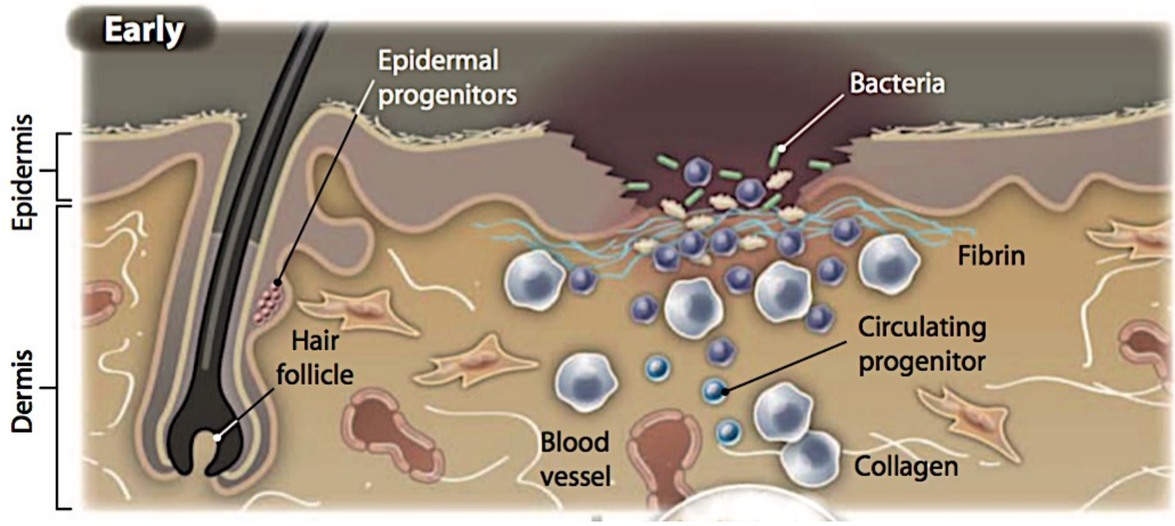
Os primeiros componentes do processo cicatricial são as plaquetas. Elas contribuem para o estágio hemostático através da aderência, agregação e degranulação. A adesão das plaquetas ao local da lesão ocorre pela exposição de colágeno e trombina (FURIE; FURIE, 2008). Essa exposição estimula a expressão de integrinas que dão suporte a adesão de plaquetas ao colágeno, fator de von Villebrand, fibrinogênio e outras plaquetas. A adesão das plaquetas a trombina leva a liberação dos mediadores serotonina e tromboxano A2 otimizando, dessa maneira, a agregação (KALISH *et al*., 2013).

A formação do tampão de fibrina, como resultado da agregação plaquetária, impede a hemorragia, estabelece uma barreira protetora e serve de reservatório para substâncias liberadas pela degranulação de plaquetas (BLIDEN *et al*., 2016). A fibrina seca ou crosta, surge através do contato dos componentes presentes no tampão com o O_2 presente no ambiente (KUMAR *et al*., 2005).

A degranulação de plaquetas é principalmente estimulada pela liberação de interleucina-1 (IL-1) e fator de necrose tumoral-alfa (TNF-α) mediado por queratinócitos adjacentes àqueles da camada epitelial que sofreu a lesão (BARRIENTOS *et al*., 2008). A degranulação envolve a liberação de numerosas citocinas, fatores de crescimento e proteínas da MEC que estão estocados nos alfa-grânulos das plaquetas. Essas substâncias promovem uma variedade de respostas celulares que darão sequencia ao processo de cicatrização como quimiotaxia de polimorfonucleares (PMNs), proliferação celular, deposição de componentes da MEC, angiogênese e remodelação (KONDO; ISHIDA, 2010).

Por conseguinte, após atingir a hemostasia, a inflamação no local da ferida torna-se mais evidente através de sinais flogísticos como calor, dor, edema e hiperemia (BROWN, 2015). Nesse sentido, células vestigiais do endotélio e mastócitos presentes no microambiente da injúria tecidual passam a liberar mediadores como histaminas, prostaglandinas e leucotrienos que são responsáveis pela vasodilatação e consequente permeabilidade vascular (NISHIKORI; SHIOTA; OKUNISHI, 2014; WERNER; GROSE, 2003). O conjunto desses eventos estimulam o surgimento dos sinais flogísticos citados anteriormente. Além de histaminas e prostaglandinas, outros agentes quimiotáticos como citocinas, fatores de crescimento e produtos bacterianos estimulam a migração celular, recrutando neutrófilos e macrófagos para o sítio da inflamação (Figura II.2.2).

Figura II.2.2: Mecanismos celulares e moleculares da regeneração em pele normal. Representação da fase inicial da cicatrização incluindo hemostasia, ativação de queratinócitos e células inflamatórias.



Fonte: EMING, S. A.; MARTIN, P.; TOMIC-CANIC, M. Wound Repair and Regeneration: Mechanisms, signaling, and translation. **Wound Healing**. v. 6, n. 265, p. 1-16, 2014.

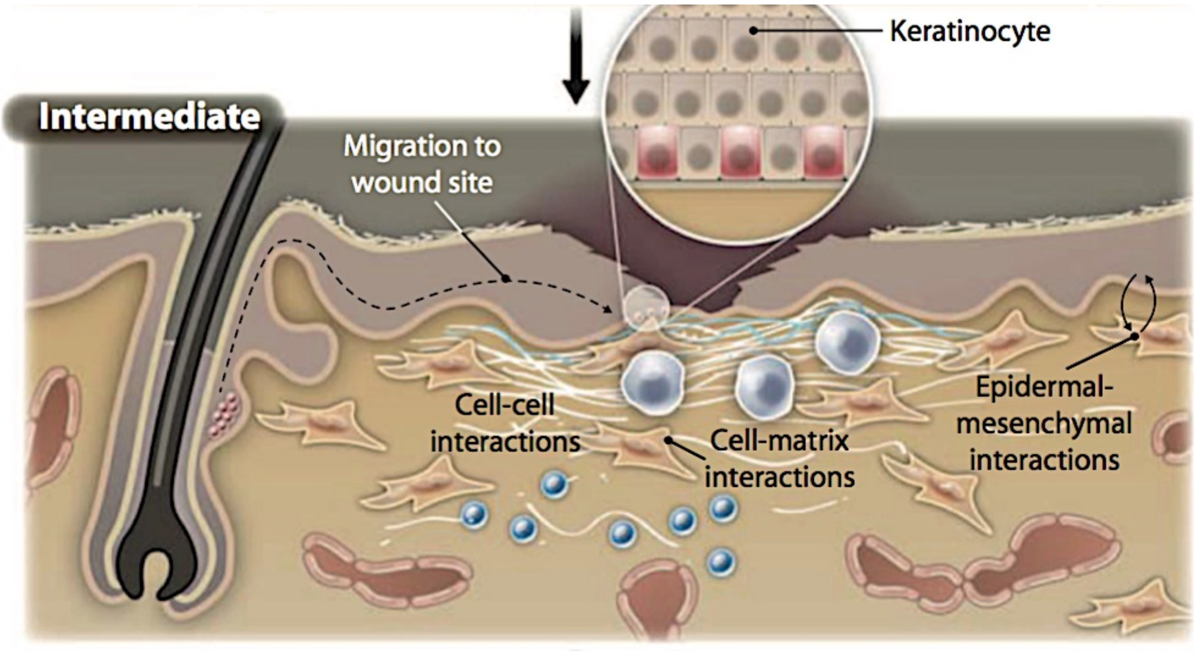
Neutrófilos são as primeiras células a chegarem no sítio da lesão pelo processo de diapedese, onde são inicialmente estimulados por fatores quimiotáticos resultante da degranulação de plaquetas como a interleucina-8 (IL-8), IL-1, TNF-α e produtos bacterianos (GOLDBERG; DIEGELMAN, 2010; ZHANG *et al.*, 2016). Primariamente, os neutrófilos, através da síntese e secreção de enzimas proteolíticas como elastase e metaloproteinase de matriz tipo 8 (MMP-8), causam danos às bactérias e degradam componentes da MEC que sofreram abrasão, evento essencial para continuidade do processo de cicatrização (GOLDBERG; DIEGELMAN, 2010). Assim, neutrófilos fagocitam bactérias mortas e restos dos componentes da MEC outrora degradados (KONDO; ISHIDA, 2010). Após realizada a “limpeza” no sítio da inflamação, neutrófilos são capazes de perpetuar a resposta inflamatória através da secreção de citocinas, predominantemente TNF-α (BARRIENTOS *et al*., 2008; MARTIN, 1997; SU; RICHMOND, 2015). TNF-α estimula a diferenciação de monócitos residentes no tecido a macrófagos e a migração de queratinócitos na tentativa de epitelização. Aproximadamente 24-36h após a abrasão, neutrófilos são, gradativamente, eliminados do local da lesão por apoptose ou fagocitados por macrófagos (PARK; BARBUL, 2004).

Em 48 a 96 horas após a abrasão, macrófagos são as células predominantes no local da ferida (TELLER; WHITE, 2009). Macrófagos são células essenciais ao processo cicatricial, onde desempenham diversas funções. Eles são o elo entre a resposta inflamatória e a fase proliferativa (WITHEREL *et al*., 2016). Além de continuarem a atividade fagocitária de restos celulares para esterilização da ferida, os macrófagos servem como uma fonte de citocinas e fatores de crescimento para controle da resposta inflamatória através da secreção de citocinas pró- e anti-inflamatórias como IL-1, interleucina-6 (IL-6) e interleucina-10 (IL-10), recrutamento e ativação de fibroblastos, consequentemente síntese de componentes da MEC, angiogenese e remodelação tecidual (WERNER; GROSE, 2003). Dessa forma, macrófagos permanecem no local da lesão até o processo cicatricial estar completo (SINGER; CLARK, 1999).

A secreção de IL-10 por macófagos no local da lesão e por queratinócitos da camada epitelial, induzem uma redução na intensidade do processo inflamatório. Como consequência há diminuição da infiltração de neutrófilos e macrófagos, bem como diminuição dos níveis de quimiocinas e citocinas inflamatórias (SATO; OHSHIMA; KONTO, 1999; WERNER; GROSE, 2003). A redução da intensidade da inflamação no local da ferida serve como sinalizador para o início da fase proliferativa e remodeleção tecidual.

A fase proliferativa é caracterizada pela intensa atividade mitogênica entre células e síntese de componentes estruturais da camada dermal. Mais especificamente, há multiplicação de fibroblastos e células endoteliais, envolvendo migração celular e alterações fenotípicas (GREAVES *et al*., 2013). Estes eventos contribuem para a fibroplasia e neovascularização, que se caracteriza pela deposição de componentes da MEC e formação de tecido de granulação (TELLER; WHITE, 2009). Fibroblastos derivados da MEC dão suporte para o influxo celular, adesão e diferenciação de outros fibroblastos das camadas epiteliais adjacentes. Nesse seguimento, a MEC passa a sofrer continua síntese e remodelação de seus componentes (Figura II.2.3).

Figura II.2.3: A fase proliferativa envolve multiplicação e migração de fibroblastos, queratinócitos, deposição de componentes da MEC e angiogênese.



Fonte: EMING, S. A.; MARTIN, P.; TOMIC-CANIC, M. Wound Repair and Regeneration: Mechanisms, signaling, and translation. **Wound Healing**. v. 6, n. 265, p. 1-16, 2014.

Fatores de crescimento, principalmente o Fator de Crescimento Derivado de Plaquetas (PDGF-BB), Fator de Crescimento de Fibroblastos-2 (FGF-2) e Fator de Crescimento Transformante-beta (TGF-β), interagem com fibronectina e fibrinogênio presentes no tampão de fibrina resultante do processo hemostático que funciona como uma matriz extracelular provisória, estimulando a proliferação, expressão de receptores específicos de integrina e migração de fibroblastos até o local da lesão (CHRISSOULI *et al*., 2010; NAFIU; RAHMAN, 2015).

Uma vez no local da lesão, fibroblastos sofrem efeito autócrino de TGF-β1 para proliferação e iniciam a síntese de colágeno reticular ou tipo III, ativados por PDGF, para a remodelação da nova MEC, que gradualmente substituirá a matriz provisória composta de fibrina (BIEFELD; AMINI-NIK; ALMAN, 2013; CLARK, 2001). Além disso, fibroblastos presentes no local da ferida são capazes de produzir MMPs dos tipos 1, 2, 3 e 13, o que permite a degradação da matriz de fibrina que temporariamente obstrui a migração de fibroblastos e queratinócitos (MILLS *et al*., 2014; STAMENKOVIC, 2003; TELLER; WHITE, 2009).

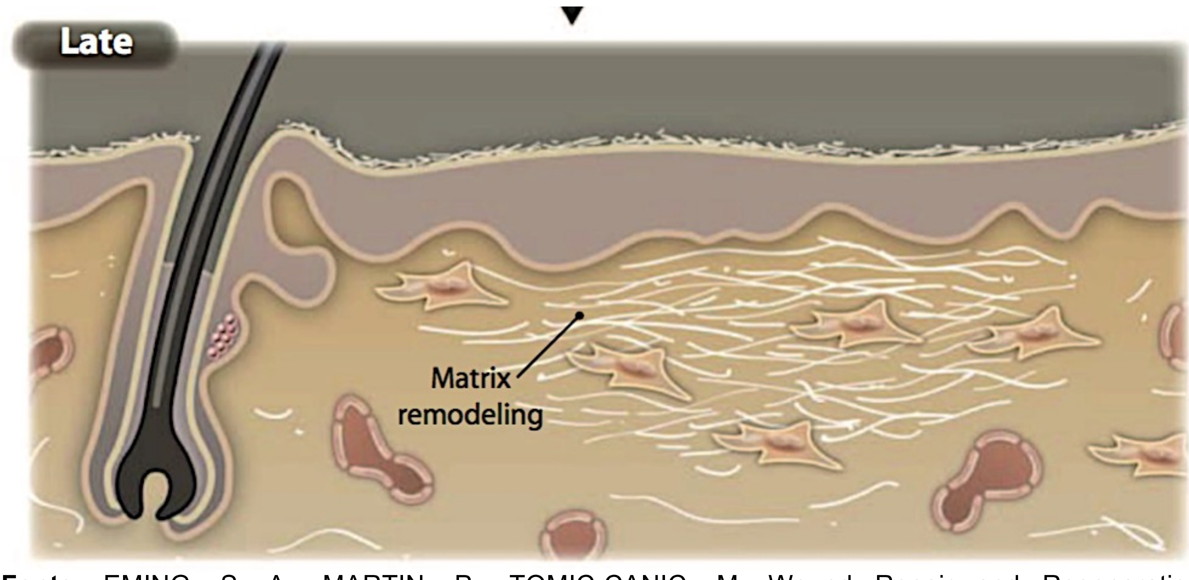
No mesmo tempo da fibroplasia, ocorre o processo de epitelização com a finalidade de reestabelecer a função primária da pele como barreira externa. A secreção de Fator de Crescimento Epidermal (EGF) e TGF-β1 por macrófagos, Fator de Crescimento de Queratinócitos (KGF) por fibroblastos e TGF-α de maneira autócrina estimulam a proliferação e migração centrípeta de células epiteliais resultando no início da epitelização (BARRIENTOS *et al*., 2008; KONDO; ISHIDA, 2010; KOSKELA *et al*., 2010). A migração de queratinócitos ocorre sobre a matriz provisória de fibrina e termina através do contato entre as células, cessando a atividade migratória. Assim, uma nova monocamada do epitélio é criada sobre a lesão. Após atingir 100% de epitelização, os queratinócitos sofrem estratificação e diferenciação para restaurar a barreira (BIEFELD; AMINI-NIK; ALMAN, 2013; O´TOOLE, 2001).

Durante a fase proliferativa, a microvasculatura no local da lesão é reconstituída para reestabelecer o suprimento de nutrientes devido a elevada taxa metabólica no tecido que está em regeneração, promovendo a fibroplasia e prevenindo a hipóxia tecidual (SOTTILE, 2004; YIP, 2015). Células endoteliais são ativadas por TNF-α e FGF secretados inicialmente por macrófagos no local da lesão (STAVRI *et al*., 1995; WILLENBORG *et al*., 2012). Células endoteliais migram para o local da lesão e sua replicação resulta em novos capilares. O Fator de Crescimento Vascular Endotelial (VEGF) é vital ao processo de neovascularização ou angiogênese (LI *et al*., 2014; RAHMAN *et al*., 2016). VEGF é predominantemente secretado por fibroblastos, macrófagos e queratinócitos durante o processo cicatricial (NAFIU; RAHMAN, 2015). A alta afinidade por fibrina e fibrinogênio, mantém o VEGF presente na matriz provisória da lesão, que associado a degradação proteolítica realizada por MMPs, facilita o avanço dos novos vasos por toda a área lesionada (GREAVES *et al*., 2013; SAHNI; FRANCIS, 2004). A densa população de células composta por fibroblastos, macrófagos e células endoteliais intricadas ao colágeno, fibronectina e GAGs recém produzidos formam o tecido de granulação em resposta a intensa atividade de citocinas e fatores de crescimento da fase proliferativa (DIEGELMAN; EVANS, 2004).

Na transição entre fase proliferativa e remodelativa, fibroblastos são capazes de modular sua atividade assumindo um fenótipo contrátil, o miofibroblasto, na qual realizam a contração da ferida por exercer tração física sobre as fibras colágenas e posterior remodelação. Essa mudança fenotípica é modulada por TGF-β (CRIDER *et al*., 2011). Miofibroblastos possuem em seu citoplasma fibras compostas de actina alfa similares as encontradas na musculatura lisa (α-SMA) (MURPHY *et al*., 2011; TOMASEK *et al*., 2013).

Nessa perspectiva, o processo cicatricial é finalizado através da atividade de inibidores tissulares de metaloproteinases (TIMPs) responsáveis por reduzir os níveis de MMPs que outrora degradaram a matriz provisória de fibrina (GILL; PARKS, 2007; GOLDBERG; DIEGELMAN, 2010). No momento em que o fechamento da lesão é realizado por completo, miofibroblastos passam a sofrer apoptose (MOULIN *et al*., 2004). Entretanto, fibroblastos continuam o processo remodelativo que aumenta a força tênsil do tecido cicatricial (MURPHY *et al*., 2011). A matriz provisória composta por fibrina desorganizada é remodelada com novos componentes da MEC como GAGs, proteoglicanos e fibronectina. O colágeno tipo III inicialmente sintetizado na fibroplasia é gradativamente substituído por colágeno fibrilar ou tipo I com a finalidade de aumentar a espessura, força e organização do novo tecido conjuntivo (Figura II.2.4) (GOLDBERG; DIEGELMAN, 2010).

Figura II.2.4: A fase de remodelativa envolve remodelação da MEC, resultando na formação de cicatriz e restauração da barreira epitelial.



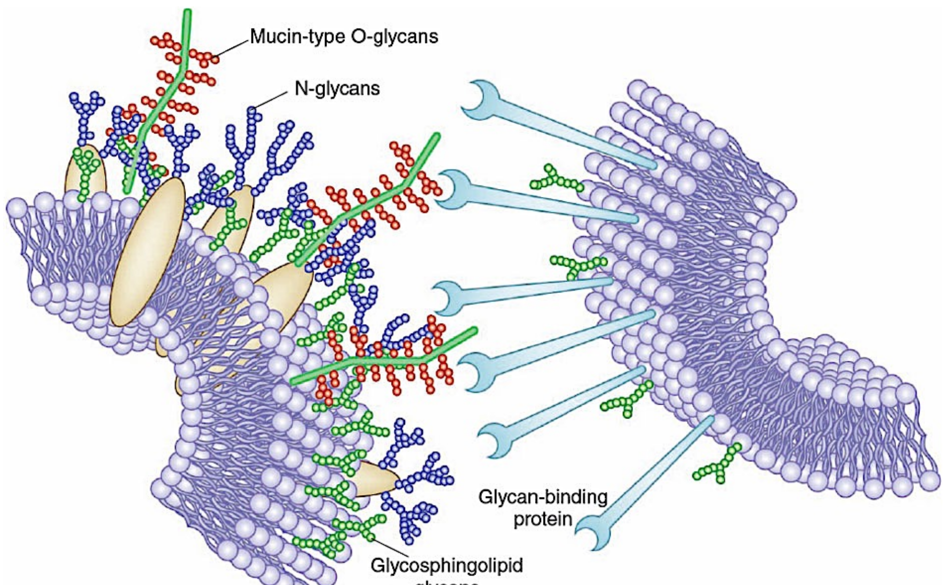
Fonte: EMING, S. A.; MARTIN, P.; TOMIC-CANIC, M. Wound Repair and Regeneration: Mechanisms, signaling, and translation. **Wound Healing**. v. 6, n. 265, p. 1-16, 2014.

II.3 Câncer: Definição, tumorigênese e glicosilação, incidência e mecanismos de apoptose

Câncer é um termo genérico utilizado para designar um grupo diversificado de doenças que podem afetar qualquer parte do corpo humano. Outros termos são também aplicáveis, como tumores e neoplasias (NWODO *et al*., 2016). A característica que melhor define o câncer refere-se à proliferação descontrolada de células anormais, as quais crescem sem respeitar os limites das células vizinhas (KUMAR et al., 2010). Células neoplásicas conseguem invadir os tecidos adjacentes e disseminar-se para outras regiões do corpo, num processo conhecido como metástase (GIBBS, 2000; HANAHAN; WEINBERG, 2011; RASTOGI et al., 2014). Segundo a Organização Mundial de Saúde (OMS) as metástases estão relacionadas ao aumento nos casos de mortalidade em decorrência do câncer (LI; CHEN, 2012).

Carboidratos ou glicanos são macromoléculas essenciais para a produção de energia e reações metabólicas dos sistemas vivos e são distribuídos de maneira ubíqua (GHAZARIAN; IDONI; OPPENHEIMER, 2011). Além disso, os carboidratos desempenham papeis cruciais em uma variedade de processos biológicos, pois servem como moléculas sinalizadoras nos ambientes intra- e intercelular e são capazes de mediar interações entre células (Figura II.3.1) (GHAZARIAN; IDONI; OPPENHEIMER, 2011; ROTH; YHEZKEL; KHALAILA, 2012).

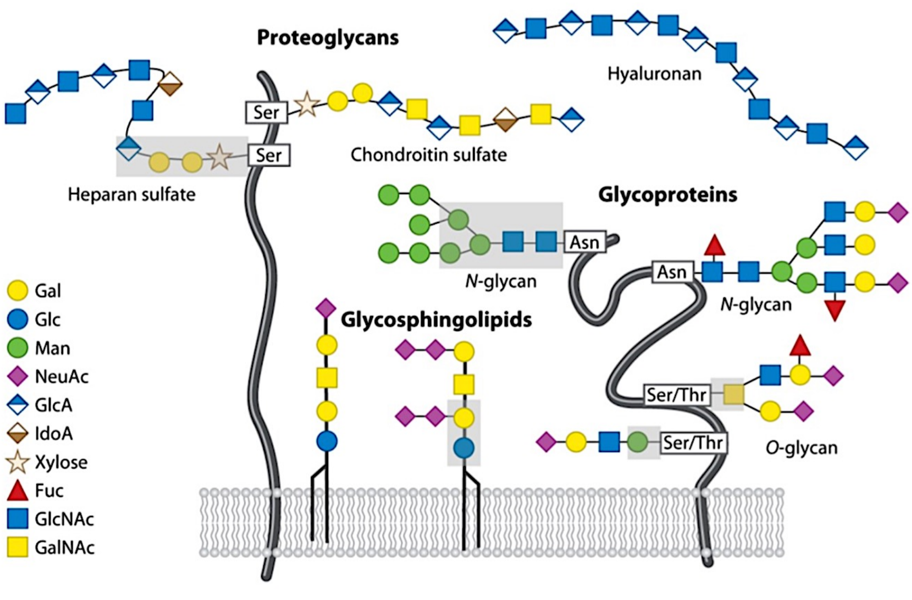
Figura II.3.1: Interações célula-célula são mediadas através da interação entre carboidratos e proteínas na superfície celular.



Fonte: ZAIA, J. At last, fuctional glycomics. **Nature Methods**., v. 8, p. 55-57, 2011.

Essas funções fisiológicas tornam-se possíveis, pois todas as células são compostas por uma densa camada de glicoconjugados em que um ou mais monossacarídeos estão ligados covalentemente a lipídeos (glicolipídeos) e/ou proteínas (glicoproteínas) (BULL; DEN BROK; ADEMA, 2014; VARKI; LOWE, 2009). A formação de glicoconjugados através da glicosilação na superfície celular é um processo de modificação pós-traducional de proteínas sendo considerado universal aos sistemas vivos, na qual refletem o estado fisiológico das células (Figura II.3.2) (JOHNSON *et al*., 2013; VARKI; ESKO; COLLEY, 2009). Em glicoproteínas, oligossacarídeos encontram-se covalentemente *N*-ligados ou *O*-ligados ao arcabouço da proteína (GAO, 2015; REIS *et al*., 2010). *N*-glicanos são oligossacarídeos que se ligam covalentemente a resíduos de asparagina no contexto de uma sequência aminoacídica do tipo Asn-X-Ser/Thr (sendo X qualquer aminoácido com exceção da prolina). Enquanto *O*-glicanos são oligossacarídeos capazes de se conjugar a resíduos de serina e/ou treonina (Ser/Thr) (GAO, 2015; VARK; SHARON, 2009). As conjugações referentes a *O*-glicosilação ocorrem de forma sequencial no aparelho de Golgi, na qual o processo é iniciado pela adição de uma N-acetilgalactosamina (GalNac) a um resíduo de serina ou treonina no arcabouço da sequencia aminoacídica, formando o antígeno *Tn* (CAZET *et al*., 2010; REIS *et al*., 2010).

Figura II.3.2: Classes de glicanos comumente encontrados na superfície celular de mamíferos.



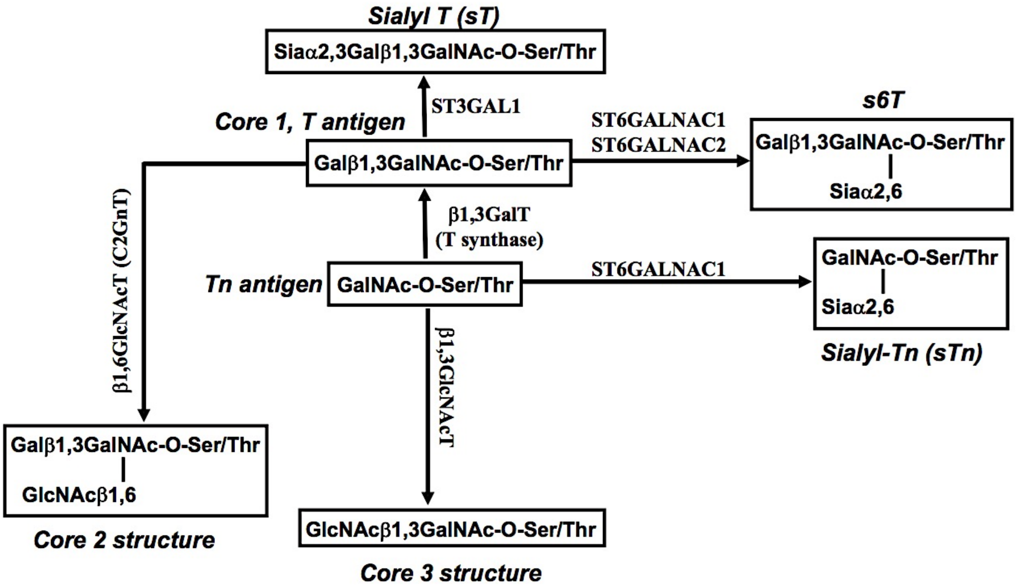
Fonte: ILLAHAN, C. D.; PAULSON, J. C. Glycan microarrays for decoding the glycome. **Annu Rev Biochem,** v. 80, p. 797-823, 2011.

Apesar de sua abundância na membrana celular, muitas funções fisiológicas dos glicanos ainda não são bem estudas. No entanto, é sabido que mudanças no padrão de glicosilação das células animais se refletem em várias condições patológicas (HAUSELMANN; BORSIG, 2014; BULL; DEN BROK; ADEMA, 2014). A glicosilação é notavelmente dinâmica e comumente modificada nas células neoplásicas levando a expressão de antígenos associados a tumorigênese (VARKI; KANNAGI, 2009). Os dois princípios básicos que guiam a alteração da "população" glicosídica na superfície de células tumorais são ou a síntese incompleta e/ou a síntese de novas estruturas glicídicas. Essas mudanças determinam quando o câncer está em estágio inicial ou estágio avançado (KANNAGI *et al*., 2004). De maneira geral, alterações na via da glicosilação de células normais ocorre devido as seguintes condições: baixa ou elevada expressão de glicosiltransferases, que pode ser desregulada a nível epigenético; disfunções na estrutura do aparelho de Golgi, onde muitas glicosiltransferases são abrigadas e alterações na expressão de aceptores em glicoconjugados (KELLOKUMPU; SORMUNEM; KELLOKUMPU, 2002; MIYAGI, 2008).

A glicosilação incompleta de *O*-glicanos, leva à formação de oligossacarídeos truncados e, consequente aumento da expressão de antígenos nomeados *Thomsen-Friedenreich* (TF). Antígenos TF são representados por *T* e *T*n além de suas formas sialiladas s*T* e s*T*n como resultado da glicosilação efetivada por sialiltransferases. Sua estrutura e biossíntese está representada na figura II.3.3 (próxima página). A expressão dos antígenos TF na forma de *T* e *T*n não é frequentemente encontrada nos tecidos normais (BROCKHAUSEN, 1999; DALLOLIO *et al*., 2014).

Assim, é de notável importância referir que a metastização desempenhada por células neoplásicas são principalmente fruto do diferenciado padrão de glicosilação em relação a células normais (HAKOMORI, 1996; PINHO *et al*., 2007; VARKI; KANNAGI, 2009). A expressão anormal de glicanos na superfície de células neoplásicas têm sido correlacionada com o potencial invasivo de vários tipos de câncer, principalmente por estruturas sialiladas como antígeno s*T*n, que são largamente expressas em carcinomas colorretal, de ovário, de mama e pancreático (BALDUS; HANISCH, 2000; KIM *et al*., 2002; LEIVONEN *et al*., 2001; CAZET *et al*., 2010; DALLOLIO *et al*., 2014). Nesse contexto, a disseminação de células cancerosas através de metástase é imensamente atribuída a suas propriedades adesivas, que são afetadas pela glicosilação anormal na superfície da membrana celular, promovendo a interação da célula com moléculas da MEC (GHAZARIAN; IDONI; OPPENHEIMER, 2011; GOETZ *et al*., 2009).

Figura II.3.3: Estrutura e biossíntese relacionados a antígenos *Thomsen-Friedenreich*.



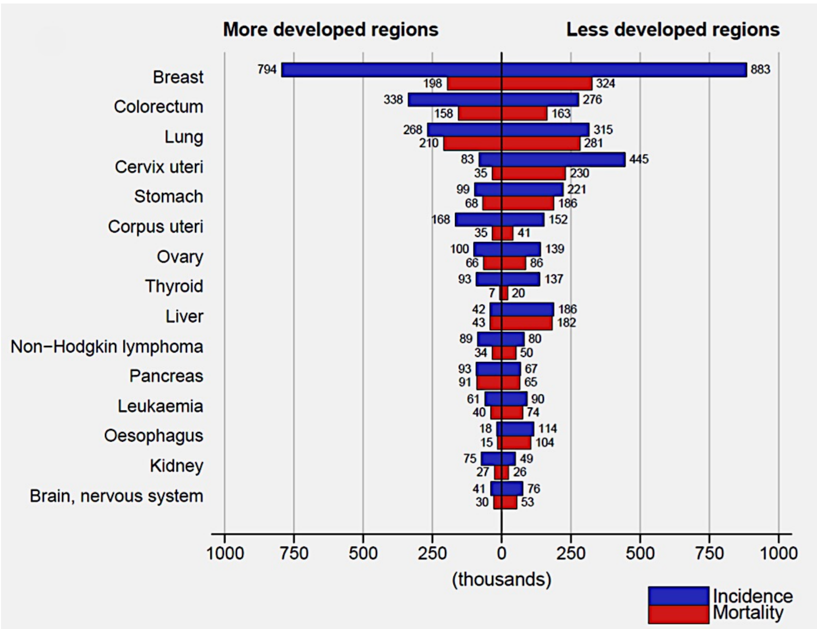
Fonte: DALL'OLIO, F. *et al.* Sialosignaling: sialyltransferases as engines of self-fueling loops in cancer progression. **Biochim Biophys Acta,** v. 1840, n. 9, p. 2752-2764, Sep, 2014.

O câncer é considerado, atualmente, um dos principais problemas de saúde pública (SIEGEL; MILLER; JEMAL, 2016). De acordo com estimativas da Organização Mundial de Saúde (OMS), o câncer, desde 2011, é a segunda principal causa de morte no mundo, ainda com números pouco inferiores em relação as mortes causadas por doenças coronarianas e superando as incidências relativas a acidentes cerebrovasculares (AVC) (FERLAY *et al*., 2015; OMS, 2011). Nesse sentido, dados epidemiológicos advindos de levantamentos anuais mostram que a problemática do câncer demanda ações intensas e urgentes. Segundo a OMS, 7,6 milhões de pessoas morreram de câncer em 2008, equivalendo a 13% do total de todas as mortes ocorridas no mundo naquele ano (OMS, 2013; NTIE-KANG *et al*., 2014). Em 2012, o número de mortes por câncer aumentou para 8,2 milhões. Face a essas estatísticas, já é constatado pela OMS que há mais de 100 tipos de câncer, bastante variados e dependem principalmente da localização, capacidade de metástase e tamanho (FERLAY et al., 2013; LI *et al*., 2015).

As contínuas transições demográficas e epidemiológicas globais sinalizam para um aumento do número de casos da doença nas próximas décadas, particularmente em países de baixa renda, com cerca de 20 milhões de novos casos expectáveis, anualmente, até 2025 (BRAY, 2014; SIEGEL; MILLER; JEMAL, 2015). Atualmente, os tipos de câncer mais comuns são: melanoma, próstata, mama, colorretal, fígado, pâncreas, próstata e câncer de pulmão, sendo este último a principal causa de morte entre os tipos de cânceres citados (SIEGEL; MILLER; JEMAL, 2016). Entretanto, a tabua de incidência e mortalidade relacionada ao câncer sofre alterações diferenciadas quando as estatísticas passam a voltar-se para as diferenças no gênero (homens ou mulheres) (SIEGEL; MILLER; JEMAL, 2015; 2016).

O câncer de mama é considerado a neoplasia mais comum em mulheres e é a segunda maior causa de morte no mundo, para indivíduos desse gênero. Cerca de 1,67 milhões de novos casos foram diagnosticados em 2012, compreendendo 25% entre todos os carcinomas (SOTIRIOU *et al*., 2003; WU; SARKISSYAN; VADGAMA, 2016). Foi constatado que a maior incidência ocorre em países subdesenvolvidos, seguido de perto por países desenvolvidos (Figura II.3.4) (FERLAY *et al*., 2015). Nesse contexto, as taxas de incidência variam em até quatro vezes em diferentes regiões do mundo.

Figura II.3.4: Estimativa de novos casos e mortalidade por tipo de câncer em mulheres em países desenvolvidos e subdesenvolvidos.



Fonte: FERLAY, J. *et al.* Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. **Int J Cancer,** v. 136, n. 5, p. E359-86, Mar 1 2015.

Na América do Sul, estimou-se, para 2012, a ocorrência de 1,1 milhão de novos casos de câncer. Nesse sentido, é possível observar a existência de um perfil de magnitude similar aos dos países desenvolvidos (FERLAY *et al*., 2013). A estimativa para o Brasil, no biênio 2016-2017, aponta para o surgimento de 600 mil novos casos de câncer (INCA, 2016). Excetuando-se o câncer de pele não melanoma, ocorrerão 420 mil novos casos (INCA, 2016). Em 2016, são esperados aproximadamente 58 mil novos casos de câncer de mama, com um risco estimado de 56,20 casos a cada 100 mil mulheres (Tabela II.3.1).

Tabela II.3.1: Estimativas para o ano de 2016 das taxas brutas de incidência por 100 mil habitantes no Brasil.

Localização Primária da Neoplasia Maligna	Estimativa dos Casos Novos							
	Homens				Mulheres			
	Estados		Capitais		Estados		Capitais	
	Casos	Taxa Bruta	Casos	Taxa Bruta	Casos	Taxa Bruta	Casos	Taxa Bruta
Próstata	61.200	61,82	13.940	64,93	-	-	-	-
Mama Feminina	-	-	-	-	57.960	56,20	18.990	79,37
Colo do Útero	-	-	-	-	16.340	15,85	4.550	19,07
Traqueia, Brônquio e Pulmão	17.330	17,49	4.430	20,59	10.890	10,54	3.230	13,49
Cólon e Reto	16.660	16,84	5.560	25,80	17.620	17,10	6.210	25,95
Estômago	12.920	13,04	3.130	14,54	7.600	7,37	2.180	9,07
Cavidade Oral	11.140	11,27	2.780	12,95	4.350	4,21	1.230	5,04
Laringe	6.360	6,43	1.600	7,50	990	0,94	320	0,97
Bexiga	7.200	7,26	2.110	9,79	2.470	2,39	830	3,21
Esôfago	7.950	8,04	1.460	6,75	2.860	2,76	610	2,27
Ovário	-	-	-	-	6.150	5,95	2.170	8,92
Linfoma de Hodgkin	1.460	1,46	450	1,74	1.010	0,93	400	1,33
Linfoma não Hodgkin	5.210	5,27	1.550	7,15	5.030	4,88	1.670	7,02
Glândula Tireoide	1.090	1,08	350	1,27	5.870	5,70	1.800	7,46
Sistema Nervoso Central	5.440	5,50	1.290	5,86	4.830	4,68	1.250	5,20
Leucemias	5.540	5,63	1.370	6,38	4.530	4,38	1.180	4,88
Corpo do Útero	-	-	-	-	6.950	6,74	2.530	10,47
Pele Melanoma	3.000	3,03	840	3,86	2.670	2,59	740	2,96
Outras Localizações	51.850	52,38	11.890	55,45	47.840	46,36	11.820	49,33
Subtotal	**214.350**	**216,48**	**52.750**	**245,63**	**205.960**	**199,57**	**61.710**	**257,55**
Pele não Melanoma	80.850	81,66	17.370	80,90	94.910	91,98	21.910	91,65
Todas as Neoplasias	**295.200**	**298,13**	**70.120**	**326,51**	**300.870**	**291,54**	**83.620**	**348,99**

Fonte: Instituto Nacional do Câncer José Alencar Gomes da Silva (2016)

Durante a tumorigênese dos carcinomas de mama, há uma interrupção da expressão de GlcNacβ1,6 (core-2, Figura II.3.3) e consequente acumulo de *T* e *sTn*. Essa mudança está relacionada a superexpressão da sialiltransferase ST3Gal1, que converte o antígeno *T* em sialil-T (*sT*), inibindo a síntese de GlcNacβ1,6 que é encontrada em células normais da mama (MARCOS *et al*., 2003). A super-expressão de ST3Gal1 está associada a condições que caracterizam o crescimento tumoral na mama, contribuindo também para sua progressão (PICCO *et al*., 2010; SOLATYCKA *et al*., 2012).

Nesse contexto, a detecção precoce do câncer é uma etapa crucial, e na maioria das vezes, constitui-se como condição importante para que ocorra o sucesso durante o tratamento (WHELAN *et al*., 2009). Atualmente, as principais formas de tratamento contra o câncer são o procedimento cirúrgico, a quimioterapia e a radioterapia. Muitos tipos de câncer têm se mostrado resistentes aos tratamentos convencionais, pois a maior efetividade da quimioterapia, por exemplo, só ocorre nos estágios iniciais da doença, pois é capaz de impedir a progressão de tumores primários bem delimitados (TEH *et al*., 2013). No entanto, o mesmo não se aplica a metástase devido ao seu envolvimento sistêmico. A resistência ao processo de apoptose está intimamente relacionada a tumorigênese e essa etapa é a principal causa da resistência de células tumorais as terapias anticâncer (JAATTELA, 1999; VALASTYAN; WEINBERG, 2011).

A morte celular programada não envolve apenas a tradicional morte modulada pela via apoptótica, mas sim por múltiplas mortes programadas, incluindo a apoptose tipo II ou autofagia (BIALIK *et al*., 2010). A apoptose (tipo I) é o principal tipo de morte celular e ocorre quando o dano ao DNA é irreparável. Duas vias distintas são capazes de induzir ao processo apoptótico. A via extrínseca é ativada através do receptor de morte e a via intrínseca é iniciada a partir de danos causados a mitocôndria no citoplasma celular (AMELIO; MELINO, KNIGHT, 2011; EUM; LEE, 2011).

A via extrínseca é iniciada a partir de um estímulo extracelular que induz a ligação de FAS, o receptor de morte presente na membrana plasmática, e seu ligante específico extracelular denominado FAS-ligante ou FAS-L (BIALIK *et al*., 2010; KERR; WYLLIE; CURRIE, 1972). Esse estimulo é realizado, principalmente pelo acoplamento de ligantes similares como o CD95 a CD95L ou pela ligação de TNF ao seu receptor TNFR-1. Essa combinação FAS/FAS-L, promove o recrutamento de adaptadores de proteínas intracelulares conhecidos como FADD (*Fas-associed death domain*) que se ligam a várias moléculas de procaspase-8 para ativá-las (ORAL *et al*., 2015). Consequentemente, após ativada, a caspase-8 já como caspase iniciadora, comumente ativa a procaspase-3 para a execução do processo apoptótico (OUYANG *et al*., 2012). Além da caspase-3, as caspases-6 e -7 são exemplos de caspases efetoras sobre o processo de apoptose por via extrínseca (KIM; JAYAPRAKASHA; PATIL; SHOJA *et al*., 2015).

A família Bcl-2 é uma família de proteínas indutoras ou sepressora da apoptose e que participam ativamente da regulação do processo apoptótico. Bcl-2 e

Bcl-XL são membros inibidores da apoptose, pois previnem a liberação de citocromo *c,* inibem a formação de espécies reativas de oxigênio (EROS) e estabilizam o potencial de membrana nas mitocôndrias (BORNER, 2003; VANDER; THOMPSON, 1999). Bax, Bid e Bak são proteínas pró-apoptóticas, pois induzem de forma positiva a morte celular por apoptose (HENGARTNER, 2000). Nesse contexto, a homeostasia é mantida pelo balanço da expressão de proteínas pró- e anti-apoptóticas (GRIVICICH; REGNER; ROCHA, 2007).

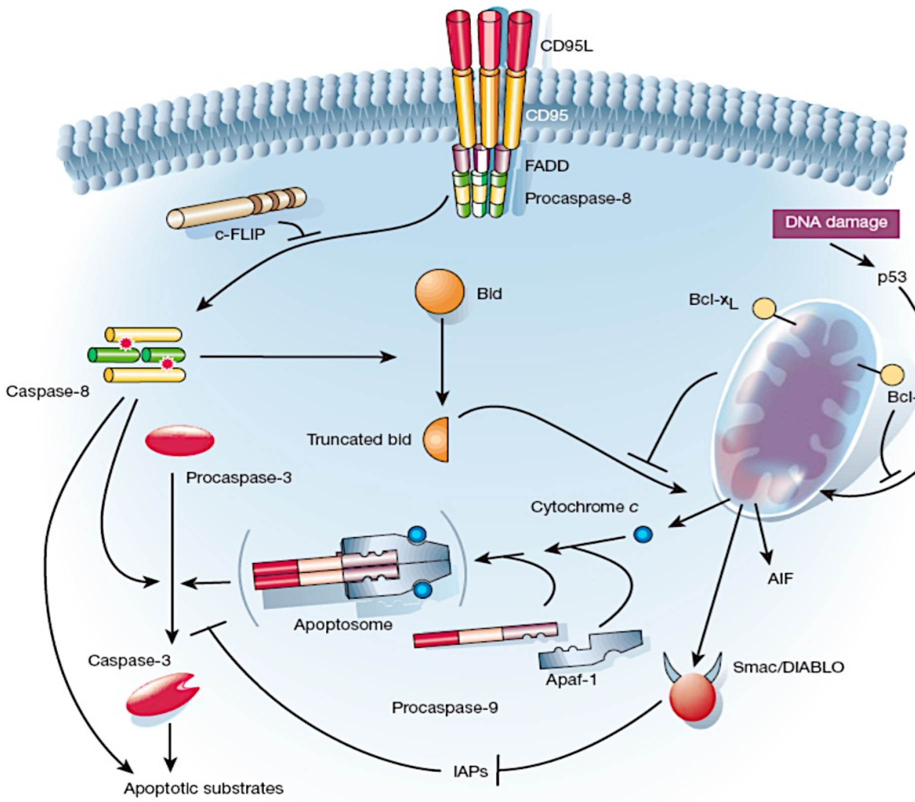
Na via intrínseca, a apoptose é induzida a partir de vários estímulos extra- ou intracelulares, como a deprivação de fatores de crescimento, danos ao DNA, ativação de oncogenes ou hipóxia (ORAL *et al*., 2015). Os estímulos citados anteriormente, induzem a uma maior expressão de proteínas pró-apoptóticas, como Bax que se liga a membrana externa da mitocôndria e como resultado ocorrem alterações no potencial da membrana interna da mitocôndria, levando a interrupção da síntese de ATP, aumento da produção de EROS, bem como, aumento da permeabilidade ao citocromo *c*, como consequência do desacoplamento da cadeia respiratória (GOTTLIEB, 2001; GUPTA, 2003; MURPHY *et al*., 2000). O acumulo de citocromo *c* no citoplasma, recruta o cofator Apaf-1 que se liga especificamente a procaspase-9 para compor o apoptossomo e promover a clivagem da procaspase-9, liberando a caspase-9 ativa. Por conseguinte, a caspase-9 ativa a caspase-3 promovendo a apoptose (GHOBRIAL; WITZIG; ADJEI, 2005; OUYANG *et al*., 2012) (Figura II.3.5, próxima página).

Em muitos adenocarcinomas, a proteína Bcl-2 é superexpressa no sentido de dar maior longevidade as células tumorais e impedir de qualquer maneira que a atividade das células tumorais sejam suprimidas (KOLLEC et al., 2016). Embora o câncer possa exibir características heterogêneas, todos os tumores malignos adquiriram a propriedade de crescer além dos limites de células normais. De fato, a análise do processo de tumorigênese demonstra que sua capacidade de resistir à morte pode ser adquirida por diferentes mecanismos durante o desenvolvimento tumoral.

A apoptose na prática clínica é alvo para compreensão de mecanismos de resistência à tratamento convencionais. Dessa forma, a elucidação de alguns mecanismos moleculares abrem à possibilidade de novas estratégias para a modulação do processo apoptótico, ou mesmo da pesquisa por novos compostos com potencial biotecnológico para o tratamento do câncer.

Nessa perspectiva, vários trabalhos realizados na última década, deixam bem documentado que a morte celular programada (apoptose) pode ser induzida por uma variedade de produtos naturais, em alternativa aos métodos convencionais já citados, em uso clínico, com diversas estruturas químicas e diferentes mecanismos de ação, como por exemplo a degradação do DNA genômico (gDNA) e a outra forma de morte celular programada, conhecida como autofagia (LEE *et al*., 2016). Assim, a habilidade de células tumorais em responder e ativar o processo apoptótico podem, em parte, determinar o sucesso do uso de produtos naturais como estratégia terapêutica.

Figura II.3.5: Representação das duas principais vias de apoptose em mamíferos.
A via extrínseca inicia após ligação de CD 95 ao CD95L promove o recrutamento de múltiplas procaspase-8 via FADD, induzindo a ativação de caspase-8 que pode ser bloqueada pelo recrutamento de c-FLIP. No mecanismo mitocondrial ou via intrínseca, estímulos extra- ou intracelulares como danos ao DNA (*i.e. ilustrado*), aumentam a expressão de proteínas pró-apoptóticas da família Bcl-2. A interação de Bax com a mitocôndria, desestabiliza a membrana levando a liberação de citocromo c e conseguintes ativações das caspases-9 e -3. A regulação da ativação das caspases na via intrínseca é mediada pelas proteínas IAP (*Inhibitor of Apoptosis Protein*) e smac/DIABLO.



Fonte: HENGARTNER, M. O. The biochemistry of apoptosis. **Nature,** v. 407, n. 6805, p. 770-6, 2000.

Outro tipo de morte celular programada, ou apoptose tipo II, a autofagia vem se tornando cada vez mais evidente por se tratar de um mecanismo multifacetário relacionado a progressão ou supressão de células tumorais. A autofagia é o principal regulador de mecanismos catabólicos que ocorrem em células neoplásicas. Nessa perspectiva, quando a relação entre autofagia e câncer são analisados, um desafio comum é determinar se o processo autofágico tem fator protetor ou contribui para morte celular de células neoplásicas (DUFFY *et al*., 2015).

Apesar de ser pouco frequente em comparação a apoptose tipo I, a autofagia é fisiopatologicamente relevante, pois participa da degradaçãoo retinal (GUIMARAES *et al*., 2003), elimina *Salmonella* de macrófagos infectados (HERNANDEZ *et al*., 2003) e em alguns casos é responsável por mediar a morte tumoral por quimioterapia (KANZAWA *et al*., 2003; 2004; OGIER-DENIS et al., 1996). Além disso, muitas pesquisas têm demonstrado que apoptose tipo I e autofagia são eventos interconectados, e a morte de células neoplásicas pode ser considerada como um balanço dinâmico entre as vias (KESSEL; REINERS, 2007).

A autofagia é caracterizada pelo acumulo de vacúolos acidicos no citoplasma celular e por ser crucial para sobrevivência celular em condições extremas, principalmente pela deprivação de nutrientes, onde a degradação de macromoléculas no ambiente intracelular é importante para o fornecimento de energia (BOYA *et al*., 2005; WANG *et al*., 2011). Nesse sentido, a ativação do processo autofágico pode exercer funções importantes para a progressão do tumor nos estágios iniciais. Por outro lado, a autofagia pode agir como supressor tumoral através da regulação de genes pró-autofágicos como beclina-1 (ATG 6-*Autphagy-related gene-6*), Bcl-2 e p53 (KUNDU; THOMPSON, 2008; WHITE, 2012;). Assim, a modulação da autofagia através de genes ou mesmo através da aplicação de novos compostos com a finalidade de ativar o processo autofágico no sentido da supressão tumoral, emergem como novos alvos terapêuticos para o tratamento de neoplasias.

II.4 Produtos Naturais

Nos últimos anos, o aumento do número de pacientes portadores de doenças crônicas como câncer e/ou diabetes Mellitus têm assolado os sistemas de saúde pública de diversos países desenvolvidos e em desenvolvimento. O constante aumento na incidência de número de casos que decorre quase que paralelamente ao número de mortes, como citado anteriormente, está longe de ser acompanhado por soluções terapêuticas. Estimativas indicam que aproximadamente 6 milhões de pessoas sofrem com feridas crônicas atualmente. Nos Estados Unidos, é suposto que se gaste cerca de US$ 1.000 por dia com tratamento de lesões crônicas (HSU *et al*., 2015). Feridas cutâneas em pacientes diabéticos quase sempre são acompanhadas por um elevado número de morbimortalidades, e as alternativas terapêuticas em muitos casos geram gastos elevados, entretanto, com pouca efetividade. Como já citado anteriormente, os tratamentos convencionais contra o câncer não acompanham, em termos de efetividade, o fator progressivo da doença, deixando claro a extrema necessidade de recursos medicamentosos para esses tipos de patologia.

Segundo a OMS cerca de 80% da população mundial ainda utiliza primariamente plantas medicinais para os cuidados com a saúde (KHALIL; AFIFI; AL-HUSSAINI, 2006). Esse fato fica mais evidenciado em regiões em que a saúde pública mostra-se mais deficiente. Por necessidade ou opção, muitas pessoas recorrem a este tipo de terapia. Produtos naturais são compostos ou substâncias que são produzidos naturalmente por organismos vivos e têm uma “longa história” na prevenção e tratamento de várias doenças (NOBILI *et al*., 2009) A prospecção de novas moléculas advindas de produtos naturais são comumente derivadas de gêneros alimentícios como plantas e são considerados seguros para seres humanos (GOSH; SIL, 2013). Produtos naturais são fontes de moléculas que podem ser utilizadas como agentes para o tratamento de várias doenças com forte relação epidemiológica (PHILLIPSON, 2007). Para tratamento de desordens dermatológicas ou mesmo cicatrização de feridas, o uso de plantas não tem sido diferente, já que há indícios que as plantas são utilizadas desde a antiguidade para tratar especialmente, feridas, cortes e queimaduras (HORINOUCHI et al., 2013).

Os oceanos cobrem cerca de 71% da área total do planeta e abrigam 34 dos 36 Filos animais identificados taxonomicamente (ARRIETA; ARNAUD-HAOND; DUARTE, 2010). Uma enorme diversidade biológica e química de habitats marinhos fornece novas fontes de agentes com potencial terapêutico (CHEUNG et al., 2016). Dessa forma, a diminuição do número de drogas sintéticas efetivas contra várias doenças como o câncer e o aumento do número de produtos naturais derivados de organismos marinhos em estudos clínicos e pré-clínicos aumentam a perspectiva do desenvolvimento de novos insumos com potencial biotecnológico (MOLINSKI et al., 2009). Além do mais, alguns produtos naturais isolados de animais marinhos já foram aprovados para o tratamento contra o câncer, na qual muitas vezes são conjugados a anticorpos, onde, como destaque pode-se citar o Adcetris® que contém dolastatina 10 isolada da lebre do mar *Dolabella auricularia* (FLAHIVE; SRIRAGAM, 2012; NEWMAN; CRAGG, 2016).

Compostos biologicamente ativos com grande potencial terapêutico formam as bases moleculares para muitas drogas em uso clínico (PESSOA *et al*., 2006). Um elevado número de estudos descrevendo às potencialidades de produtos naturais como estratégias terapêuticas na medicina tradicional, tem estado em evidência na literatura. Nesse contexto, a maioria das pessoas residentes em países desenvolvidos têm buscado terapias a partir da medicina popular, em virtude da diminuição dos efeitos colaterais (KUMAR *et al*., 2007).

Plantas e organismos marinhos produzem uma grande variedade de biomoléculas e compostos químicos, como lectinas e metabólitos secundários, que exibem um amplo espectro de atividades biológicas, como: atividade antineoplásica, pró-cicatrizante, anti-inflamatória, antimicrobiana e antiviral (CHEUNG *et al*., 2015; 2016; KOHARUDIN; GRONENBORN, 2014; LONGHI-BALBINOT *et al*., 2012; LA e*t al*., 2013; MOURA *et al*., 2013; RABELO *et al*., 2012; SATO *et al*., 2011; TEIXEIRA *et al*., 2006), e vêm sendo largamente explorados como moléculas biologicamente ativas e, portanto, possuem um forte amparo biotecnológico.

Dessa maneira, torna-se ainda mais solicito o advento de novos recursos medicamentosos para o tratamento de doenças crônicas. De fato, a sociedade necessita de respostas urgentes para os principais problemas de saúde pública atualmente, as quais podem ter seu diagnóstico e/ou tratamento fortemente beneficiado pela descoberta de novas moléculas advindas de plantas e organismos marinhos.

II.4.1 Metabólitos secundários

Por conveniência de investigação, há a distinção no metabolismo das plantas. Nomeadamente, metabolismo primário e metabolismo secundário. O metabolismo primário refere-se ao processo de produção de compostos essenciais para a sobrevivência do organismo, como ácidos carboxílicos do ciclo de Krebs, α-aminoácidos, carboidratos, proteínas, lipídeos e ácidos nucleicos (SHEEN, 2014). Esses produtos são produtos das vias metabólicas que são comuns a todos os organismos vivos. Por outro lado, os metabólitos secundários, são produzidos por vias biosintética diferentes das estabelecidas para metabólitos primários. Contudo, ambos os metabolismos encontram-se interligados, onde o metabolismo primário fornece moléculas que servem de base para a síntese de estruturas complexas originadas pelo metabolismo secundário (GANDHI; MAHAJAN; BEDI, 2015).

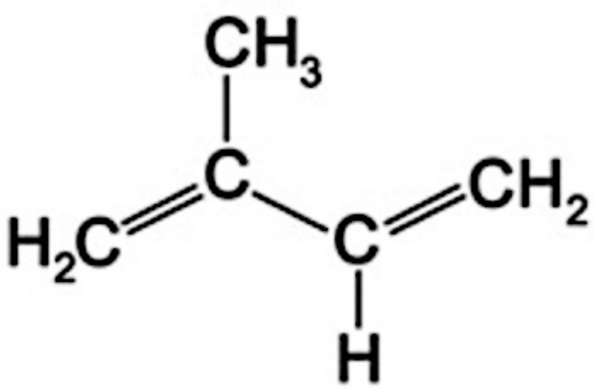
Por décadas, os metabólitos secundários foram considerados como subprodutos sem função aparente, a não ser a de armazenamento de carbono em excesso fixado pela fotossíntese (IASON, 2005). No entanto, essa visão foi refutada, e atualmente é bem estabelecido que metabólitos secundários desempenham papel fundamental no desenvolvimento das plantas e sua interação com o meio ambiente (PUSZTAHELYI; HOLB; PÓCSI, 2014). Os metabólitos secundários são responsáveis por desempenhar atividades importantes na bioquímica e fisiologia dos vegetais (SHEEN, 2014). Muitas classes de produtos naturais constituem grupos importantes de metabólitos secundários, estando envolvidos em muitos processos fisiológicos respondendo, principalmente a estímulos ambientais (LIMA; GOMES, 2014; STOBIECKI *et al*., 2003).

Uma importante classe entre os metabólitos secundários são os terpenos. Terpenos, representam uma extensa e diversa classe de compostos químicos produzidos por plantas. As plantas empregam terpenos para uma variedade de funções no crescimento e desenvolvimento. Contudo, sua participação é majoritariamente desempenhar interações químicas e proteção em ambientes bióticos e abióticos. Tradicionalmente, terpenos tem sido usados em indústrias alimentícias e química, além de serem contemplados por indústrias farmacêuticas no desenvolvimento de novos medicamentos. Existem mais de trinta mil, terpenos e terpenoides, identificados e esses compostos apresentam variadas funções fisiológicas nas plantas (ZWENGER; BASU, 2008).

Os terpenos são basicamente hidrocarbonetos, enquanto os terpenóides que, também são conhecidos como isoprenoides e podem compreender mais de 20 mil moléculas distintas, podem conter funções químicas como álcool, aldeído, ácidos, cetonas e são caracterizados por ciclizações, rearranjos (KRIEF et al., 2005; TA; ARNASON, 2015).

A estrutura química dos terpenos é constituída pela união de duas, quatro, seis, oito ou mais unidades de isopreno (Figura II.4.1) (ROSENSTIEL *et al*., 2002). Essa é uma característica particular dos terpeno que apresentam a fórmula geral $(C_5H_{10})_n$. Dessa forma, esses compostos são classificados de acordo com o número de unidades de isopreno presente no seu arcabouço de carbono: hemiterpenos (n=1), monoterpenos (n = 2), sesquiterpenos (n = 3), diterpenos (n = 4), triterpenos (n = 6), tetraterpenos (n = 8) e politerpenos (n > 8).

Figura II.4.1: Estrutura química da molécula de isopreno.



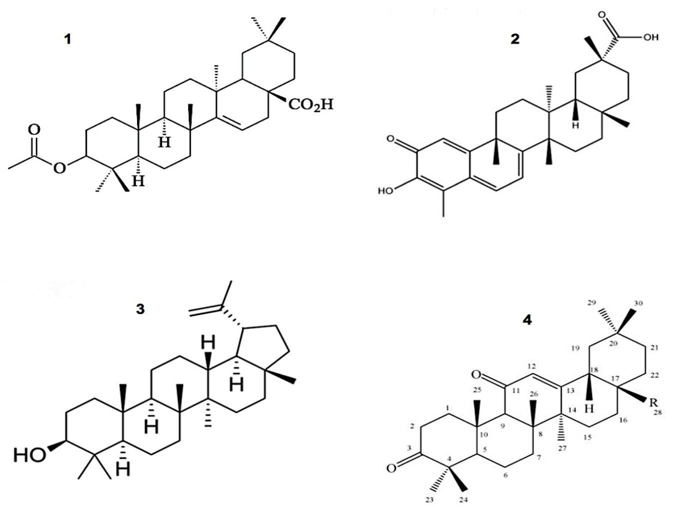
Fonte: ROSENSTIEL, T. N. *et al.* Differential accumulation of dimethylallyl diphosphate in leaves and needles of isoprene- and methylbutenol-emitting and nonemitting species. **Plant Physiol,** v. 129, n. 3, p. 1276-84, Jul 2002.

Na medicina popular, assim como na terapêutica, plantas contendo derivados terpênicos tem sido usadas como antimicrobianos, anti-inflamatórios, antiulcerogênicos, tranquilizantes (PASSOS *et al*., 2009; SOUSA *et al*., 2007). Muitos óleos essenciais e extratos etanolico e hexânicos possuem uma grande variedade de propriedades farmacológicas como: anticonvulsivante, antinociceptiva, anti-inflamatória e anti-hemorrágica. Silveira, Andrade e Sousa (2015), relatam que as atividades farmacológicas muitas vezes estão ligadas a terpenóides.

Particularmente, os triterpenos são compostos presentes em materiais como resina, látex, ceras e cutícula de plantas. Além disso, essa classe de terpenoides apresentam comportamento anfipático, ou seja, possuem uma parte hidrossolúvel (glicose) e outra lipossolúvel (triterpeno), esta última pode explicar o fato da internalização de alguns triterpenos através da membrana plasmática de células tumorais (VIAU *et al*., 2014). Nas plantas, as saponinas desempenham papel protetor contra insetos (PERES, 2004).

A literatura relata que triterpenos já isolados de plantas, são possuidores de várias atividades biológicas importantes. Dentre essas atividades, podemos citar: atividade antineoplásica, antiparasitária, neuroprotetora, anti-inflamatória e antinociceptiva (LIMA *et al*., 2015; NAGASE *et al*., 2003; NEUKIRCH *et al*., 2005; SETHI *et al*., 2007). As estruturas dos triterpenos relacionados as atividades biológicas citadas anteriormente são: (1) ácido acetil aleuritólico (AAA); (2) celastrol; (3) lupeol e (4) oleanano e estão representados na figura II.4.2. Contudo, uma limitada quantidade de produtos ou compostos isolados de plantas tem sido cientificamente explorado no contexto da regeneração tecidual de feridas agudas e crônicas (TEH *et al*., 2013). Assim, face as várias aplicabilidades biotecnológicas possíveis, terpenos e terpenoides tem chamado a atenção de diversos pesquisadores.

Figura II.4.2: Estruturas dos triterpenos com atividades biológicas relevantes para saúde.



II.4.1.1 Potencialidades biotecnológicas de Metabólitos secundários na cicatrização de feridas

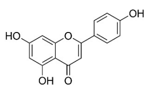
Aproximadamente um terço de todos os medicamentos derivados de plantas é utilizado para o tratamento de feridas cutâneas ou outras doenças de pele, quando comparado a medicamentos convencionais de uso clínico (KHALIL; AFIFI; AL-HUSSAINI, 2007). Apesar do crescente uso de plantas na cicatrização, apenas uma pequena quantidade de espécies tem sido investigada com relação ao seu potencial no âmbito da descoberta de novos medicamentos (SERVAT- MEDINA *et al*., 2015). Nesse contexto, cerca de 1-3% das drogas listadas na farmacopeia ocidental são próprias para o uso na pele ou sobre a cicatrização de feridas (KUMAR *et al*., 2007), deixando claro a necessidade de estudos mais aprofundados sobre metabólitos bioativos isolados de plantas.

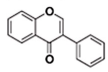
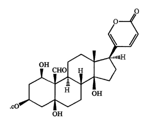
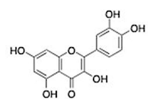
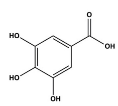
Rane e Mengi (2003), avaliaram o efeito do extrato hidroalcoólico do caule de Terminalia arjuna e concluíram que o extrato demonstrou efeitos promissores sobre a apitelização das lesões em ratos. Posteriormente, Chaudhari e Mengi (2006) avaliaram o efeito da aplicação tópica de diferentes fitoconstituintes (frações I, II e III) fracionados do extrato hidroalcoólico do caule de *Terminalia arjuna* em modelo *in vivo* de cicatrização. O efeito das frações foram avaliados através de ensaios para determinar força tênsil da lesão, epitelização, tecido de granulação e atividade antimicrobiana. Foi observado que o extrato hidroalcoólico de *T. arjuna* consistia principalmente de taninos, açucares redutores e saponinas. As lesões tratadas com a fração I (taninos) mostraram maior força tênsil quando comparada as frações II (açucares) e III (saponinas) e o controle positivo. Além disso, a fração I induziu epitelização em 9 dias, enquanto que a fração III apenas no dia 20 após indução da lesão. A fração I (constituída de taninos) também induziu atividade antimicrobiana relativamente a *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* e *Streptococcus pyogenes*, indicando que taninos podem promover a cicatrização por prevenir infecções e aumentar a contração da lesão.

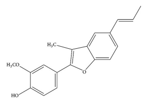
Por conseguinte, ensaios realizados com um extrato de triterpenos isolados do caule de *Betulae cortex*, árvore popularmente conhecida como vidoeiro, é capaz de induzir a diferenciação e migração de queratinócitos *in vitro* e acelerar a cicatrização em modelo *ex vivo* (METELMANN *et al*., 2012). Além disso, uma pomada constituída por óleo-gel semissólido a base de triterpenos isolados de *B. cortex* (OG-S10) encontra-se em estudo clínico fase II. A pomada é constituída por ácido betulinico, lupeol, eritrodiol e ácido oleanólico. O tratamento com OG-S10 é capaz de acelerar a cicatrização e prevenir cicatriz fibrótica, melhorando a aparência estética (METELMANN *et al*., 2014).

O mecanismo pela qual a maioria dos metabólitos bioativos promovem a cicatrização ainda não é bem elucidado. Além disso, o efeito sobre o processo cicatricial está melhor descrito na literatura, através da ação sinérgica entre os compostos presentes nos extratos ou infusões, em sua maioria, de partes aéreas das plantas (FACUNDO *et al*., 2005). Schmidt e colaboradores (2009), realizaram estudo de plantas usadas na medicina popular brasileira como agentes cicatrizantes. Os extratos etanólico e hexânico das partes aéreas de 12 plantas pertencentes a flora brasileira foram testadas em vários ensaios para se determinar seus efeitos citotóxicos sobre células e citocinas inflamatórias envolvidas no processo cicatricial (Tabela II.4.1).

Tabela II.4.1: Plantas da flora brasileira e seus efeitos sobre o tratamento de feridas in vitro e seus metabólitos secundários conhecidos.

Nome Científico	Órgão	Atividade Biológica	Estrutura/ Metabólito Secundário	Referência
Brugmansia suaveolens	Folha	ND	Alcaloide Tropano	ALVES *et al*., (2007)
Eupatorium laevigatum	Folha	Cicatrizante, anti-fúngico	ND*	MAIA *et al*., (2002)
Galingosa parviflora	Folha	Cicatrizante	 Flavonoides: apigenina	PLECKANOV *et al*., (1978)

Iresine herbstii	Folha	Cicatrizante, anticâncer	 Isoflavona: betacianina	CAI *et al*., (2005)
Kalanchoe tubiflora	Folha	Cicatrizante, anti-inflamatório	 Briofilina A	SUPRATMAN *et al*., (2001)
Pativeria alliacea	Folha, Flores e Raiz	Cicatrizante, anti-inflamatório	ND*	KUBEC *et al*., (2002)
Pluchea sagitalis	Folha	Cicatrizante	 Flavonóides	MARTINO *et al*., (1979)
Piper regnellii	Folha	Cicatrizante, anti-inflamatório	 Sesquiterpens	PEREZ-GARCIA *et al*., (2005)

Schinus molle	Folha	Cicatrizante, anti-inflamatório	 Ácido gálico	MARZOUK *et al*., (2006)

Fonte: SCHIMDT, C. *et al.* Biological studies on Brazilian plants used in wound healing. **Journal of Ethnopharmacology,** v. 122, p. 523-532, 2009.
ND*: Não determinado.

II.4.1.2 Plantas do gênero Combretum

Plantas medicinais têm sido utilizadas praticamente em todas as culturas, desde os primórdios como medicamentos, e são de grande importância para a saúde das comunidades (EDEOGA; OKWU; MBAEBIE, 2005). Com o passar dos anos, a medicina tradicional vem sendo usada em várias partes do mundo e está crescendo com relação a sua importância econômica, sobretudo em países desenvolvidos.

O uso de plantas da família Combretaceae na medicina popular, é bem documentado na literatura (MCGAW *et al*., 2001; MUTHU *et al*., 2006). Esta família é constituída por aproximadamente 20 gêneros onde possuem cerca de 600 espécies. Os maiores gêneros são Combretum e Terminalia, com 370 e 200 espécies catalogadas, respectivamente (PIETROVSKI *et al*., 2006). Os membros da família Combretaceae são distribuídos, principalmente, em regiões tropicais e subtropicais, como África e Brasil (LIMA *et al*., 2012).

Estudos fitoquímicos envolvendo plantas do gênero Combretum vem, proporcionalmente, aumentando nos últimos anos. Estes estudos revelaram a ocorrência de muitas classes de compostos incluindo triterpenos, flavonoides, aminoácidos não proteicos, entre outros (PIETROVSKI *et al*., 2006). Nesse contexto, vários trabalhos envolvendo espécies do gênero Combretum têm evidenciado várias atividades biológicas importantes, demonstrando que esses fitoconstituintes possuem potencial promissor na geração de novos fármacos. *Combretum micrathum*, uma espécie endêmica da África possui atividade antimicrobiana e antidiabética (ABREU *et al*., 1999; CHIKA; BELLO, 2010). Uma proantocianidina isolada do extrato hidroalcóolico de *Combretum mucronatum* apresentou atividade anti-helmintica (SPIEGLER *et al*., 2015). Uma ecacetina isolada do extrato aquoso de folhas de *Combretum vendae* demonstrou atividade anti-oxidante (KOMAPE *et al*., 2014). Já o extrato etanólico de *Combretum adenogonium* induz atividade anti-HIV-1, contudo ainda não se sabe que componente é capaz de exercer atividade anti-viral (MUSHI *et al*., 2012).

No Brasil, *Combretum leprosum*, é uma planta que vem sendo bastante estudada (Figura II.4.3, próxima página). Plantas dessa espécie são conhecidas popularmente como mofumbo, mufumbo, pente-de-macaco, carne-de-vaca ou cipoaba, e além de ser uma espécie melífera, está distribuída nos estados do Norte e Nordeste, Mato Grosso e Mato Grosso do Sul (CHAVES *et al*., 2007). *C. leprosum* é bastante utilizada na medicina popular, principalmente pela preparação de infusões de suas partes aéreas que proporcionam o tratamento deferidas e irritações cutâneas, ação expectorante, anti-hemorrágico, sedativo e analgésico (AGRA *et al*., 2007; FACUNDO *et al*., 2005).

Nos últimos anos, vários estudos envolvendo fitoconstituintes, principalmente isolados de extratos das partes aéreas de *C. leprosum*, vem demonstrando potencialidades biotecnológicas promissoras. Lopes e colaboradores (2012), isolaram o flavonoide (-) epicatecina (EPI) do extrato hidroalcoólico da casca do caule de *C. leprosum*. EPI demonstrou atividade antinociceptiva em camundongos, reduzindo os efeitos causados pelo glutamato. A atividade antinociceptiva foi revertida por naloxona e glibenclamida, sugerindo a participação de receptores opioides e canais de potássio dependentes de ATP. Em outro estudo, Facundo e colaboradores (2005), isolaram o composto denominado ácido arjunólico (Figura II.4.4, próxima página) do extrato etanólico de raízes de *C. leprosum* e observaram que o composto apresenta atividade anti-inflamatória ao reduzir edema de pata em ratos, induzido por carragenina, quando administrado por via oral . Fernandes e colaboradores (2014), constataram que o mesmo ácido arjunólico (AJ) foi capaz de reduzir os efeitos do veneno de *Bothrops jararacussu* e *Bothrops jararaca* em modelos *in vitro* e *in vivo*, sugerindo que AJ pode ser um potencial antidoto antiofídico no futuro.

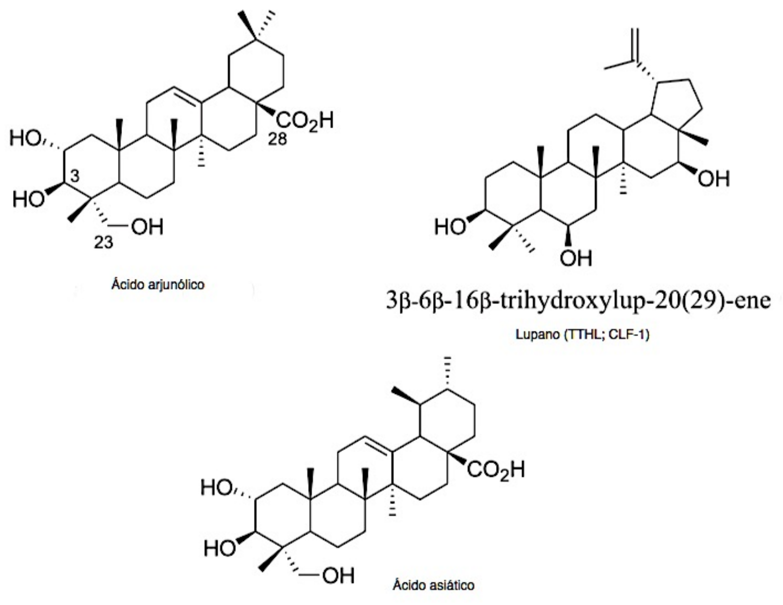
Figura II.4.3: Partes aéreas do Mufumbo (*Combretum leprosum*).

Fonte: http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB6906

O triterpeno isolado do extrato etanólico de flores de *C. leprosum*, conhecido como lupano ou TTHL induziu atividade antinociceptiva em camundongos, reduzindo a nocicepção causada por glutamato (PIETROVSKI *et al*., 2006) . Outros trabalhos já descritos com TTHL (lupano), mostram que o triterpeno possui atividade antiinflamatória onde reduziu os níveis de TNF-α e IL-1β, sem alterar os níveis de IL-10. Além disso, reduziu a infiltração leucocitária induzida por ácido acético (LONGHI-BALBINOT *et al*., 2012). Além disso, a atividade antinociceptiva também foi descrita para o triterpeno (LONGHI-BALBINOT *et al*., 2011). Outro importante efeito de TTHL é a atividade antitumoral descrita por Viau e colaboradores (2014). TTHL foi capaz de induzir apoptose de células do adenocarcinoma de mama MCF 7 de modo tempo dependente. Além disso, o triterpeno é capaz de causar fragmentação do DNA de MCF 7 através da geração de espécies reativas de oxigênio (EROS) ou pela internalização e interação do triterpeno com o DNA das células tumorais. TTHL possui atividade antileishmanial, onde causa a redução da viabilidade das formas promastigotas de *Leishmania amazonensesis* (TELES *et al*., 2011). Mais recentemente, o triterpeno isolado do extrato etanólico das folhas de *C. leprosum* (CLF-1) apresentou atividade antimicrobiana sobre bactérias Gram-positivas (EVARISTO *et al*., 2014).

Nesse contexto, *Combretum leprosum* pode ser uma importante opção para a medicina tradicional e clínica, pois vem demonstrado ser fonte potencial de novos fármacos. Além disso, apesar das diversas atividades biológicas apresentas até o momento, por diferentes constituintes, não existem dados que comprovem sua eficácia sobre o processo cicatricial, bem como qual/quais composto(s) podem induzir de forma efetiva a cicatrização.

Figura II.4.4: Estrutura química dos triterpenos isolados de *Combretum leprosum* (Lupano; ácido arjunólico e ácido asiático).



Fonte: Adaptado de FACUNDO, V. A. *et al.* Arjunolic Acid in the Ethanolic Extract of *Combretum leprosum* Root and its Use as a Potential Multi-Functional Phytomedicine and Drug for Neurodegenerative Disorders: Anti-Inflammatory and Anticholinesterasic Activities. **Journal of the Brazilian Chemical Society,** v. 16, n. 6B, p. 1309-1312, 2005.

II.4.2 Lectinas

II.4.2.1 Aspectos históricos e conceito

Em organismos vivos, existe um grupo de proteínas extremamente difundido, as lectinas, estando presente de maneira ubíqua em todas as classes e famílias de serves vivos, desde micro-organismos até organismos superiores (LANOO; VAN DAMME, 2010).

Charcot e Robin (1853), observaram a presença de inclusões em tecidos patológicos, conhecidas como cristais de Charcot-Leyden, demonstrando que essa classe de biomoléculas já era bem documentada. Esses cristais possuem a habilidade de se ligar a carboidratos e compreendem uma forma parcialmente pura de uma lectina, na qual foi denominada galectina-10 (SWAMINATHAN, 1999).

Pouco tem depois, Mitchell em 1960, observou que certos venenos de cobra possuíam a habilidade de aglutinar eritrócitos, contudo não definiu quais constituintes eram responsáveis por tal efeito (NILSSON, 2007).

As atividades biológicas de lectinas foram descobertas inicialmente no fim do século XIX, quando uma proteína chamada ricina foi encontrada em sementes de mamona (*Ricinus communis*) (VAN DAMME; LANNOO; PEUMANS, 2008). Peter Hermann Stillmark (1988), em sua tese de doutorado, referiu essas proteínas como hemaglutininas ou fitoaglutininas devido a potente capacidade dos extratos dessa planta em aglutinar eritrócitos, na qual esta lectina estava presente,. Contudo, ao mesmo tempo que são capazes de exercer atividade hemaglutinante, se mostraram altamente tóxicas (SHARON; LIS, 2004).

A ideia de que a toxicidade é uma propriedade intrínseca das lectinas foi abandonada no início do século XX, quando Ladsteiner e Raubistscheck (1907) relataram pela primeira vez a presença de uma lectina não tóxica nas leguminosas *Phaseolus vulgaris* (feijão), *Pisum sativum* (ervilha), *Lens culinaris* (lentilha) e *Vicia sativa*. Após esse trabalho muitas outras hemaglutininas vegetais não tóxicas foram descobertas. A partir de então, tornou-se evidente que lectinas estão difundidas nos vários reinos e que o aspecto que infere toxicidade é exceção, e não regra (VAN DAMME *et al*., 1998).

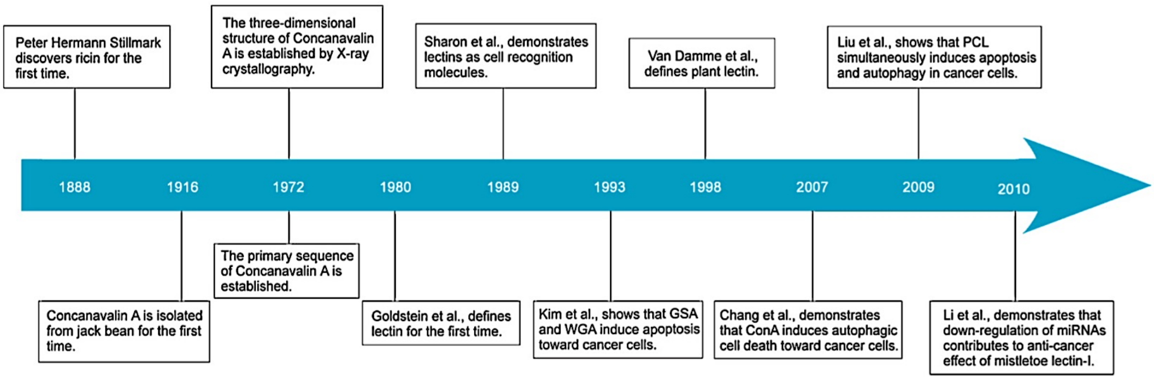
Watkins e Morgan (1952), demonstraram, pela primeira vez, que a ligação a carboidratos era a principal mediação no processo de hemaglutinação de lectinas. Anteriormente a esse estudo, Summer e Howell (1936) observaram que o açúcar da cana inibia a atividade hemaglutinante de uma aglutinina isolada de sementes de *Canavalia ensiformis*, a Concanavalina A ou Con A.

O termo lectina (do latim, *legere* – escolher, selecionar) foi inicialmente proposto por Boyd e Shapleihg (1954) para descrever aglutininas presentes em plantas que exerciam atividade hemaglutinante sobre eritrócitos humanos dependendo de seu grupo sanguíneo ABO (SHARON; LIS, 1972; NILSSON, 2007). Nesse contexto, posteriormente foi mostrado que esta seletividade exercida por lectinas é baseada na sua capacidade de se ligar a diferentes tipos de carboidratos presentes nas membranas eritritárias.

A partir disso, muitas atividades biológicas passaram a ser performadas, passando a fazer parte do *hall* do potencial biotecnológico dessas proteínas. Peter

C. Nowell (1960), demonstrou que a lectina de *Phaseolus vulgaris* (PHA) possui a habilidade de estimular mitogenicidade em linfócitos, e que essa atividade e inibida por monossacarídeos específicos (SHARON; LIS, 2004). Posteriormente, Joseph C. Aub demonstrou que a lectina isolada em gérmen de trigo, *Wheat germ agglutinin* (WGA), tem a habilidade de aglutinar preferencialmente células neoplásicas (AUB *et al*. 1963; 1965). A figura II.4.2, demonstra do ponto de vista histórico, as pesquisas realizadas com lectinas de plantas, deixando claro seu potencial na pesquisa de novas drogas no combate ao câncer.

Figura II.4.5: Linha do tempo. A história de lectinas na pesquisa contra o câncer.



Fonte: FU, L. L. *et al.* Plant lectins: targeting programmed cell death pathways as antitumor agents. **Int J Biochem Cell Biol,** v. 43, n. 10, p. 1442-9, Oct 2011.

Por décadas, lectinas tem sido um paradigma na área diretamente relacionada as interações proteína-carboidrato. E mesmo a base molecular do reconhecimento de carboidratos por lectinas tem sido elucidados a partir de uma variedade de técnicas como cristalografia de raios-X, Ressonância Magnética Nuclear (RMN), espectrometria de massas e calorimetria (LORIS *et al*., 1998; CAVADA *et al*., 2001). A especificidade a carboidratos tem permitido os uso dessas biomoléculas em muitas aplicações bioquímicas e biotecnológicas como caracterização de carboidratos complexos e glicoconjugados (*Lectin microarray*). Dessa forma, lectinas tem sido utilizadas como poderosas ferramentas em imunologia e glicobiologia devido a suas variadas especificidades por carboidratos complexos (TATENO *et al*., 2008).

Com base nesse conhecimento, Peumans e Van Damme (1995) definem lectinas como (glico)proteínas de origem não-imune com pelo menos um domínio não catalítico que se ligam reversivelmente a mono ou oligossacarídeos de forma específica. Entretanto, esse conceito tem criado controvérsias entre vários pesquisadores, devido a descoberta recente de lectinas que participam ativamente do sistema imunológico em animais como a MBL (*Mannan Binding Lectin*) ou as selectinas que compartilham propriedades similares as lectinas tipo-C. Dessa forma, lectinas podem também ser definidas como proteínas que possuem pelo menos um domínio não catalítico que se ligam especifica e reversivelmente a epítopos de carboidratos sem alterar sua estrutura (PANDA *et al*., 2014).

II.4.2.2 Classificação das lectinas animais

Estruturas complexas de oligossacarídeos estão dispostas na superfície celular, sendo incorporadas na MEC e/ou presentes em glicoconjugados. Esses oligossacarídeos executam funções estruturais, podem mediar o endereçamento de glicoconjugados para a superfície celular, ou agir como moléculas sinalizadoras mediando interações entre células ou a MEC. Contudo, as funções não-estruturais de glicanos geralmente requerem lectinas, em que a resposta a ligação ao açúcar podem ser atribuídas ao(s) domínios de reconhecimento de carboidratos (DRCs) presentes nessas biomoléculas (DRICKAMER; TAYLOR, 1998).

Lectinas animais estão divididas em famílias distintas estruturalmente de acordo com seu DRC, e têm sido encontradas no citoplasma, no núcleo e associadas a membranas em uma diversidade de organelas e tipos celulares. Ainda que, o número de estudos, isolamento e caracterização de lectinas animais continue a crescer, essas lectinas podem ser classificadas em diferentes famílias com base em suas características físico-químicas, função e sequencia primária (KILLPATRICK, 2000).

A classificação de lectinas animais pode ser inserida em um dos cinco principais grupos: lectinas tipo C (dependentes de Ca^{2+}), lectinas específicas a β-galactosídeos (tipo S), receptores de manose-6-fosfato (tipo P), Siglecs (ligantes a ácido siálico) e lectinas animais relacionadas a lectinas de leguminosas (tipo L) (DRICKAMER, 2006). Além desses grupos, lectinas animais podem ser divididas em outras 14 famílias, perfazendo 19 no total (CARNEIRO, 2013). Dentre esses grupos, oito são bem estabelecidos, na qual quatro contém lectinas que são predominantemente intracelulares (calnexina, famílias tipo M, tipo L e tipo P) e outras quatro que são encontradas fora da célula (famílias tipo C, tipo R, Siglecs e galectinas). De acordo com Carneiro (2013), famílias de lectinas animais são apresentadas como segue:

Anexinas são proteínas estruturalmente relacionadas, dependentes de Ca^{2+} e específicas a fosfolipídios. Essas proteínas possuem de quatro a oito domínios repetidos com 70 resíduos de aminoácidos. Acredita-se que seu domínio N-terminal possa interferir na diversidade funcional, pois a atividade de ligação a fosfolipídio na presença de Ca^{2+} está presente em cada unidade de repetição (ISHITSUKA *et al*., 1998). As anexinas podem realizar funções como: inibição da fosfolipase A2, coagulação sanguínea, regulação do transporte de substâncias pela membrana, ligação à proteínas do citoesqueleto, sinalização intracelular como substrato de quinase (SWAIRJO *et al*., 1994).

Calnexinas são proteínas que compreendem um sistema de chaperonas do retículo endoplasmático (RE) e asseguram o *folding* de glicoproteínas sintetizadas no citoplasma. Além disso, calnexinas e sua proteína homologa (calreticulina), possuem especificidade por glicoproteínas regida por um domínio lectínico que reconhece o oligossacarídeo precursor $Glc_1Man_9GlcNac_2$, encontrado em proteínas recém-formadas. Calnexina e calreticulina possuem sítios de ligação para ATP, Ca^{2+} e ERp57, uma conhecida enzima que atalisa a formação de pontes de sulfeto (WILLIAMS, 2006). Em humanos e muitos outros mamíferos, já foram encontradas quatro proteínas da família das calnexinas: calreticulina, calmegina, calreticulina e calreticulina 2 (LIU *et al*., 2015).

As kaliklectinas são encontradas em peixes teleósteos, onde são mais precisamente secretadas no muco, por exemplo em *Platycephalus indicus*. Essas proteínas são estruturalmente semelhantes às cadeias pesadas das claricreínas, que são proteases séricas encontradas no plasma de mamíferos. Kaliklectinas são específicas a manose, dependentes de Ca^{2+} e apresentam-se como dímeros ligados por pontes de sulfeto (TSUTAUI *et al*., 2012).

Outra família de lectinas animais são as do tipo H. A lectina isolada do caracol terrestre *Helix pomatia* (HPA) e do octocoral *Sinularia lochmodes* (SLL-2) são representantes dessa família. HPA é uma proteína constituída de seis subunidades idênticas em um trímero de dímeros ligados por pontes de sulfeto. HPA isolada da glândula albúmen é específica a N-acetil-galactosamina e vem sendo utilizada em estudos de identificação relacionadas a mudança no padrão de glicosilação para o potencial metastático de tumores sólidos (HAMMARSTROM *et al*., 1997; MARKIV *et al*., 2011).

As lectinas tipo I ou imunoglobulinas ligantes de ácido siálico (Siglecs – *Sialic acid binding Ig-type lectins*) são proteínas de membrana do tipo I caracterizadas por um domínio tipo imunoglobulina no N-terminal que contém o sítio de ligação a ácido siálico (VASTA; AHMED, 2008). A função das Siglecs pode variar consideravelmente, visto que sua especificidade por ácidos siálicos é significativamente diferente. Entretanto, Siglecs parecem estar envolvidas em processos de infecção e evasão de mamíferos por patógenos. Alguns patógenos, incluindo bactérias e protozoários não possuem ácidos siálicos em sua superfície, entretanto possuem a habilidade de expressar esse açúcar como modo de disfarce para evasão da resposta imune, prevenindo o reconhecimento por leucócitos. Além disso, muitos vírus possuem grande quantidade de ácido siálico em seus capsídeos. Dessa forma, vírus podem utilizar ácidos siálicos não somente como proteção, mas também para facilitar sua entrada na célula utilizando a capacidade de ligação das Siglecs (VMIR *et al*., 2004).

Lectinas da família tipo L são caracterizadas por possuírem um DRC muito similar ao DRC presente em lectinas de leguminosas. Contudo, lectinas tipo L são proteínas de membrana envolvidas no sistema de secreção do RE, enquanto que as lectinas de leguminosas são proteínas solúveis (ITIN *et al*., 1996).

Já as lectinas pertencentes a família tipo M tem elevado grau de similaridade com as α-manosidases. Essas lectinas ligam-se aos glicanos de manose presentes em glicoproteínas do lúmen do RE, mas não tem função catalítica (DRICKAMER, 2006). Mamíferos possuem três classes de lectinas tipo M: EDEM1, EDEM2 e EDEM3 (VASTA; AHMED, 2008).

Em lectinas tipo P o DRC foi inicialmente identificado em dois tipos de proteínas transmembrenares, os receptores de manose-6-fosfato (M6P) dependentes de cátions e independentes de cátions (CD-MPR e CI-MPR, respectivamente). Esses receptores estão presentes em vertebrados e cordados evolutivamente mais antigos, como ascídias (DRICKAMER, 2006). As MPRs são essenciais na formação dos lisossomos, orientando hidroláses ácidas solúveis recém sintetizadas contendo manose-6-fosfato para os lisossomos (VASTA; AHMED, 2008).

As lectinas do tipo R, se assemelham as proteínas tóxicas de vegetais, como ricina e abrina. Em invertebrados, a lectina hemolítica do pepino-do-mar Cucumaria echinata (CEL-III) é dependente de Ca^{2+}, sendo específica a GalNac é capaz de hemolisar eritrócitos humanos. Nesse contexto, a capacidade de hemolisar células foi inibida pelo açúcar específico, o que demonstra que a capacidade hemolítica da lectina depende do reconhecimento do glicídio específico (HATAKEYMA *et al*., 1995). Além disso, CEL-III apresentou toxicidade a células da linh MOCK, mas não contra CHO (*Chinese Hamster Ovary*), sugerindo que o efeito de CEL-III é mediado pela especificidade celular (ODA *et al*., 1997).

Já as lectinas pertencentes a família tipo X são dependentes de Ca^{2+}, contudo, não apresentam DRC encontrado nas lectinas da família tipo C. A primeira lectina dessa família foi a XL35, identificada nos oócitos e embriões do sapo *Xenopus laevis*. XL35 é uma lectina específica a galactose/GalNac e não apresenta similaridade com lectinas da família tipo C (LEE *et al*., 2004).

As lectinas ligantes de ramnose (RBL-*L-rhamnose binding lectin*) são uma família de lectinas animais que se ligam especificamente a L-ramnose e D-galactose (HILPATRICK, 2002). As RBLs tem sido encontradas principalmente em peixes ósseos, ouriços, ostras e ascídias (GASPARINI *et al*., 2008; HOSONO *et al*., 1993; NAGANUMA *et al*., 2006). Essas lectinas são descritas como componentes importantes do sistema imune dos animais citados, atuando como proteínas de reconhecimento padrão (PRPs). Atualmente, somente a RBL isolada de ovos de salmão (*chum salmon lectin* – CSL3) tem sua estrutura tri-dimensional resolvida. Além disso, CSL3 apresenta atividade antineoplásica por causar perturbações em junções aderentes de células do adenocarcinoma colorretal caco-2 (NEMOTO *et al*., 2015). Outra RBL, isolada de *Oncorhynchus tshawytscha* (chinook salmon) também exibiu atividade antineoplásica sobre as linhas celulares de hepatoma (Hep G2) e câncer de mama (MCF 7) (BAH *et al*., 2011).

Lectinas de esponjas marinhas estão inseridas nas famílias de lectinas tipo C, tipo S (galectinas), tachylectinas e lectinas tipo F (GARDÈRES *et al*., 2015).

Lectinas tipo S, mais conhecidas como galectinas são restritas ao reino animal e demonstram uma seletividade preferencial por β-galactosídeos. Galectinas são divididas em 3 subfamílias de acordo com sua arquitetura estrutural: prototipo, tipo quimera e tipo tandem (HIRABAYASHI; KASAI, 1993). A função das galectinas é diversa e varia de acordo com a estrutura e localização. Essas proteínas são capezes de regular a tumorigênese através da modulação de oncogenes e do ciclo celular (Galectinas 1 e 3). Além disso, também podem induzir morte celular por apoptose através das vias extrínseca e intrínseca e através da redução da expressão de porteínas adesivas como integrinas (SANCHEZ-RUDERISCH *et al*., 2011). Galectinas de esponjas diferem das galectinas encontradas na maioria dos animais por conter características estruturais específicas. Essas galectinas podem criar complexos na presença de Ca^{2+} (não envolvido na ligação a carboidratos) e mostram extrema afinidade por N-acetil-galactosamina presente em sacarídeos (MULLER *et al*., 1997). Seis lectinas de esponjas foram identificadas como galectinas: *Geodia cydonium*, *Suberites domuncula* (galectinas 1 e 2), *Halichondria okadai* (galectina 30) e *Cinachyrella* sp. (galectinas 1 e 2).

Lectinas tipo C são bastante encontradas em bactérias, plantas e animais. Essa classe de lectinas inclui as colectinas (lectina ligante a manose (MBL), ficolinas, conglutininas e surfactantes pulmonares), selectinas, receptores endociticos e receptores de macrófafagos, deixando claro sua importância para o sistema imune. A ligação dessas proteínas ao seu açúcar específico está condicionada a presença de Ca^{2+} no ambiente (VASTA, 2012). Seis lectinas de esponjas foram classificadas como membros da família tipo C: *Pellina semitubulosa*, *Aplysina archeri*, *Aplysina lacunosa*, *Aphrocallites vastus*, *Cliona varians* e *Lubomirskia baicalensis*, as duas últimas como lectinas MBL.

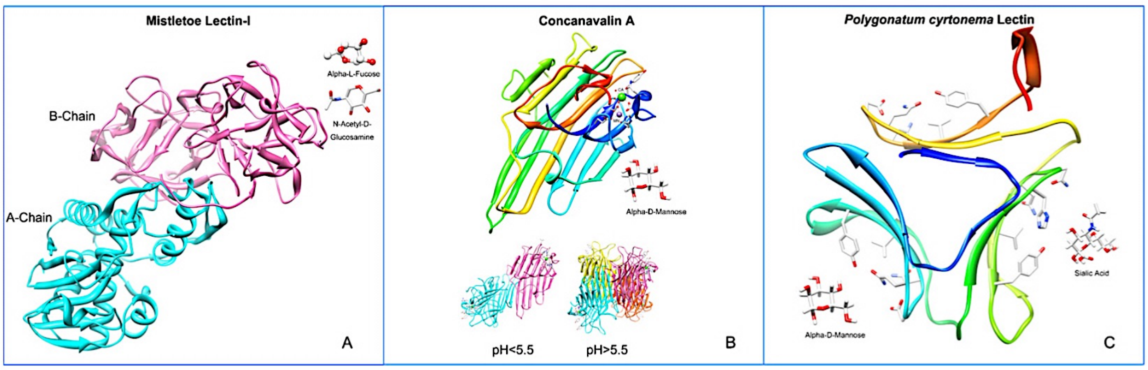
Tachylectinas são proteínas que foram primeiramente descritas no crustáceo *Tachypleus tridentatus* (Tachylectina-1) e são dotadas de alta similaridade com L6 e Tachylectina-P também isoladas do crustáceo citado. Essas lectinas possuem a capacidade de ligar-se a lipopolissacarídeos (LPS) (KILPATRICK, 2002). Dessa maneira, essas lectinas frequentemente exibem atividade antimicrobiana, provavelmente por sua ligação ao LPS das bactérias (SAITO *et al*., 1995; SCHRODER *et al*., 2003; FUNAYAMA *et al*., 2005). Duas lectinas isoladas das esponjas *Ephydatia fluviatilis* (EfL) e *Suberites domunculata* (SdL) estão inseridas nessa família.

Lectinas tipo F constituem um grupo recente caracterizado pela presença de um DRC que reconhece especificamente fucose. Essas lectinas também são encontradas em vertebrados e protocordados. Dois subgrupos distintos podem ser classificados com relação aos DRCs dessas lectinas, onde um grupo possui DRC simples e outro com DRC em repetição em tandem. Nessa família, o Ca^{2+} não é necessário para a ligação a carboidratos, contudo, esse íon é necessário para estabilizar a proteína (VASTA *et al*., 2012). A lectina CcL isolada da esponja marinha *Crambe crambe* exibiu especificidade a fucose e apresentou muitos aminoácidos básicos em sua estrutura primária, fato incomum entre lectinas de esponjas (PAJIC *et al*., 2002).

II.4.2.3 Breve insight sobre atividade anticâncer de lectinas

Lectinas podem apresentar diferentes atividades biológicas importantes no contexto de sua potencialidade biotecnológica para o surgimento de novas drogas e utilização para saúde humana. Entre elas, uma das mais importantes é o já bem documentado potencial anticâncer (LIU *et al*., 2010). Nas últimas duas décadas, lectinas tem sido usadas para reconhecer e diferenciar tumores malignos de benignos a partir de seu padrão de glicosilação e sua associação com o processo metastático. Várias lectinas estão sendo adotadas como alternativa terapêutica para o tratamento do câncer. Entre elas, podem-se destacar três principais famílias: lectinas de leguminosas, proteínas inativadoras de ribossomos tipo II (RIPs II) e as lectinas pertencentes as relacionadas a família *Galanthus nivalis* (GNA), inclusive com algumas em uso clínico (VAN DAMME; LANNOO; PEUMANS, 2008; LIU *et al*., 2010) (Figura II.4.6).

Figura II.4.6: Estruturas tridimensionais de lectinas de plantas. (A) Mistetloe-I (ML-I) que representa a família das RIPs II; (B) lectina de Canavalia ensiformis (Con A), representa a família de leguminosas; (C) lectina de Polygonatum cyrtonema (PCL) que representa a família GNA.



Fonte: FU, L. L. *et al.* Plant lectins: targeting programmed cell death pathways as antitumor agents. **Int J Biochem Cell Biol,** v. 43, n. 10, p. 1442-9, Oct 2011.

Células tumorais apresentam estrutura superficial diferente de células normais. Por esse motivo, sofrem diretamente efeitos específicos de lectinas, devido a seletividade e interação com carboidratos desempenhados por essas biomoléculas (POWLESLAND *et al*., 2009). De fato, a grande diversidade e a elevada complexidade dos glicanos expressos na superfícies de células neoplásicas, estimulam a interação direta e/ou indireta de lectinas disparando o processo de apoptose por via superficial ou mesmo por internalização (intracitoplasmática) (FU *et al*., 2011).

Apesar dos vários avanços nas pesquisas com lectinas, o mecanismo de ação dessas proteínas sobre o processo apoptótico, ainda não foi esclarecido por completo. Assim, pelo menos três hipóteses podem lançar luz do mecanismo de lectinas e seu efeito anti-proliferativo: 1) a inativação de ribossomos no citoplasma celular após a internalização da lectina (SEIFERT *et al*., 2008) ; 2) endocitose dependente e localização seletiva a algumas organelas, como mitocôndria, no citoplasma de células tumorais (CHANG *et al*., 2007); 3) a interação com glicanos específicos da superfície de células tumorais (LIU *et al*., 2013). Baseado nessas três vias, lectinas podem matar células tumorais induzindo as várias formas de morte celular por apoptose como a morte celular programada e/ou autofagia, que por sua vez, podem fornecer novos alvos terapêuticos com potencial antitumoral e descoberta de novas drogas (SHI *et al*., 2016).

II.4.2.4 Lectinas de Poríferos: tendências e potencialidades biotecnológicas

Organismos marinhos vem sendo bastante explorados nas últimas décadas como fontes potenciais de compostos biologicamente ativos (CHEUNG *et al*., 2015). Lectinas derivadas de organismos marinhos são estruturalmente diversificadas, diferindo daquelas presentes em organismos terrestres (CARNEIRO *et al*., 2013), devido a sua baixa imunogenicidade, pois são relativamente menores, além de serem mais estáveis, caracterizadas pela presença de pontes de sulfeto (CHEUNG *et al*., 2015), além disso tem maior especificidade por glicoconjugados complexos em comparação a açucares simples.

A abundância de lectinas que tem sido isoladas do ambiente terrestre, particularmente de plantas, entra em tremendo contraste com as já isoladas de organismos marinhos (CHEUNG *et al*., 2015). Ainda assim, nos últimos anos, um amplo espetro de lectinas isoladas de várias espécies que vivem nos oceanos tem sido obtidas (NASCIMENTO *et al*., 2012). As lectinas isoladas de espécies marinhas são relativamente novas. Pesquisas sobre as funções fisiológicas e aplicações biotecnológicas dessas lectinas tem ganhado cada vez mais destaque como fontes de novas drogas (CHERNIKOV *et al*., 2013).

O filo Porífera é composto de 8500 espécies descritas, na qual possuem vasta distribuição geográfica. Esponjas marinhas possuem uma habilidade especial de sintetizar moléculas com funções diversas de defesa e comunicação que as torna uma fonte de moléculas com potencial na área biomédica (ROUÉ *et al*., 2012; VAN SOEST *et al*., 2012).,O interesse em lectinas de esponjas marinhas aumentaram a partir de 1968, com o estudo dos extratos de esponjas que eram capazes de aglutinar eritrócitos (DODD; MACLENNAN; ALYOSHKIN, 1968; BRETTING et al., 1981). Extratos de 48 espécies de esponjas coletados no mar vermelho, em barreiras de corais australianos e ao longo da costa da Flórida nos EUA, foram investigados. 42% dos extratos exibiram atividade hemaglutinante para eritrócitos humanos do grupo ABO (MEBS; WEILER; HEINKE, 1985). Quantidade similar da efetividade de extratos de esponjas colhidas no mar Adriatico, confirmaram que lectinas de esponjas estão distribuídas ubiquamente. Estudos com lectinas de esponja iniciaram de fato nos anos 80, a partir do qual, sua capacidade de aglutinar eritrócitos foi constatada. A partir disso, variados trabalhos demonstrando atividade mitogênica, antimicrobiana, citotóxica e neuromodulatória foram descritas (GARDÈRES *et al*., 2015).

Diversos estudos tem demonstrado que lectinas de esponjas exercem diferentes funções fisiológicas, estando envolvidas na morfogênese e interações celulares, espiculogênese e biomineralização, defesa contra predadores ou comunicação com micro-organismos associados. Lectinas de esponja são capazes de promover interações célula-célula a partir da interação com carboidratos. Nesse contexto físico foi constatado que a galectina da espécie *Geodia cydonium* está envolvida na interação entre esponjas dessa espécie através de uma ligação complexa, na presença de Ca^{2+}, com o fator de agregação (FA) localizado na membrana citoplasmática da esponja (CONRAD *et al*., 1981; SCHUTZE *et al*., 2001).

Lectinas de esponjas são capazes de diferenciar bactérias Gram-positivas e Gram-negativas demonstrando respostas diferentes em contato com os diferentes tipos de bactéria. A lectina de *Cliona varians* (CvL) inibe a proliferação de bactérias Gram-positivas, enquanto *Suberites domuncula* (SdL) prefenrencialmente induziu atividade anti-bacteriana contra bactérias Gram-negativas, ligando-se especificamente ao LPS da bactéria (WIENS *et al*., 2005; 2007). SdL foi superexpressa em resposta ao tratamento com LPS (MOURA *et al*., 2006; SCHRODER *et al*., 2003). Dessa forma, a habilidade de reconhecer diferentes carboidratos sugerem que lectinas participam da defesa de esponjas marinhas contra bactérias.

A associação de lectinas de esponjas na comunicação entre esponjas marinhas e micro-organismos, ainda não é bem descrita. A lectina de *Halichondria panicea* é a única lectina de esponja que parece ser capaz de estimular a proliferação in vitro de sua bactéria comensal *Pseudomonas insólita*, entretanto não teve o mesmo efeito sobre micro-organismos não comensais (HOOPER; FALK; GORDON, 2000; MULLER *et al*., 1981).

Como anteriormente relatado, além das funções fisiológicas, uma variedade de atividades biológicas já foram investigadas e descritas para esponjas marinhas. Ao contrário do que ocorre com lectinas de leguminosas, contudo, no tocante a atividade antineoplásica, a utilização de lectinas de esponjas para o reconhecimento e identificação ou mesmo atividade citotóxica sobre células tumorais, ainda é pouco explorado. Entretanto, os poucos trabalhos existentes na literatura pontam para o fato do elevado potencial de lectinas de esponjas marinhas no reconhecimento de células do câncer e a indução de apoptose por diferentes vias (GARDÈRES *et al*., 2015). Os efeitos de lectinas de esponjas marinhas contra células tumorais, está descrito na tabela II.4.2.

Tabela II.4.2: Efeitos citotóxicos de algumas lectinas de esponja marinha contra células neoplásicas

Espécie	Linha celular	IC_{50}	Referência
Haliclona cratera	HeLa (melanoma)	9 µg/mL	PAJIC *et al*., 2002
Cliona varians	K562 (leucemia) Jurkat (leucemia)	70 µg/mL 100 µg/mL	QUEIROZ *et al*., 2012
Cinachyrella apion	HeLa	10 µg/mL	RABELO *et al*., 2012
Halichondria okadai	K562	ND*	MATSUMOTO *et al*., 2012

ND*: Não determinado

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Capítulo III

Effect of the triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene isolated from leaves of Combretum leprosum Mart. on cutaneous wounds in mice

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Effect of the triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene isolated from the leaves of *Combretum leprosum* Mart. on cutaneous wounds in mice

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ABSTRACT

Ethnopharmacological relevance: Combretum leprosum Mart. is a native species of the Caatinga, an important biome in the Brazilian semi-arid region. This species is commonly used in Brazil as a healing agent, as well as for the treatment of skin diseases.

Aim of the study: This study investigated the healing potential of the ethanolic extract (EECL) and the bioactive triterpene 3β, 6β, 16β-trihydroxylup-20 (29)-ene (CLF-1) isolated from the leaves of *C. leprosum*.

Materials and methods: Skin wounds (1 cm^2) were created in the dorsal zone of mice with a scalpel blade number 15. The treatment consisted in a daily topical application of 100 µl of 150 mM NaCl, EECL and CLF-1 (at 10 µg/100 µl) for 12 days. The lesions were then macro and microscopically evaluated.

Results: On postoperative day (POD) 2, the lesions treated with EECL and CLF-1 showed a moderate presence of vessels of the granulation tissue progressing in the dermis. The same effect was not observed in the control group. The treatment with EECL and CLF-1 stimulated angiogenesis, resulting in a rapid deposition of extracellular matrix (ECM). Moreover, the animals treated with EECL and CLF-1 showed smaller lesions on POD 7, primarily due to the contraction in the reticular dermis induced by organization of myofibroblasts, which was not observed in the group treated with NaCl. In addition, the lesions treated with EECL and CLF-1 showed ECM restructuration and presence of epithelium coating, which was not observed in the group treated with NaCl, in which the lesions showed no epithelial lining, suggesting delayed healing.

Conclusion: CLF-1 isolated from the leaves of *C. leprosum* may be considered to be an important molecule for the treatment of skin lesions. However, further investigations are necessary to establish its role in chronic lesions and to elucidate the mechanism of action involved in the cutaneous healing process. To the best of our knowledge, this is the first report describing the pro-healing activity of the ethanolic extract and the triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene isolated from leaves of *C. leprosum*.

1. Introduction

Wound healing is defined as a complex process involving pathophysiological and immunological mediators to restore cellular integrity of the damaged tissue as well as its functionality through three distinct and overlapping stages: inflammation, proliferation/regeneration and tissue fibroplasia (Kalish et al., 2013). Physiologically, wound healing mainly depends on the interaction between a variety of cells, biochemical mediators and extracellular matrix molecules (Singer and Clark, 1999).

Several factors can interfere with and hence delay the regenerative process. Among them are age, health status of the patient, site of injury and nutrition (Guo and Dipietro, 2010). Some patients cannot be subjected to surgical treatment because of their poor general conditions, thus the opened wounds are susceptible to serious health problems, including ischemia,

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infection by microorganisms, fasciitis and osteomyelitis, possibly leading to life-threatening complications such as disseminated intravascular coagulation (Yamaoka et al., 2014). Different methods have been developed for the treatment of acute and chronic lesions, including antimicrobial therapy and enzymes for debridement of extracellular matrix, among others. However, several cases of failure are associated with the high costs that directly impact the progression of treatment.

Indeed, the costs related to the traditional treatment of wounds encourage the search for new drugs and dressings that are capable of interacting with the injured tissue and aim to accelerate healing processes, thus reducing the associated costs (Mendonça and Coutinho-Netto, 2009). Recent studies have reported positive effects of compounds isolated from plants on tissue repair through the release and modulation of inflammatory mediators, growth factors, extracellular matrix proteins and mitogenic effects on fibroblasts (Labat-Robert, 2012; Yadav et al., 2014).

The shrub *Combretum leprosum* Mart. (Combretaceae) is a species that is commonly found in Northeast Brazil, where it is commonly known as "mofumbo" (de Sousa Lira et al., 2002). Different parts of this plant, such as stems, leaves and flowers are used in folk medicine as a healing agent, to prevent rashes, and to clean wounds (Horinouchi et al., 2013). Pharmacological studies using extracts from different parts of the plant suggest that the biological activities of *C. leprosum* include anti-inflammatory, antinociceptive and anti-ulcerogenic effects (Agra et al., 2007; de Albuquerque et al., 2007; Facundo et al., 2005; Fernandes et al., 2014; Longhi-Balbinot et al., 2012; Nunes et al., 2009).

According to phytochemical analysis, *C. leprosum* is rich in pentacyclic triterpenoids (arjunolic and molic acid and 3β, 6β, 16β-trihydroxylup-20(29)-ene), cycloartane triterpenoids and flavonoids (3-O-methylquercetin and quercetrin), and some of these substances have important biological activities (Facundo et al., 1993; Fernandes et al., 2014; Longhi-Balbinot et al., 2012; Pietrovski et al., 2006). However, the components present in the ethanolic extract of *C. leprosum* that are responsible for the healing activity remain unknown. In addition to *C. leprosum*, many plants in the family Combretaceae have been evaluated in regard of their pro-healing potential. Bosisio et al. (1997) showed that elastase (an enzyme present in high concentration in chronic wounds) activity was inhibited by components present in the ethanolic extract of *Guiera senegalensis*. Regarding *in vivo* assays, Chaudhari and Mengi (2006) observed that topical applications of different phytoconstituents derived from hydro alcohol extracts of the bark of *Terminalia arjuna* accelerated wound healing in rats.

Therefore, this study aimed to evaluate the effects of topical applications of the ethanolic extract (EECL) and the bioactive triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene (CLF-1) isolated from leaves of *C. leprosum* on the healing of wounds in a murine model.

2. Material and methods

2.1. Plant material

Fresh *C. leprosum* leaves were collected in June 2009 in Salgado dos Machados district (3°46′43″S 40°20′27″W), located 15 km away from the city of Sobral, Ceará, Brazil. The plant authentication was performed by Professor Elnatan Bezerra de Souza, a plant taxonomist from Universidade Estadual Vale do Acaraú (Sobral, Brazil), and a voucher specimen (id: 4573) has been deposited at the herbarium Francisco José de Abreu Matos (Sobral, Brazil).

2.1.1. Sample preparation

The ethanolic extract (EECL) and the triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene (CLF-1) were prepared as previously reported by Evaristo et al. (2014). Briefly, CLF-1 was solubilized in sterile saline at a concentration of 100 μg/mL. The ethanolic extract was used at the same concentration and the negative control was performed using sterile saline only.

2.2. Animals and experimental wounds

2.2.1. Animals

The assays employed 6-week-old male Swiss albino mice (*Mus musculus*) weighing 30.0 ± 5.0 g, supplied by the animal facility of the Universidade Federal do Ceará (BIOCEN/UFC). During the experimental procedures, the animals were kept in individual cages in a controlled environment (circadian cycle, 25 ± 2 °C, 55 ± 10% humidity, food and water *ad libitum*) at the Department of Pathology and Legal Medicine. All animals were treated in compliance with the recommendations of the Brazilian College of Animal Experimentation (COBEA) and the Guide for the Care and Use of Laboratory Animals of the US Department of Health and Human Services (NIH publication No. 85–23, revised 1985).

2.2.2. Surgical procedure and wound treatment

The experimental protocol was approved by the animal ethical committee of Universidade Federal do Ceará, Brazil, under the entry #77/2013. All efforts were made to reduce the stress and pain of animals during experimental procedures. Prior to the surgical procedure, the animals were randomly distributed to 1 of 3 groups ($n = 12$) according to the topical treatment administered: G-I (sterile saline); G-II (EECL at 100 μg/mL); and G-III (CLF-1 at 100 μg/mL).

The mice were then anesthetized using a subcutaneous administration of 2% xylazine plus 10% ketamine hydrochloride (10 mg/kg and 115 mg/kg, respectively), followed by shaving and antisepsis of the dorsal thoracic region. After marking the skin with a sterile mold (1.00 cm^2), circular skin wounds were surgically created by incisions with a surgical scalpel blade number 15 followed by resection of the subcutaneous tissue with dissection tweezers.

Immediately after the surgical procedure, the wounds were topically treated for 12 days with a single dose per day (100 μl) of the respective substance (150 mM NaCl, EECL or CLF-1).

2.2.3. Evaluation of the healing effect

The wounds were macroscopically evaluated regarding the clinical signs presence of edema, hyperemia, scabbing and healing. The observations and measurements were carried out every 48 h to establish the healing potential of each treatment. A digital camera (Olympus, VG-110, Japan) was used to monitor the changes in wound area. Afterwards, the images were transferred to a computer and the wound area was determined using ImageJ software (Schneider et al., 2012). The data from the measurements of the wound areas were expressed as the mean ± SEM. The wound contraction rate was calculated as percentage of the reduction in wounded area (Suntar et al., 2010).

The animals were anesthetized prior to the collection of histopathological material and subsequently euthanized. Samples of injured tissue were collected on the 2nd, 7th and 12th postoperative days (POD), fixed in 10% formaldehyde (v/v) buffered in 0.01 M PBS (pH 7.2), cut into 5 μm thick sections for routine histological analysis and stained with Hematoxylin & Eosin (H&E). The histopathological assessment included the following parameters: presence of scabs, sores, inflammatory infiltrate,

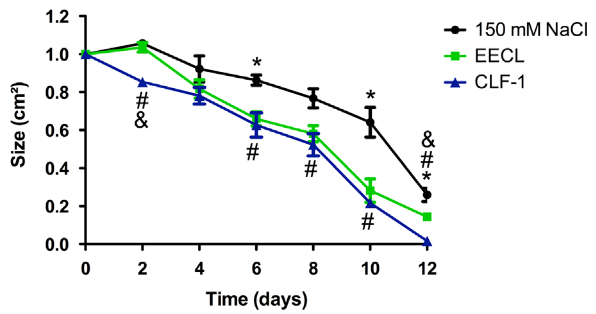

Fig. 1. Effects of topical application of EECL, CLF-1 and 150 mM NaCl (negative control) on wound area of surgically induced skin wounds in mice. Data are expressed as the mean ± SEM. #$p<0.05$ for CLF-1 compared to the negative control; *$p<0.05$ for EECL compared to the negative control; &$p<0.05$ for CLF-1 compared to EECL and negative control.

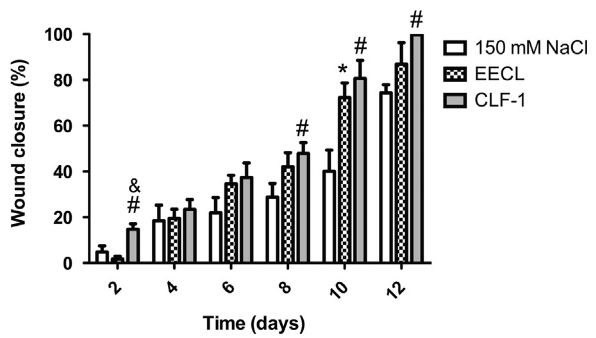

Fig. 2. Effects of topical application of EECL, CLF-1 and 150 mM NaCl (negative control) on wound contraction rate (%) of surgically induced skin wounds in mice. Data are expressed as the mean ± SEM. #$p<0.05$ for CLF-1 compared to the negative control; *$p<0.05$ for EECL compared to the negative control; &$p<0.05$ for CLF-1 compared to EECL.

monocytes, fibroblast proliferation, re-epithelialization, collagen deposition, neovascularization and exudate.

Microscopic analyses were performed using a Zeiss light microscope (Primo Star iLED) at objective of 4, 10, 20 and 40 × magnification.

2.3. Statistical analysis

Significant differences in the means of wound areas and closure percentages were evaluated using a *t*-test. The data were processed with the statistics software GraphPad Prism v. 5.00 for Windows®. Values are given as the mean ± SEM. Data were considered statistically significant when $p<0.05$.

3. Results

3.1. Macroscopic assessment

Treatment with EECL and CLF-1 resulted in decreased wound areas at almost all evaluated days (Fig. 1). Significant differences between EECL and CLF-1 were observed only on POD 2. Average wound areas on POD 2 for the groups treated with EECL and CLF-1 were 1.036 ± 0.025 cm^2 and 0.853 ± 0.023 cm^2, respectively. As expected, the treatment with 150 mM NaCl produced the smallest reduction in wound areas on all evaluated days. Interestingly, between POD 4 and POD 10, both EECL and CLF-1 showed similar effects on wound areas (Fig. 1). Both groups were significantly different from the control on PODs 6, 8 and 10 ($p<0.05$). On POD 12, the animals treated with EECL and CLF-1 showed scar tissue. However, those treated with EECL still possessed scab fragments in the wound bed. In contrast, only 25% of the animals in the group treated with 150 mM NaCl exhibited scar tissue on POD 12 and the majority of lesions remained open at the end of the treatment.

Regarding the rate of epithelialization/contraction shown in Fig. 2, the results revealed that EECL is less effective than CLF-1 on wound closure. This effect was primarily observed in the inflammatory phase of the wound healing process (POD 2). Both EECL and CLF-1 exhibited similar effects during the following days of the experiment, as no significant differences were found between the groups. Nevertheless, CLF-1 action on the first days of the treatment suggests a more rapid and effective closure of lesions (99.65 ± 0.20%) compared to the treatment with EECL (86.88 ± 9.37) and 150 mM NaCl (74.25 ± 3.59%).

To evaluate the clinical signs that arise from the evolution of the healing process, animals were observed daily before each treatment. Lesions treated with 150 mM NaCl displayed an acute inflammatory response, presence of edema, severe hyperemia and slight exudation in the early days of the postoperative period. In contrast, no significant inflammation was observed in animals treated with EECL and CLF-1. Only slight edema and hyperemia were noticeable in some of the animals.

3.2. Histopathology

To perform a histopathological assessment of wounds, fragments of skin from the thoracic dorsal region of all animals were removed on POD 2, 7 and 12. On POD 2, all animals treated with 150 mM NaCl showed a thick scab covering the wound and presence of inflammatory infiltrate at a higher prevalence relative to the animals treated with EECL and CLF-1. Animals treated with EECL and CLF-1, however, exhibited formation of granulating tissue on POD 2, with angiogenesis in adipose tissue and mild inflammatory infiltrate (Fig. 3A and B). On POD 7, animals treated with 150 mM NaCl presented proliferation and migration of epidermal cells at the edges of the injured area, progressing towards the bed of the lesion (Fig. 3F). Those animals treated with EECL showed hemorrhage in the papillary dermis with the presence of few vessels arising from the process of angiogenesis (Fig. 3D). Interestingly, animals treated with CLF-1 showed a large amount of vessels in the dermis, indicating a strong prevalence of angiogenesis to facilitate the nourishment of all skin layers (Fig. 3E).

On POD 12, animals treated with 150 mM NaCl presented open wounds with scabbing in the wound bed and proliferation of epidermal cells at the borders of the lesion towards the bed, as well as intense angiogenesis in the dermis (Fig. 3I). In contrast, animals treated with EECL presented wound closure with the presence of epidermal hyperplasia and formation of corneal plates with traces of granulating tissue in the dermis (Fig. 3G). Regarding the group treated with CLF-1, wound closure with keratin production and adipose tissue retraction was observed, making the arrangement of layers of the skin (Fig. 3H).

4. Discussion

In this study, the healing process of mouse skin wounds was clinically and histopathologically described during the first twelve days of treatment with ethanolic extract and a triterpene isolated from the leaves of *Combretum leprosum*. Few studies have explored the potential of this plant as an anti-inflammatory, antinociceptive and anti-ulcerogenic agent. To the best of our knowledge, this is the first report describing the wound healing activity of ethanolic extract and triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene isolated from leaves of *C. leprosum*.

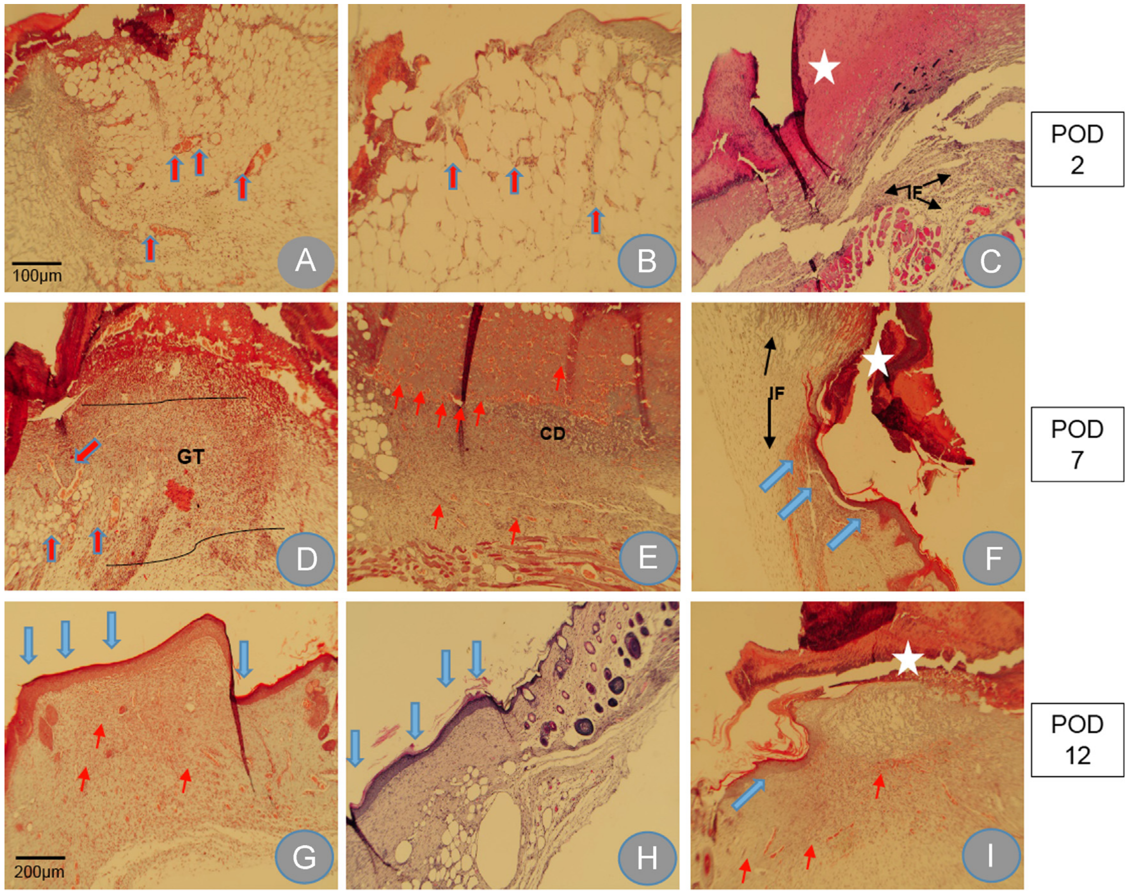

Fig. 3. Photomicrographs of surgically induced skin wounds in mice treated with topical administration of EECL (A, D and G), CLF-1 (B, E and H) and 150 mM NaCl (C, F and I). The images were acquired on postoperative days (POD) 2, 7 and 12. The arrows indicate events that occurred during the healing process: red – vessels of angiogenesis; black – inflammatory infiltrate; blue – re-epithelialization. White star – scab, IF – inflammatory cells, GT – granulation tissue, CD – collagen deposition. Scale bar = 100 μm (A–F) and 200 μm (G–I). Method: H&E staining.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Plants are the oldest known source of medicines used for wound care and management and remain promising candidates for novel drugs in this respect (Kasote et al., 2015). The healing properties of several drugs of plant origin are described in traditional texts of systems of medicine (Kumar et al., 2007). In this context, some species of the family Combretaceae have been considered for isolation of new molecules with pro-healing activity.

The process of contraction and epithelialization of lesions primarily depends on the ability of repair, the tissue type, patient's health status and extension of the lesion (Anuar et al., 2008). According to Ghosh et al. (2012), compounds isolated from plants can promote the contraction of lesions more effectively and in a shorter period of time relative to other conventional topical treatments, especially by stimulating epithelial cell proliferation and subsequent epithelialization. Thus, our results suggest that CLF-1 can act on tissue-resident cells or platelets, thereby stimulating the proliferation of epidermal cells in the inflammatory phase.

The tissue repair component of the wound healing process is regulated by a powerful inflammatory response, which is characterized by an intense inflammatory infiltrate of polymorphonuclear neutrophils (PMN), followed by macrophages (Martin and Leibovich, 2005). When properly stimulated by prostaglandins, these cells produce inflammatory mediators such as IL-1, IL-6, TNF-α, and proteoglycans such as PF4, which cause a remodeling of the extracellular matrix (Teller and White, 2009).

Although many drugs are marketed for the purpose of limiting inflammation, some anti-inflammatory drugs delay the wound healing process. Treatment with non-steroidal anti-inflammatory drugs (NSAIDs) in animal models has caused an anti-proliferative effect on fibroblasts, weakening the tensile strength, and ultimately reducing angiogenesis and epithelialization (Kant et al., 2014; Krischak et al., 2007). Therefore, CLF-1 may constitute an important approach to overcome the problem associated with the use of anti-inflammatory drugs on wound healing.

Horinouchi et al. (2013) showed that ethanolic extract from the flowers of *C. leprosum* reduced IL-6 levels in mice, thereby promoting an anti-inflammatory action. In addition, the triterpene isolated from the flowers of the same plant reduced the levels of IL-1β and TNF-α without altering the levels of IL-10, thus demonstrating anti-inflammatory and antinociceptive effects (Longhi-Balbinot et al., 2012; 2011). Thus, it is possible that these findings may explain the results of this study, in which treatment with EECL and CLF-1 induced a mild inflammatory response, compared to the control group. Furthermore, it is important to note that the treatment with CLF-1 accelerated the epithelialization. This effect was observed even in the presence of an evident anti-inflammatory response.

The process of angiogenesis is associated with the proliferative phase of wound healing, in which new vessels emerge on the third or fourth day after the occurrence of the injury and is closely related to the formation of granulating tissue. The formation of new vessels depends on the control of pro- and anti-angiogenic factors (Bao et al., 2009). One of the major pro-angiogenic factors is vascular endothelial growth factor (VEGF), which is closely related to the proliferation and migration of endothelial cells to form new blood vessels (Johnson and Wilgus, 2012). In addition, another essential growth factor is transforming growth factor-β (TGF-β), which promotes the formation of granulating tissue by proliferating fibroblasts, and may stimulate the production and deposition of proteoglycans from the extracellular matrix (Werner and Grose, 2003). Therefore, the results achieved in this study suggest that CLF-1 may be acting on the production of VEGF and TGF-β, thus stimulating angiogenesis, collagen production by fibroblasts and keratinocyte migration, collectively resulting in a rapid closure of the lesions.

5. Conclusion

As a summary, this study provided evidence of the role of *Combretum leprosum* on wound healing. Treatment with CLF-1 improved the formation of new blood vessels, structuring the extracellular matrix for migration of keratinocytes and increasing tensile strength of the injury, thus leading to a faster and more effective closure. Because both EECL and CLF-1 showed similar effects on the healing of lesions, it is possible to suggest the use of CLF-1 as an alternative therapeutic model for the treatment of cutaneous wounds. However, further studies are necessary to establish the role of CLF-1 on chronic lesions, as well as to elucidate its specific mechanism of action.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Capítulo IV

Effect of the triterpene 3β,6β,16β-trihydroxylup-20(29)-ene isolated from the leaves of Combretum leprosum Mart. on in vitro scratch wound assay

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Article Type: Research Paper

Keywords: Natural products; Combretum leprosum; triterpene; scratch assay; fibroblasts

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Abstract: Ethnopharmacological relevance: Combretum leprosum Mart. is a native specie of the Caatinga, used in Brazilian folk medicine to treat wounds. The bioactive triterpene 3β,6β,16β-trihydroxylup-20(29)-ene (CLF-1) was isolated from leaves and flowers of C. leprosum and was shown to improve healing of mouse skin wounds. However, the mechanism of action involved in the healing process is unknown and there is scarce data to support the potential therapeutic application of CLF-1 on wound healing.
Aim of the study: To investigate the in vitro effect of CLF-1 in human dermal fibroblasts (HDF).
Materials and Methods: The effect of CLF-1 on HDF viability was assessed by using the MTS colorimetric assay. The CLF-1 effect on HDF proliferation/migration was evaluated using an in vitro scratch wound healing assay in 24h treatment. Alteration on the expression of growth factors induced by CLF-1 was analysed by real-time qPCR.
Results: The treatment of HDF cells with 0.1 to 2.5 μg/ml of CLF-1 did not affected cell viability, while treatment with 5 and 10μg/ml decreased cell viability in 63.53 ± 1.63% and 62.30 ± 8.02%, respectively. At 2.5 μg/ml, CLF-1 induced a 97% closure of the wound scratch showing a positive trend to induce migration of fibroblasts. qPCR experiments showed that CLF-1 significantly upregulated TGF-β1 expression and induced the TNF-α expression, as compared to untreated cells.
Conclusions: Treatment with CLF-1 improves the ability of fibroblast cells to heal wound scratches, and upregulates the expression of TGF-β1. Reduction of TNF-α expression levels suggest that CLF-1 has anti-inflammatory properties. While further investigations are needed to better understand the precise mechanism of action of CLF-1 on chronic wounds, our study suggests that CFL-1 accelerates tissue repair by increasing TGF-β1 expression and simultaneous anti-inflammatory activities, which is of great interest for a variety of dermatological treatments.

LABORATÓRIO INTEGRADO DE BIOMOLÉCULAS

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Dear Editor,

Could you please consider the article "**Effect of the triterpene 3β,6β,16β-trihydroxylup-20(29)-ene isolated from the leaves of *Combretum leprosum* Mart. on *in vitro* scratch wound assay"** to be published in the **Journal of Ethnopharmacology.** It is important to say that (i) all authors contributed and approved this manuscript. (ii) It represents an original work that complement a previous work published in JEP and (iii) it was never submitted for publication, (iv) the work aimed to determine the effect of a triterpene isolated from *Combretum leprosum* (a shrub widely used in the Brazilian folk medicine) on an *in vitro* wound model.

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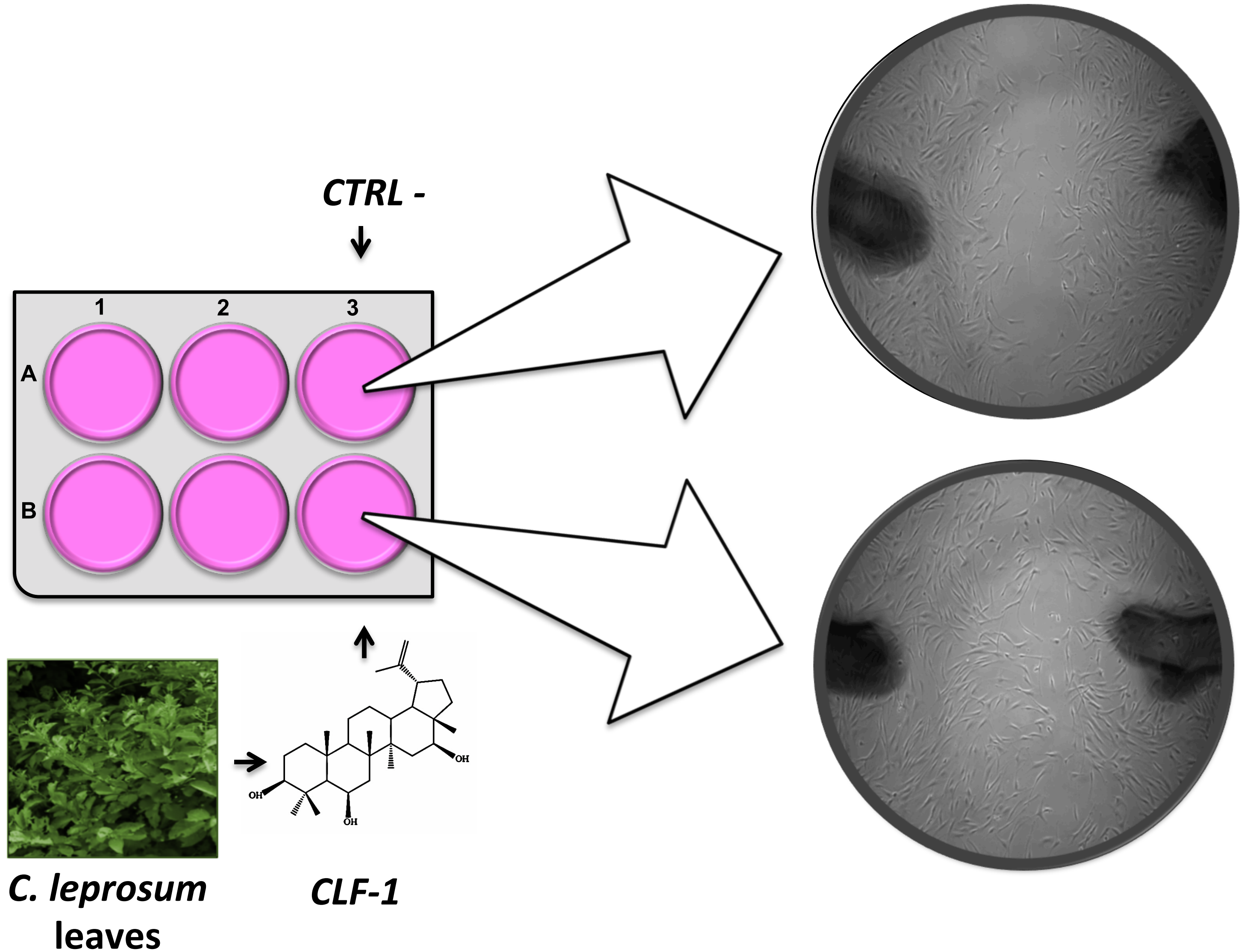
*Graphical Abstract



Abstract

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Effect of the triterpene 3β,6β,16β-trihydroxylup-20(29)-ene isolated from the leaves of *Combretum leprosum* Mart. on *in vitro* scratch wound assay

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Keywords: Natural products, *Combretum leprosum*, triterpene, scratch assay, fibroblasts.

1. Introduction

Skin fibroblasts play a key role in tissue remodelling that takes place during wound healing (Adetutu et al., 2011). In the skin, acute wound healing starts immediately after wound injury and it broadly includes four stages: haemostasis, inflammatory, proliferative, and finally the remodelling, which determines the appearance of the healed tissue (Bielefeld et al., 2013; Singer and Clark, 1999).

During the proliferation phase, fibroblasts proliferate and migrate to the injured tissue, where they proliferate and promote the regeneration of blood vessels, the deposition of collagen-based extracellular matrix (ECM) and the formation of granulation tissue (Bainbridge, 2013; Felice et al., 2015). Fibroblasts secrete a number of growth factors, key for the healing process, such as the transforming growth factor beta (TGF-β1), the vascular endothelial growth factor A (VEGF-A) and the fibroblast growth factor two (FGF-2). Pro-inflammatory cytokines play also an important role in both inflammatory and proliferative phase by increasing cell proliferation and vascular permeability. Yet, excessive inflammation can be detrimental to the control of wound healing. Moreover, a large number of fibroblasts express thick actin bundles as myofibroblasts which contributes to dermis reshuffle (Fronza et al., 2009; Gurtner et al., 2008).

Several plant species are traditionally used in Brazil to improve both wound healing, and due to their alleged anti-inflammatory properties (Geller et al., 2015). Since ancient times, people have used plants and preparations to accelerate the wound healing process (Schmidt et al., 2009). Thereafter, many authors have presented *in vitro* and *in vivo* evidences to support the use of plant materials as topical agents to enhance wound healing (Balekar et al., 2012; Perumal Samy et al., 2006).

Combretum leprosum Mart. (Combretaceae) is a native shrub that grows in the northeast of Brazil, where is known as *mufumbo*, *mofumbo*, *cipoaba* or *pente-de-macaco* (Alves Filho et al., 2015), which is popularly used in the treatment of wounds in bleeding or as an anti-inflammatory agent. Infusions prepared with the aerial part (stems, leaves and flowers) and roots of *C. leprosum* are used in folk medicine for the treatment of as a sedative and anti-ulcerogenic (Lira et al., 2002). In phytochemical studies (Facundo et al., 1993), it was isolated 3β,6β,16β-trihydroxylup-20(29)-ene, arjunolic and molic acids of roots. Arjunolic acid has potent anti-inflammatory and antinociceptive properties (Facundo et al., 2005), however.

Recently, we reported for the first time, that the bioactive triterpene 3β,6β,16β-trihydroxylup-20(29)-ene (CLF-1), isolated from leaves of *C. leprosum* (Evaristo et al., 2014), has remarkable *in vivo* wound healing activity. In mice, the treatment of wounds with CLF-1 stimulates angiogenesis, resulting in a rapid deposition of ECM. Moreover, lesions treated with CFL-1 become significantly reduced due to the contraction in the reticular dermis induced by organization of myofibroblasts. These data suggested that the CLF-1 could stimulate the release of growth factors, such as VEGF and TGF-β by fibroblasts leading to a faster wound healing (do Nascimento-Neto et al., 2015). However, to the best of our knowledge, it is still unknown how CFL-1 functions in fibroblast.

Here, we aimed to investigate the effect of CFL-1 in the viability and proliferation of human dermal fibroblasts in vitro. We also addressed how CFL-1 modulates the ability of fibroblasts to heal in vitro scratch wounds and to express growth factors. Understanding how natural compounds accelerate cutaneous tissue repair is of great interest for a potential therapeutic application of such compounds.

2. Material and Methods

2.1 Chemicals

Phosphate buffer solution (PBS), Dulbelcco´s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), L-glutamine (200mM), trypsin/EDTA solution and penicillin-streptomycin were purchased from Gibco® by life Technologies (Bleiswijk, Netherlands). Dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich® (Saint Louis, MO, USA). CellTiter 96® Aqueous MTS Reagent Powder was purchased from Promega (Madison, Wi, USA).

2.2 Cell line and culture conditions

Primary human dermal fibroblasts (HDF) were purchased from ATCC® (PCS-201-012™, Manassas, VA, USA) and kindly supplied by professor Alexandra R. Fernandes (Department of Life Sciences, Faculty of Sciences and Technology, NOVA University of Lisbon). HDN were maintained in T-25 flasks containing DMEM medium supplemented with 10% of FBS, 1% of L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2. Medium was routinely changed every 3rd day or when cells reached 90% confluence and cells were subcultured by trypsinization (0.05% trypsin/EDTA). In all experiments cells were used between 4 and 10 passage number.

2.3 Plant material, compound isolated and sample preparation

Fresh *C. leprosum* leaves (2.5 Kg) were collected in October 2014 in Salgado dos Machados district (3°46´43"S 40°20´27"W), located 15km away from the Sobral city and 232 km away from the Fortaleza, Ceará, Brazil. The plant authentication was performed by Dr. Elnatan Bezerra de Souza, a plant taxonimist from Universidade Estadual Vale do Acaraú (Sobral, Brazil), and a voucher specimen (id:4573) has been deposited at the herbarium Francisco José de Abreu Matos at the same university.

The isolation of CFL-1 was performed as previously reported (Evaristo et al., 2014) and stock solutions were prepared freshly prior to use. Specifically, CLF-1 was dissolved at 10 mg/ml in 50% DMSO and 50% PBS and stored at -20°C (Reyes et al., 2006). For the in vitro assays, the stock solution was diluted in cell culture medium, and the final concentration of DMSO never exceeded 0.1% (v/v).

2.4 Cell viability assay

With the purpose of evaluating the effect of CFL-1 in HDF viability and proliferation, HDF (1,5 x 10^4 cells/well) were seeded in a 96 well flat bottom plates in DMEM containing 10% FBS and incubated overnight. Different doses of CLF-1 (0.1; 1; 2.5; 5 and 10 µg/ml) were added to and incubated at 37 °C, for 24 h.

The viability of the HDF was determined using the CellTiter 96® Aqueous MTS Reagent Powder (Promega), following the manufacturer´s instructions. Three independent experiments were performed in triplicate. The optical density was read at 490 nm on a microplate absorbance reader (iMark™, Bio-Rad). The viability was calculated according to the formula:

$$\text{Fibroblast cell viability (\%)} = \frac{\text{average OD 490nm (CLF1)}}{\text{average OD 490nm (control)}} * 100$$

2.5 Scratch wound healing assay

HDF were seeded in 6-well plates (5 x 10^5 cells/well) and grown until to reach 100% confluence in DMEM with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. Next, the in vitro wound healing procedure was carried out as previously reported (Rodriguez et al., 2005; Liang et al., 2007; Felice et al., 2015). A straight scratch was made with a P200 pipette tip, to simulate a wound. The cell debris was removed by washing twice with PBS 1x. The experimental wound was exposed to CLF-1 (2.5 µg/ml) for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The untreated cells were exposed to DMSO (0,1% v/v) only and were used as control.

After 0 h and 24 h of scratch formation, the scratch closure was observed. Photomicrographs were taken by a phase contrast microscope (10x and 40x magnification; Axiovert 40 CFL, Carl Zeiss Microscopy, LLC, USA) and the wound width at 0 h and 24 h was determined. Each well was marked below the plate surface by drawing a vertical line, to allow identification of the same scratched area in order to take consistent pictures. Scratch area was measured using ImageJ software (Schneider et al., 2012). Three independent experiments were performed in triplicate and the HDF migration was expressed as percentage of scratch closure on an initial area basis.

2.6 Gene expression analysis by real-time PCR

Gene expression was evaluated by reverse transcriptase-real time polymerase chain reaction (RT-qPCR) as previously reported (Videira et al., 2008). Briefly, after 24 h of treatment or not fibroblasts (10^6 cells) were trypsinized and total RNA was extracted, using GenElute Mammalian total RNA Purification kit (Sigma Aldrich, St. Louis, MO, USA) and DNAse treatment was performed to eliminate genomic DNA (Nzytech). 1µg of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed using TaqMan Fast Universal PCR Master Mix, primers and Taqman probes acquired from Applied Biosystems and used following the manufacturer´s instructions. For each gene, the primer/probe set were the following: TGF-β1 (Hs00171257_m1); TNF-α (Hs00174128_m1); VEGF-A (Hs00900055_m1); and FGF-2 (Hs00960934_m1). Data were normalized against the arithmetic average of the expression of endogenous controls β-actin and GAPDH gene. The relative mRNA levels were calculated according to Videira et al. (2009); Videira et al. (2007), using the adapted formula $2^{-\Delta ct}$ x 1000, which infers the number of mRNA molecules of the gene of interest per 1000 molecules of the endogenous controls. The amplification reaction´s efficiency for each primer/probe was above 95%, as suggested by the manufacturer.

2.7 Statistical analysis

Data are presented as mean ± SEM of three independent experiments and processed with the statistics software GraphPad Prism v. 5.00 for OS X v.10.11. Significant differences in the means of scratch closure were evaluated using a *t*-test or by ANOVA with Tukey *post hoc* when appropriate. Data were considered statistically significant when $p < 0.05$.

3. Results

3.1 Effect of the bioactive triterpene (CLF-1) on the viability of primary human dermal fibroblasts

In order to determine the potential effect of CLF-1 on the viability of HDF cells, MTS cell proliferation assay was used. No cytotoxic effects were seen after 24 h treatment using CLF-1 at 0.1-2.5 µg/ml (Figure 1). However, there was a decrease in the cell viability of HDF at doses 5 µg/ml (63.53 ± 1.63%) and 10 µg/ml (62.30 ± 8.02%) compared to control. As the 50% inhibition did not occur at the highest concentration, the IC_{50} value was not determined and the concentration of 2.5 µg/ml was used for subsequent experiments.

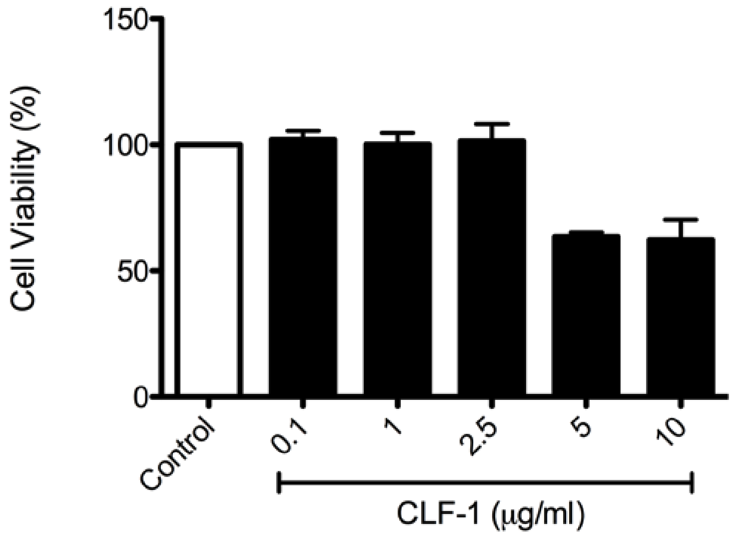

Figure 1: Effect of CLF-1 on human dermal fibroblasts viability. Human fibroblasts were incubated with CLF-1 at different concentrations during 24 h. Cell viability was assessed by MTS cell proliferation assay (OD 490 nm) and is represented as percentage of viable cells, as compared with untreated cells. Data were expressed as mean ± SEM (n=3).

3.2 CLF-1 induces a positive trend to migration of fibroblasts

In vitro scratch wound healing assays were performed in order to observe the effects of CLF-1 on the healing process of HDF. It can be observed in figure 2 that, in the course of 24 h of treatment with CLF-1, fibroblasts migrated more towards the scratched area to close the wound (Figure 2A). CLF-1 treated HDF cells close 96.9 ± 3.1% of the scratched area (Figure 2B) (p< 0.01), while the untreated cells closed 77 ± 4.5%.

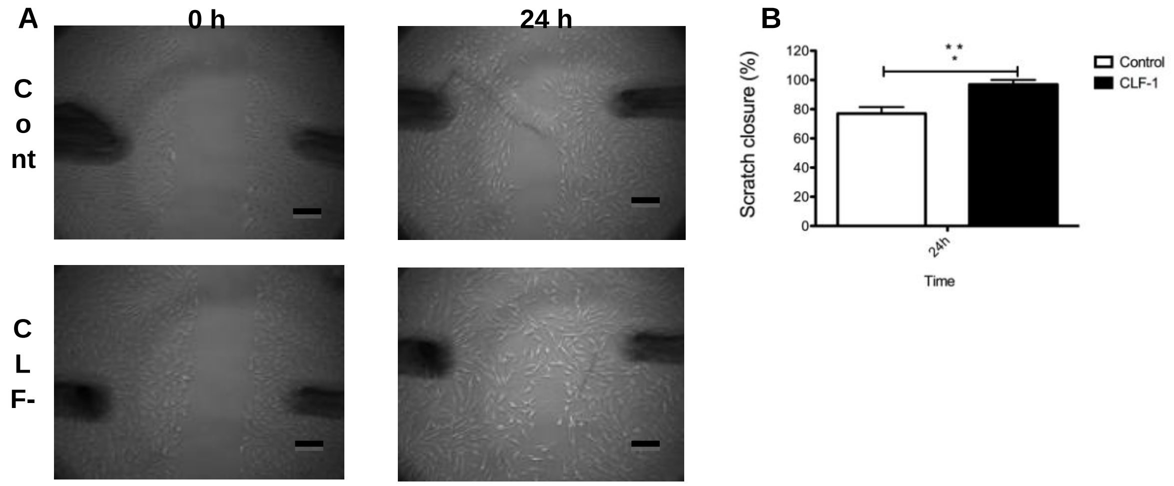

Figure 2: Evaluation of *in vitro* wound closure after scratch formation in a HDF monolayer culture. **A**: Representative fotomicrographs of the scratch of cells treated with CLF-1 at 2.5 μg/ml and untreated cells, after 24 h after scratch (10x magnification). **B**: Bar graph represents the scratch closure (%) of treated and untreated cell area when compared to initial area by Image J software. Data were expressed as mean ± SEM (n=3). Statistically significant differences between samples are indicated by *p< 0.05 and **p< 0.01.

3.3 CLF-1 increases expression of TGF-β1

We investigated the effect of CLF-1 in the expression of genes related to the healing process in HDF. The expression of several growth factors, such as TGF-β1, FGF-2, VEGF-A and the cytokine TNF-α was analysed. The TGF-β1 expression was significantly increased (p=0.029) in cells treated with CLF-1, when compared to untreated control cells (Figure 3). In contrast, the gene expression of TNFα was significantly downregulated in treated cells (p=0.002) compared to untreated cells. The expression of FGF-2 and VEGF-A did not show any significant difference between treated and untreated cells.

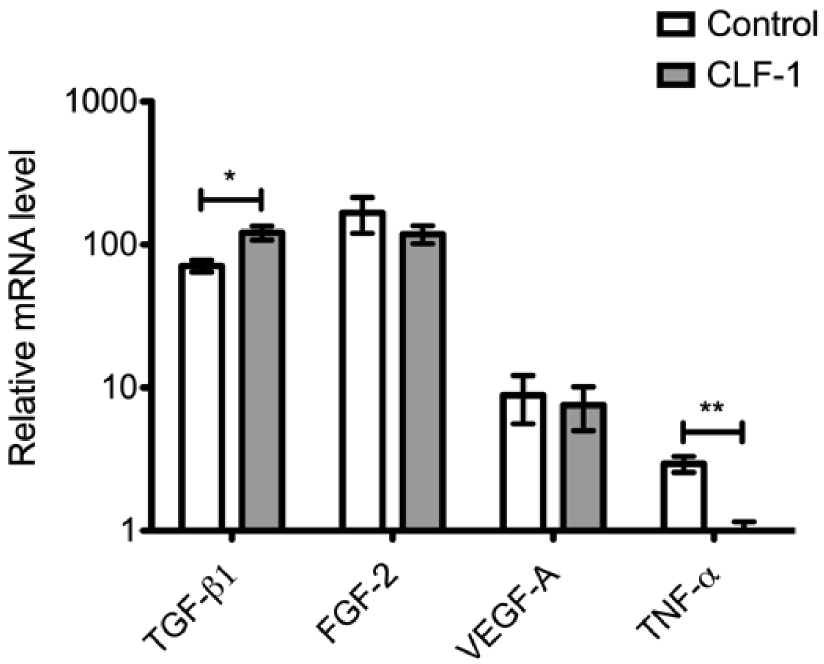

Figure 3: Effect of CLF-1 on the expression of genes associated with wound healing. Treatment with CLF-1 upregulates TGF-β1 and downregulates TNF-α expression level. The HDF were incubated with CLF-1 (2.5 μg/ml) during 24 h. Aftherwards, the total mRNA was extracted and the expression of TGF-β, FGF-2, VEGF-A and TNF-α genes was evaluated by real-time qPCR. For each gene, the relative mRNA levels are expressed as the permillage (‰) of the expression of the endogenous positive controls (β-actin/GAPDH). Statistically significant differences between samples are indicated by *p<0.05.

4. Discussion

Skin wounds affect normally more than 139 million people, which causes an economic burden in the health institutions and vulnerable communities (Thomas et al., 2016). Moreover, chronic wounds are a growing medical problem that cause high rates of morbidity and mortality, costing the healthcare industry in the world millions of dollars annually (Clinton and Carter, 2015). To identify potential new pro-healing compounds, several pharmaceutical companies are currently evaluating extracts from plants, among them new natural triterpenoids and their derivatives (Adetutu et al., 2011; Kanopleva et al., 2004). Some plant extracts are used to improve wounds via their antimicrobial, anti-inflammatory, antioxidant, and mitogenic activities (Koca et al., 2009). Often their use is merely based on folk medicine, without any scientific evidence of efficacy and knowledge about bioactive compounds or their mode of actions (Fronza et al., 2009; Reuter et al., 2009).

Wound healing is a complex and dynamic process, in which the migration and the activity of fibroblasts plays an essential role in wound repair (Dulmovits and Herman, 2012).

The proliferative phase of wound healing is characterized by fibroblasts proliferation and the granulation tissue formation (Diegelmann and Evans, 2004). Therefore, therapeutic bioactive agents that are able to stimulate fibroblast growth and proliferation may be also able to improve or promote wound healing (Chi and Trinkaus-Randall, 2013) as in the case of our present study.

In our previous work, we observed that CLF-1 promotes the *in vivo* wound healing (do Nascimento-Neto et al., 2015). Analysis of the surgically induced skin wounds in mice treated with CLF-1 showed a faster deposition of ECM and better contraction in the reticular dermis induced by organization of myofibroblasts, which leaded us to the hypothesis that CFL-1 could activate fibroblast activity. In this work, to address this question, we initially assessed the influence of CLF-1 on fibroblast viability. Doses of CLF-1 up to 2.5 μg/ml did not affect the fibroblast cell viability when compared to untreated cells. It is clear that the CLF-1 triterpene is not potentially toxic to the fibroblast cells.

Recently, Lacouth-Silva et al. (2015) evaluated the effect of lupane triterpene on peripheral blood mononuclear cells (PBMCs). The results showed that lupane at concentrations below 1.5 μg/ml was not toxic to the PBMCs. Moreover, lupane triterpene was not able to active cellular functions and did not alter the production of IL-10 and TNF-α.

It was already suggested that CLF-1 may be acting on the production of important growth factors, thus stimulating several important events as collagen production and fibroblasts migration (do Nascimento-Neto et al., 2015). In this study, our results show that CLF-1 induces the wound scratch closure in more 22% than untreated cells. Furthermore, our data showed that fibroblasts exposed to CLF-1 have increased levels of TGF-β1 when compared to untreated cells.

In normal wound healing, TGF-β has a central role. There are 3 isoforms of TGF-β, which include TGF-β1, TGF-β2 and TGF-β3 (Goldberg and Diegelmann, 2010). TGF-β1 is released in large amounts from fibroblasts and platelets immediately after wounding (Assoian et al., 1983), acting as chemoattractant for several cellular types, such as fibroblasts (Werner and Grose, 2003). Additionally, TGF-β1 has an autocrine role as a very potent stimulator of the expression of ECM proteins and integrins (Bielefeld et al., 2013; Maarof et al., 2016). The observed increased expression of TGF-β1 may explain the effect of CLF-1 in closing more effectively the scratched region, in the wound scratch assay.

CLF-1 in the mice cutaneous wounds was able to lower inflammatory response in wound sites when evaluated macroscopically (do Nascimento-Neto et al., 2015). Here, we observed that HDF treated with CLF-1 have remarkable lower expression of TNF-α, when compared to untreated cells.

Collagen-matrix deposition and collagen lattice contraction was demonstrated be TGF-β1 dependent (Eckes et al., 2010). TNF-α suppresses TGF-β-induced proliferation of fibroblasts and expression of myofibroblasts phenotypic genes by reducing TGF-β mRNA stability, with resultant reduced collagen production and contraction (Goldberg et al., 2007). Thus, wound tension combined with balanced cytokine levels is important in order to determine ECM properties during wound healing (Greaves et al., 2013).

TNF-α is a pleiotropic cytokine released by a variety of cell types and modulates several processes at the wound site, including the stimulation of keratinocytes and fibroblasts proliferation (Barrientos et al., 2008; Geller et al., 2015). TNF-α is increased in the inflammatory phase of wound healing. However, it has a destructive effect on wound repair in higher concentrations (Singer and Clark, 1999). Thus, our results can explain the anti-inflammatory effect of CLF-1 in the *in vivo* wound healing and also in the accelerating scratch closure rate. Furthermore, another works already show that bioactive CLF-1 isolated from flowers of *C. leprosum* have anti-inflammatory and antinociceptive effects (Horinouchi et al., 2013; Lacouth-Silva et al., 2015; Longhi-Balbinot et al., 2012).

To our knowledge, this is the first study that examines the effects of CLF-1 on fibroblast migration and proliferation using an *in vitro* scratch wound healing assay.

5. Conclusion

Our findings demonstrate, for the first time that the bioactive triterpene CLF-1 isolated from leaves of *Combretum leprosum* Mart., promoted fibroblasts viability and migration in an artificial wound using the scratch assay. This is related with increased expression of TGF-β1. Interestingly, CLF-1 lowered TNF-α expression, which can be related with observed anti-inflammatory properties. Natural products that are capable of accelerating cutaneous tissue repair with simultaneous anti-inflammatory activities are of great interest for a variety of dermatological treatments. Thus, treatment with CLF-1 can be a potential strategy to promote wound healing.

Conflict of interest

The authors declare that there are not conflicts of interest.

Acknowledgments

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Figure 1

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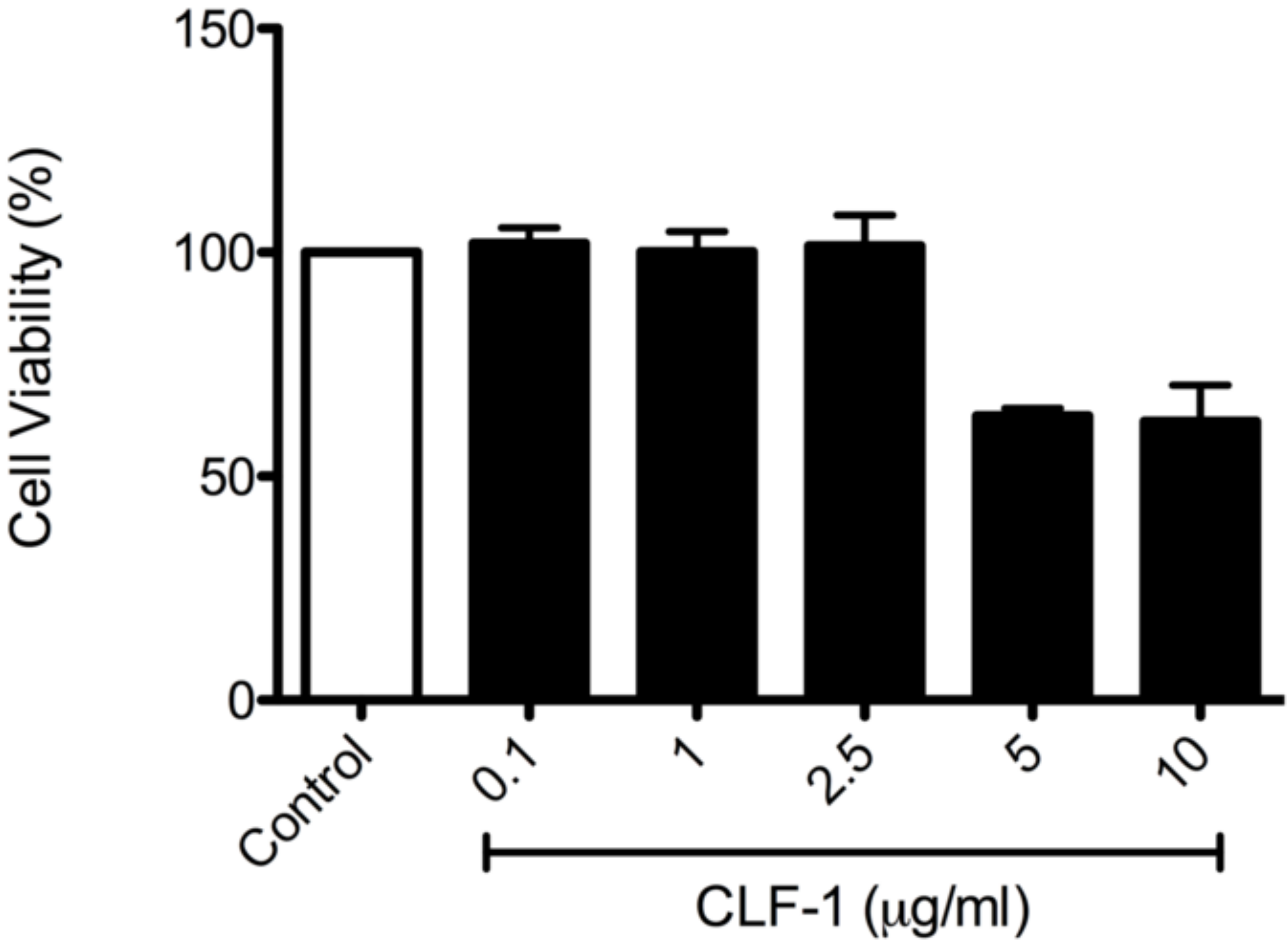

Figure 2
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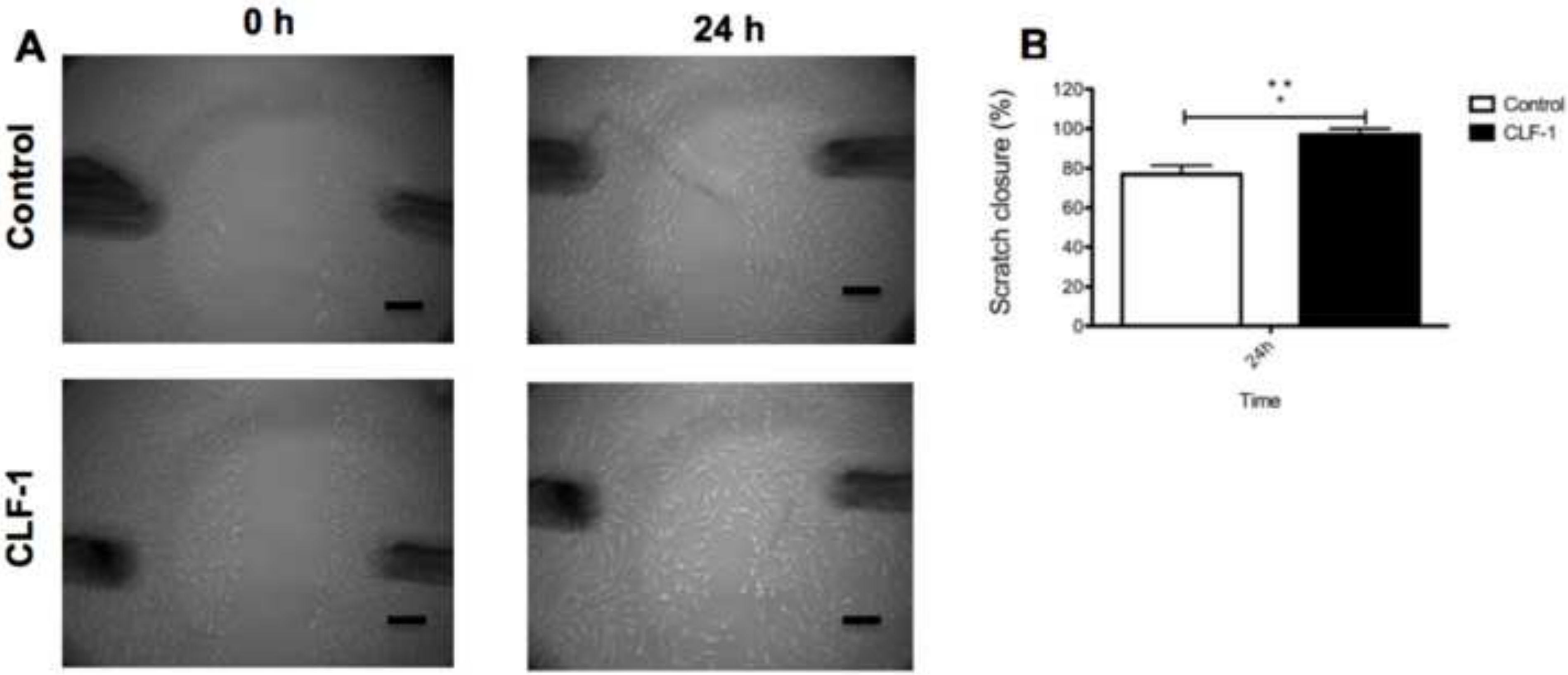

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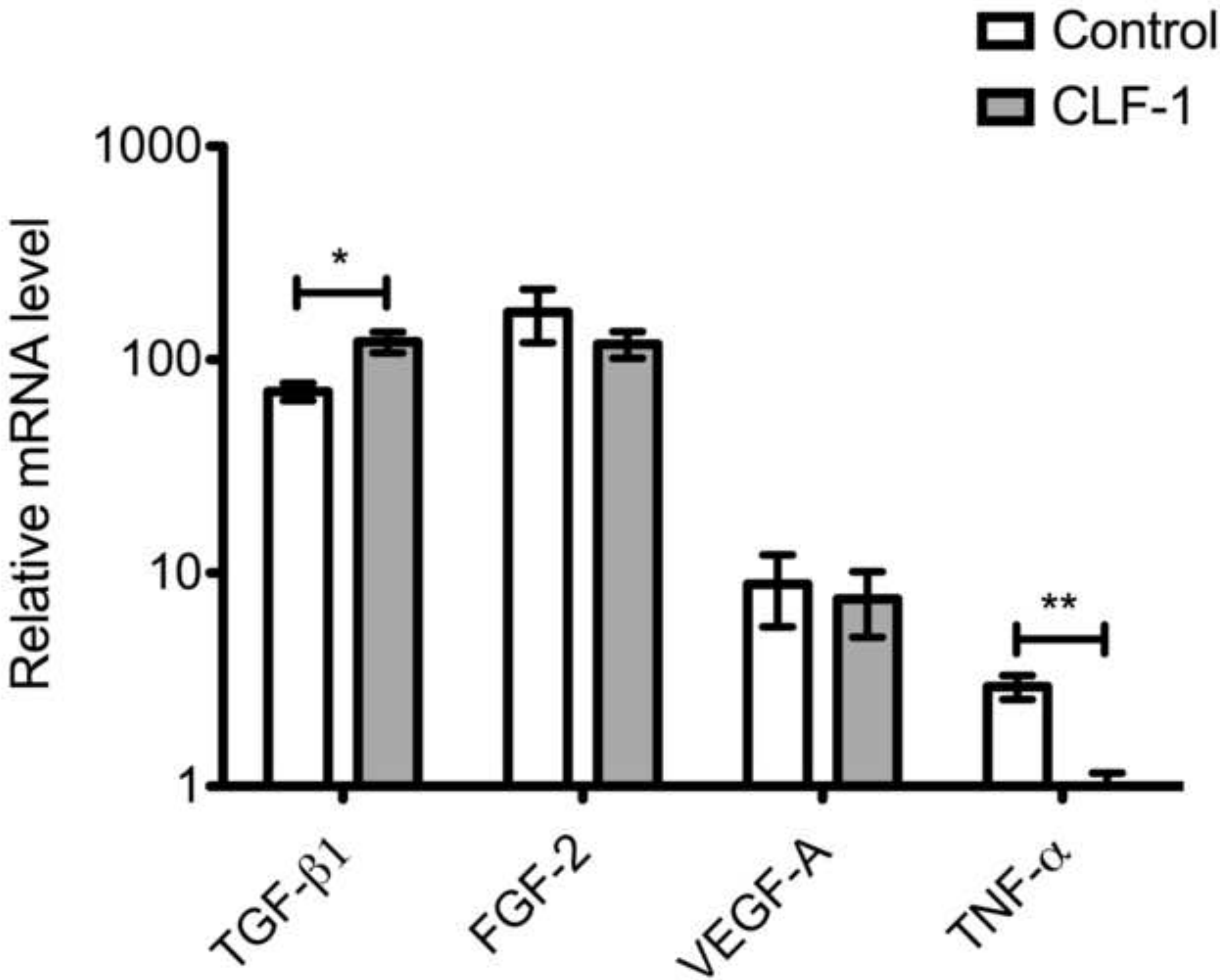

Capítulo V

Halilectin-3, a lectin from marine sponge Haliclona caerulea, induces apoptosis and autophagy in human breast cancer MCF7 cells through a caspase-9 and LC3 pathway

Artigo III – Submetido: Cancer Letters

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Article Type: Original Research Paper

Keywords: Breast cancer; apoptosis; lectins; marine sponges; Haliclona caerulea.

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FACULDADE DE
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Doctor Manfred Schwab
Editor-in-Chief of
Cancer Letters Journal

Lisbon, May 30th 2016

Dear Doctor Manfred Schwab

Could you please consider the article "**Halilectin-3, a lectin from the marine sponge** *Haliclona caerulea***, induces apoptosis and autophagy in human breast cancer MCF7 cells through a caspase-9 and LC3 pathway"** to be published in **CANCER LETTERS.**

This manuscript comprises our recent efforts in understanding the biological role of a lectin isolated from a marine invertebrate as a triggering agent of apoptosis and autophagy in breast cancer cells. The literature shows that the potential therapeutic application of lectins from marine invertebrates is far less studied in comparison to plant lectins, where some already reached clinical trials. The lack of studies is mainly due to limitations in obtaining the marine lectins. After our recent report on the successful isolation of H3, a new lectin from the marine sponge Haliclona caerulea, here we assessed its biological effect. Therefore, we believe that our findings are innovative in addressing for the first time the anti-tumor potential of the H3 marine lectin and it may contribute to the development of new strategies for breast cancer combating.

In addition, it is important to say that all authors contributed and approved this manuscript. We look forward to hearing from you in due course.

Yours sincerely,

Professor Paula A. Videira

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Highlights

- H3 lectin reduces viability of MCF7 breast cancer cells.
- H3 induces apoptosis and autophagy of MCF7.
- H3 inhibit the MCF7 cell adhesion.

Halilectin-3, a lectin from the marine sponge *Haliclona caerulea*, induces apoptosis and autophagy in human breast cancer MCF7 cells through a caspase-9 and LC3 pathway

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Abstract

An ideal strategy for cancer treatment is the specific induction of tumor cell death, sparing normal cells. Marine sponges are a rich biological reservoir of biomolecules, especially lectins, which have attracted considerable attention due to potential biological effect in human cells. Lectins are proteins that bind specific carbohydrate signatures and some gained further interest for their capacity to bind tumor associated carbohydrates antigens and induce tumor cell apoptosis. The present study aimed to evaluate the antitumor potential of H3, a lectin, recently reported from marine sponge *Haliclona caerulea*, on the human breast cancer cell line MCF7. H3 was able to reduce MCF7 cell viability with an IC_{50} of 100 μg/ml, but not in normal cells. At 24h, this concentration of H3 induced a significant arrest in the G1 cell cycle phase. Consistently, almost 50% of the cell were in early apoptosis and showed remarkable increased expression of *CASP-9* (caspase-9). Interestingly, H3 impaired dramatically the adhesiveness of MCF7 cells in culture. Assays conducted with Lysotracker Red showed increased organelle acidity, suggesting autophagic cell death, which was further supported by increased expression of microtubule-associated protein light chain 3 (*LC3*). In conclusion, the apoptotic effect of H3 may be related to a balance between apoptosis and autophagic cell death mediated by increase of expression of *CASP-9* and *LC3*. This is the first report that a marine sponge lectin can induce both apoptosis and autophagy in breast cancer cell line MCF7.

Keywords: Breast cancer; apoptosis; lectins; marine sponges; *Haliclona caerulea*.

1. Introduction

Cancer is a disease characterized by uncontrolled growth and spread of abnormal cells, representing one of the most common disease-related cause of death in the world [1, 2]. Breast cancer is the most common cancer found in women and second cause of cancer deaths among women living in developed and less developed countries. It is estimated that about 508.000 women die per year due to breast cancer [3]. Although surgery, chemotherapy and radiotherapy are typically used for cancer treatment, they are often not effective and specific, and have been associated with several side effects [4]. A strategy for cancer treatment is the induction of tumor cell apoptosis, and the use of triggering agents with this ability has attracted a higher interest in recent years [5].

Lectins are non-immunoglobulin proteins ubiquitous in nature, which possess at least one non-catalytic domain that binds reversibly to specific carbohydrates without modifying their structure [4, 6]. Previous studies reported that several plant lectins are responsible for triggering apoptosis and autophagy in tumor cells [7, 8]. In addition, lectins can potentially interfere with metastatic properties of tumor cells, a fact that has been attributed to their capacity to bind aberrant tumor-associated carbohydrates [5, 9]. Some plant lectins have even been proposed as alternative therapies in cancer due to their anti-tumor capacity [10-12]. Less frequently, the literature reports marine sponge lectins with cytotoxic effects towards cancer cell lines, mainly because the properties of these lectins remain poorly explored [13-16]. Until today, three sponge lectins were reported having antiproliferative activity and proapoptotic effect against melanoma cells, erythroleukemia and leukemia cells [14, 15, 17]. However, there are not any data about the effect of sponge lectins on breast cancer cells.

Marine organisms have been extensively studied as potential sources of novel bioactive compounds. Marine lectins show minimal immunogenicity because they have a small size, its structure is more stable due to presence of sulfide bonds, and have high specificity for complex glycans instead of simple sugars [18]. Thus, efforts in marine lectin prospection and purification fields have to be fomented aiming to explore their biological activities and/or biotechnological potential [16].

Halilectin-3 (H-3 or H3) is a mucin binding lectin recently isolated from marine sponge *Haliclona caerulea* that contains three subunits on its structure, resulting of a combination of two glycosylated subunits alpha (16 KDa and 18 KDa) and one beta subunit (11 KDa). H3 contains also a rare hydrophobic chromophore interacting with lectin subunits [19].

In this study, we report for the first time that H3 can induce apoptosis and autophagy in human breast cancer cell line MCF7. The molecular mechanism behind apoptosis and autophagy induced by H3 seem to be related with an anti-adhesion effect, alteration in cell cycle and induction of caspase-9 and microtubule-associated protein light chain 3 (*LC3*) expression.

2. Material and Methods

2.1 Chemicals and reagents

Phosphate Buffered Saline (PBS; pH 7,4), Dulbelcco´s Modified Eagle Medium (DMEM), heat-inactivated Fetal Bovine Serum (FBS), L-Glutamine (200mM), trypsin/EDTA solution (TE) and Penicillin-Streptomycin were purchased from Gibco® by life Technologies. CellTiter 96® Aqueous MTS Reagent Powder was purchased from Promega. Annexin V-APC was purchased from ImunoTools. Concanavalin A (Con A lectin) from *Canavalia ensiformis*, propidium iodide solution (PI), 7-Aminoactinomycin D (7-AAD) and ribonuclease A were purchased from Sigma-Aldrich® and 1 kb plus DNA ladder was purchased from Invitrogen™. LysoTracker® red (DND-99) was purchased from Molecular Probes®. All the others reagents used in the assays were of analytical grade or the highest quality.

2.2 Animal collection and isolation of H3

Specimens of the marine sponge *Haliclona caerulea* were collected in the Paracuru Beach, Paracuru village, Ceara, Brazil. Fresh sponges were transported on ice to the laboratory and stored at -20°C until use.

Halilectin-3 (H3) was isolated from marine sponge *H. caerulea* using a combination of hydrophobic interaction chromatography and ion-exchange chromatography as previously described [19].

2.3 Cell lines and culture condition

Human breast adenocarcinoma cell line (MCF7; HTB-22™) and primary Human Dermal Fibroblasts (PCS-201-012™) were both purchased from American Type Culture Collection (ATCC®). HDF were kindly supplied by Dr. Alexandra R. Fernandes (Department of Life Sciences, Faculty of Sciences and Technology, New University of Lisbon). The cells were maintained in T-25 flasks containing DMEM medium supplemented with 10% of FBS, 1% of L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2. Medium was routinely changed every 3rd day and cells at 90% confluence were subcultured by trypsinization (0,025% trypsin/0,1% EDTA). In all experiments cells were used between 4 and 12 passage number.

2.4 Cell viability assay

The cell viability was determined using the CellTiter 96® Aqueous MTS Reagent Powder (Promega), following the manufacturer´s instructions. Briefly, cells (1,5 x 10^4/200 µl/ well) were seeded into a 96 well flat bottom microtiter plates in DMEM containing 10% FBS and incubated overnight. Afterwards, the supernatant was removed and replaced for DMEM supplemented with 2% FBS containing different concentrations of H3. MTS assay was performed at 6h, 24h and 48h for MCF7 cells and 48h only to HDF.

Three independent experiments were performed in triplicate. The optical density was read at 490nm on a microplate absorbance reader. The viability was calculated according to the formula:

$$\text{Cell viability (\%)} = \frac{\text{average OD 490nm (H3)}}{\text{average OD 490nm (control)}} * 100$$

2.5 Anti-adhesion assay

MCF7 cells (5 x 10^4/ml) were incubated with the IC_{50} (100 µg/ml) of H3 lectin for 1h at 37°C. Then, MCF7 cells treated or untreated were seeded in 24-well polystyrene plates and incubated for 8 and 24 h. The effect of H3 on adhesion of MCF7 cells was evaluated by microscopic evaluation. Three independent experiments were performed in triplicate.

2.6 Cell cycle analysis

Cell cycle analysis was performed as previously described with modifications [20, 21]. In summary, MCF7 cells (1.5×10^5/well) were seeded in 6-well plates in DMEM supplemented with 10% (v/v) FBS for 24 h. After incubation, the supernatant was replaced with DMEM without FBS and the cells were incubated for 24 h, for cell cycle synchronization. Afterwards, the supernatant was removed and the cells were incubated with H3 (100 µg/ml) in DMEM containing 2% FBS for 24 h. After treatment, cells were trypsinized, washed with PBS, centrifuged (36 *g*/10 min/4 °C) and resuspended in ice-cold 70% ethanol with PBS, and stored for 24 h at 4 °C. Ethanol-fixed cells were centrifuged (36 *g*/10 min) and resuspended in PBS containing 20 µg/ml RNAse and 50 µg/ml PI, and incubated for 20 min on dark at room temperature. The effect of H3 in the cell cycle was evaluated by determining the percentage of cells at each phase of the cell cycle (G1, S and G2/M). through flow cytometry (FCM) using an Attune acoustic focusing cytometer (Applied Biosystems).

2.7 Cell apoptosis assay

Cell apoptosis and necrosis was assessed through annexin V-APC/7AAD double staining method. Briefly, MCF7 cells (2,25 x 10^5/well) were seeded in 6-well plates containing DMEM supplemented with 10% (v/v) FBS and grown for 24 h. Then, the cells were harvested and incubated with H3 (100 µg/ml) in DMEM containing 2% FBS for 24 and 48 h. Control cells were incubated with denaturated H3 or DMEM supplemented with 2% FBS only. The denaturation process of H3 was carried out as previously described with some modifications [22]. After treatment with H3 in the aforementioned periods, the cells were detached by trypsinization and then centrifuged (206 *g*/5 min) and washed twice with PBS. The cells were resuspended in binding buffer (PBS, pH 7,4, 25mM $CaCl_2$) and 5 µl of annexin V-APC and 5 µl of 7AAD were added to each well. Cells were incubated in the dark for 20 min at room temperature and then the proapoptotic potential of H3 was determined by FCM.

2.8 Lysotracker staining

To evaluate if H3 promotes the development of acidic vesicles in the cytosol, MCF7 cells were seeded in a 6-well plate at a density of 5 x 10^4 cells/ml and incubated for 24 h. After, the supernatant was removed and the cells were treated with H3 (100 μg/ml) or Con A (100 μg/ml) as a positive control in DMEM containing 2% FBS for 6h. Control cells were incubated with DMEM supplemented with 2% FBS only. LysoTracker Red (LTR) staining was performed as previously described [23]. In short, LTR (50 nM) and 4´6-Diamidino-2-phenylindole dihydrochloride (DAPI; 1:1000 µl) were dissolved in pre-warmed DMEM and incubated on dark at 37°C until the use. After lectins treatment, the supernatant was removed and cells were washed once with PBS and stained with LTR and DAPI for 60 min. Fluorescent microscopy (ZOE™, Bio-Rad) of the LTR and DAPI staining was performed immediately after.

2.9 mRNA extraction and qRT-PCR

Gene expression was analyzed by reverse transcriptase-real time polymerase chain reaction (qRT-PCR) as previously described [24, 25]. Briefly, after 6 h and 24 h of treatment with H3 lectin, MCF7 (10^6 cells) were trypsinized and total mRNA was extracted using GenElute Mammalian total RNA Purification kit (Sigma Aldrich) and DNAse treatment was performed to eliminate DNA (Nzytech). Then, 1 µg of total mRNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). qRT-PCR was performed in a 7500 Fast system (Applied Biosystems) using TaqMan Fast Universal PCR Master Mix, primers and Taqman probes acquired from Applied Biosystems and used following the manufacturer´s instructions. The analyzed genes were *BAX* (Hs00180269_m1), *BCL-2* (Hs00608023), *BECLIN-1* (Hs00186838_m1), *CASP 3* (Hs00234387_m1), *CASP 8* (Hs01018151_m1), *CASP 9* (Hs00609647_m1), *LC3* (Hs01076567_g1) and *TP53* (Hs01034249_m1). Each reaction was performed in duplicate. All genes, including the endogenous controls (β-actin and GAPDH), and treated or untreated cells always analyzed in the same run to exclude between-run variations.

The relative expression for each gene was calculated by $2^{-\Delta\Delta Ct}$ method as previously reported [26]. The efficiency of the amplification reaction for each primer/probe was above 95%.

2.10 Statistical analysis

All the presented results were confirmed in at least three independent experiments. Statistics are presented as the mean ± SEM. Experimental data were analyzed by Student´s t-test and one-way ANOVA followed by Tukey´s post-hoc. P<0,01 and *P*<0,05 was adopted as the level of significance. The IC_{50} values were calculated by using the software GraphPad Prism® 5 to fit a variable sigmoidal-dose-response curve.

3. Results

3.1 Effect of H3 on cell viability

To assess the effect of H3 on the cell viability, we incubated MCF7 cells with increasing doses of H3 at different time points. As shown in Fig. 1A, H3 decreased the viability of MCF7 cells. Loss of 56% and 58% viability was observed at doses of 125 and 250 µg/ml, respectively. The loss of cell viability was an early event since no significant differences were seen between different time points. Afterwards, the concentration that promotes 50% inhibition of the tumor cell viability (IC_{50}) was calculated, revealing that treatment with 100 µg/ml results in 50% inhibition of MCF7 viability. To understand whether H3 had a similar effect on non-tumor cells, we also analyzed H3 effect on HDF primary cells. The viability of HDF cells treated for 48 h with H3 at 125 and 250 µg/ml was decreased only in 25% (Fig. 1B).

3.2 Induction of apoptosis and cell cycle arrest by H3

Given that MCF7 cells had their viability decreased by the treatment with H3, its effects on cell cycle and apoptosis were evaluated. The treatment with H3 at 100 µg/ml for 24h resulted in significant increase in the number of cells at G1 phase with decrease of cell population at the S and G2/M phases (Fig. 1B).

In order to evaluate the pro-apoptotic potential of H3, MCF7 cells were double stained with annexin V-APC/7-AAD. The results showed that after 24 h treatment with H3 at 100 μg/ml, 49% of cells remain viable, while approximately 46% were in early apoptosis (Fig. 1C left). The pro-apoptotic effect was enhanced with the course of time. After 48 h treatment, only 32% of cells remain viable, whereas the number of cells in early apoptosis was increased in comparison with 24 h (Fig. 1C right). The same effect was seen on the proportion of necrotic cells, which rose from 1.93 at 24 h to 8.84% after 48 h. As expected, H3 denaturation by heating caused a high reduction of H3 pro-apoptotic function.

3.3 Anti-adhesive properties of H3 on MCF7 cells

We also investigated the role of H3 in inducing the detachment of MCF7 cancer cells or preventing their adherence. Fig. 2 shows a significant inhibition of MCF7 cell adhesion to the polystyrene surface, which floated in the culture flask, when compared to untreated cells at 8 h and 24 h. In addition, it was observed that H3 was able to agglutinate MCF7 cells at both time points (Fig. 2).

3.4 Induction of autophagy in MCF7 cells

The autophagic potential of H3 was also investigated in this study. For this purpose, LysoTracker Red (LTR) staining method was carried out. LTR is a red fluorescent probe that accumulates in acidic vesicles (such as autophagosome) [23, 27]. As shown in Fig. 3A, H3 treatment induced remarkable accumulation of LTR in MCF7 cells as analyzed by fluorescent microscopy. Untreated cells presented no acidic vesicles as only a diffuse staining was observed. On the other hand, 6 hours after the treatment with Con A, it was possible to observe the formation of autophagic vesicles confirmed by accumulation of LTR in adherent MCF7 cells. However, unlike Con A, H3 induced detachment of cells. The same effect was reported above in section 3.3.

To clarify the molecular mechanisms involved on H3-induced autophagy a real-time PCR experiment was carried out to evaluate the gene expression of the *BECLIN-1* and microtubule-associated protein light chain 3 (LC3), known to be involved in autophagy. Interestingly, it was observed that despite of LTR be positive to acidic vesicles in H3 treated cells there was a down-regulation of *BECLIN-1* gene expression after 6 h (Fig. 3B). However, there was an increase in *LC3* mRNA expression when compared to untreated cells. Therefore, the results suggest that H3 treatment can induce the autophagic process through fast formation of the autolysosome by LC3 expression induction.

3.5 Effect of H3 in the expression of apoptotic genes

The effect of H3 in the expression of pro and anti-apoptotic genes in MCF7 cells was analyzed after 6 h and 24 h of treatment. The relative expression of the genes *BCL-2*, *BAX*, *CASP 3*, *CASP 8*, *CASP 9* and *TP53* was evaluated by real-time PCR. MCF7 cells treated with H3 lectin for 6 h and 24 h were compared with untreated cells. As shown in Figure 4, there was an significative over-expression of *CASP 9* (p= 0,0058), which is involved in intrinsic apoptosis pathway (Fig. 4). The expression of *CASP 9* after 24 h treatment with H3 was 7-fold increase compared to that at 6h. It was also observed an over-expression of *CASP 3* and 8 after 24 h treatment. Interestingly, after 6h, there was an over-expression of all analyzed genes, including *BCL-2*, *BAX* and *TP53*. However, after 24 h there was a decrease in the expression of the anti-apoptotic gene *BCL-2* but non in *BAX* and *TP53*. Thereby, emphasizing the role of H3 in inducing MCF7 apoptosis.

4. Discussion

Many anticancer agents have been derived from natural sources, including plants, microbes and marine organisms [28, 29]. Indeed, the abundance of drugs that have been isolated from terrestrial organisms is in sharp contrast to the scarcity of medicine or drugs obtained from marine sources [18]. Though research on the biological function and applications lectins from marine species are relatively new their biological applications are now becoming apparent [18, 30].

In this study, it was demonstrated that H3 reduces the viability of MCF7 cells (IC_{50} = 100 μg/ml), with low toxicity against normal cells (HDF). This difference may be due to lectin recognition, indeed, lectins can bind preferentially to cancer cell instead normal ones due to small changes in carbohydrate moieties found on the cancer cell surface and not on normal cells [31]. Such differences may explain the selective cytotoxic effects displayed by H3 on MCF7 cells. In fact, H3 is a mucin binding lectin, with affinity to GalNAc [19]. Breast cancer cells as MCF7 cells have aberrant expression of GalNAc containing antigens such as the Tn antigen (GalNac-O-Ser/Thr) hence it can explain the observed selective anticancer potential of H3.

The metastatic potential of cancer cells is largely attributed to their adhesive properties, which are affected by aberrant glycosylated structures, promoting adhesive interactions with other cells and extracellular matrix [32]. Our findings show that the treatment with H3 impairs MCF7 adhesion to a polystyrene surface. Interestingly, some lectins inhibit MCF7 cell adhesion by decreasing the expression of $\alpha_6\beta_1$ and also by interacting with the fibronectin receptor $\alpha_5\beta_1$ integrin and thus inducing anoikis apoptosis [5, 9]. In addition, some lectins are able in agglutinating malignant tumor cells [31]. Thus, these properties can be related to the anti-adhesion effect exhibited by H3 on MCF7 cells.

In this study we provided evidence that H3 induces both apoptosis and autophagy in MCF7 cells. Most cells undergo in early apoptosis after 24 h treatment followed by an evident increase in the effect after 48 h.

It is widely known that several antitumor agents can induce cell death by arresting a specific phase of the cell cycle. Normal cells presents cell cycle checkpoints that ensure damage repair, whereas malignant cells can be eliminated by apoptosis [29]. Our results demonstrated that treatment with H3 induces accumulation of MCF7 cells at G1 phase of the cell cycle. Moreover, the increased expression of *TP53* may explain the cell cycle arrest after 24 h treatment. Furthermore, H3-treated cells seem undergo accumulation of autophagic vacuoles after 6 h.

Previous studies have shown that a marine sponge lectin from *Cliona varians* can induce cell tumor death through caspase independent cathepsin B pathway as reported [14]. Moreover, lectin from marine sponge *Cinachyrella apion* (CaL) induced HeLa cell death by apoptosis with cell cycle arrest in S phase. Nevertheless, there was a considerable increasing of Bax protein level, thus pointing that apoptotic effect takes place probably by intrinsic pathway [15]. However, neither sponge lectin presented interconnected apoptosis and autophagy effects.

In contrast to few prior studies, we demonstrated that H3 treatment might induce MCF7 cell autophagy, an important death-promoting apoptosis type II pathway. After 6 h treatment, *BECLIN-1* gene was downregulated in MCF7 cells. *BECLIN-1* is a very important gene that regulates autophagy through autophagosome formation [33]. However, malignant breast cancer cells often show reduced expression of *BECLIN-1* level, triggering low levels of autophagy [34]. This condition is characterized as protective factor of breast tumor. Moreover, *BECLIN-1* is monoallelically deleted in many human patients with breast, ovarian and prostate cancer [35]. On the other hand, the treatment with H3 upregulated the expression of *LC3* gene. Likely, the high expression levels of *LC3* may play important roles in accumulation of autolysosome in cytosol of MCF7 cells as detected by LTR. Our results corroborate with a previous study that proposed a role for ConA-induced autophagy on HepG2 cells by increasing LC3 protein expression. Con A also seems to induce autophagic cancer cell death via a mitochondria-mediated pathway [36]. The *TP53* has many regulatory functions including autophagy induction or inhibition [37]. In fact, LC3 accumulation can be dependent of TP53 regulation [38]. A plant lectin isolated from *Polygonatum cyrtonema* (PCL) induced apoptotic and autophagic pathways regulated by ROS, p38 and p53 [8, 39]. Thus, the upregulation of *TP53* gene expression induced by H3 treatment can explain MCF7 cell autophagy at 6 h and further promotion of apoptotic cell death.

To develop lectin-based anti-cancer therapies it is important to understand the mechanism involved in tumor cell apoptosis induced by lectins [11]. Thus, we found that the treatment with H3 upregulated some pro-apoptotic genes after 6 and 24 h. Our results suggest that MCF7 cell apoptosis occurs mainly by a caspase-dependent intrinsic pathway with upregulation of *BAX* expression and downregulation of *BCL-2* at 24 h. Some lectins are efficient in inducing both intrinsic and extrinsic pathways of apoptosis [4, 40]. H3 seems also to induce apoptosis via death receptor pathway since the expression of *CASP 8* was increased. Previous reports have pointed out to several plant lectins that induce apoptosis in various cancer cells [41]. Mistletoe lectins are widely known by their remarkable anticancer effects on MCF7 cells [12]. Thus, our findings are in agreement with these previous studies.

Furthermore, tumor cell death regulation might be associated as a “set balance” between autophagy and apoptosis cell death. However, there is a complex relationship between them that may vary through physiopathological context [39, 42, 43]. Lectins have been established as antitumor agents in various studies. For instance, Mistletoe lectins as well as Con A and *Phaseolus vulgaris* lectin are included in different stages of clinical trials [11, 41]. Thus, H3 emerges as new candidate for cancer treatment.

In summary, we report for the first time that H3, a lectin from marine sponge *Haliclona caerulea*, can induce both apoptosis and autophagy in breast cancer cell line MCF7. Also, we showed that H3 induces autophagy through LC3 mechanism and further apoptosis by intrinsic pathway with involvement of caspase 9. These findings would open a new perspective on the exploration of porifera lectins as potential anticancer agents. Meanwhile, further studies are needed to clarify the mechanisms involved in the anticancer activity by H3.

Conflicts of interest

The authors declare that there are not conflicts of interest.

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Authors Contribution

P. Videira was responsible for the conception and design of the study. L. Nascimento-Neto, G. Cabral and R. Carneiro performed the acquisition, analysis and interpretation of data. L. Nascimento-Neto and V. Arruda drafting the article. E. Teixeira, C. Nagano, A. Sampaio, G. Cabral and P. Videira assisted in the revising it critically for important intellectual content. All authors reviewed content and agreed this version for publication.

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Figure Captions

Fig. 1. H3 induces apoptosis in breast cancer cell line MCF7. The MCF7 cells and HDF were treated with various doses of H3 for indicated time periods. The cell viability was determinated by MTS assay (n=3, mean ± SEM) (A). MCF7 cells were incubated with 100 μg/ml H3 for 24 h, H3 provoked G1 cell cycle arrest (n=4, mean ± SEM) (B). In addition, MCF7 cells were incubated with 100 μg/ml H3 for 24 h and 48 h. Afterwards, annexin V-APC/7-AAD double staining was performed and analysed by flow cytometry. H3 induced apoptosis in a time dependent manner (n=3, mean ± SEM) (C). (* P<0.05). (** P<0.01).

Fig. 2. H3 impairs the adhesion of MCF7 cells. MCF7 cells were incubated with H3 lectin 100 μg/ml for 1 h and then cells were cultivated for 24 h. Photomicrographs were taken by a phase contrast microscope at 8h and 24 h to evaluate the anti-adhesion effect of H3. Magnification 10x (8h) and 40x (24 h). (Scale bar: 100 μm; Black arrow: adherent cell; Red arrow: cell aggregation).

Fig. 3. H3 treatment induces autophagy of MCF7 cells. MCF7 cells were treated with H3 100 μg/ml for 6 h. After cells were incubated wuith LTR. The acid vesicles were observed under a fluorescence microscope (A). After 6 h of H3 treatment a real-time PCR was carried out to assess the H3 effect on autophagy mRNA gene expression (B).

Fig. 4. H3 treatment upregulate mRNA relative expression of pro-apoptotic genes in MCF7 cells. The cells were treated with H3 at a concentration of 100 μg/ml for 6 h and 24 h. In order, the mRNA was extracted and the cDNA convertion was carried out. Analysis of gene expression was determined by real-time PCR. ** (P< 0,01).

Fig. 1.

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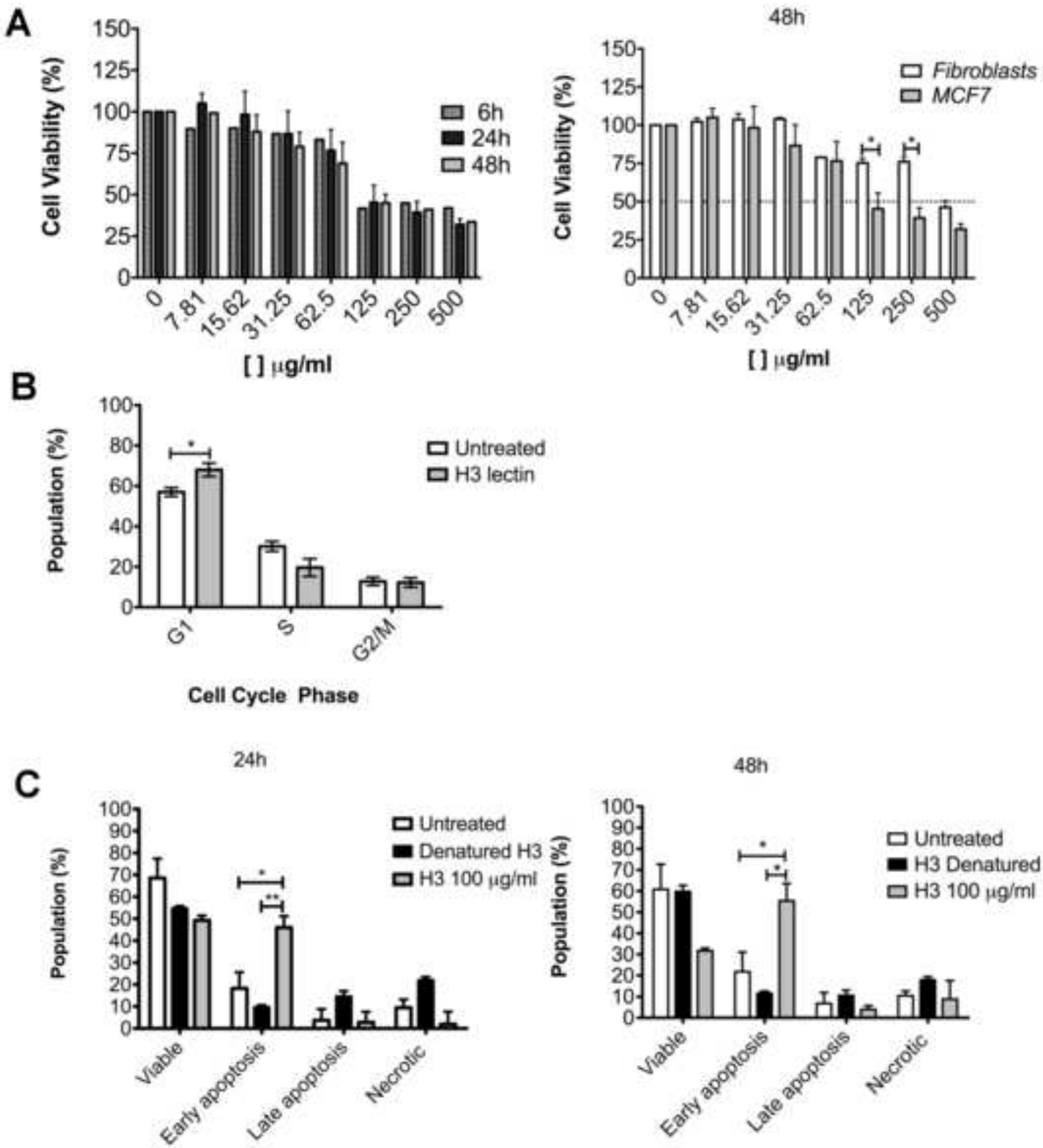

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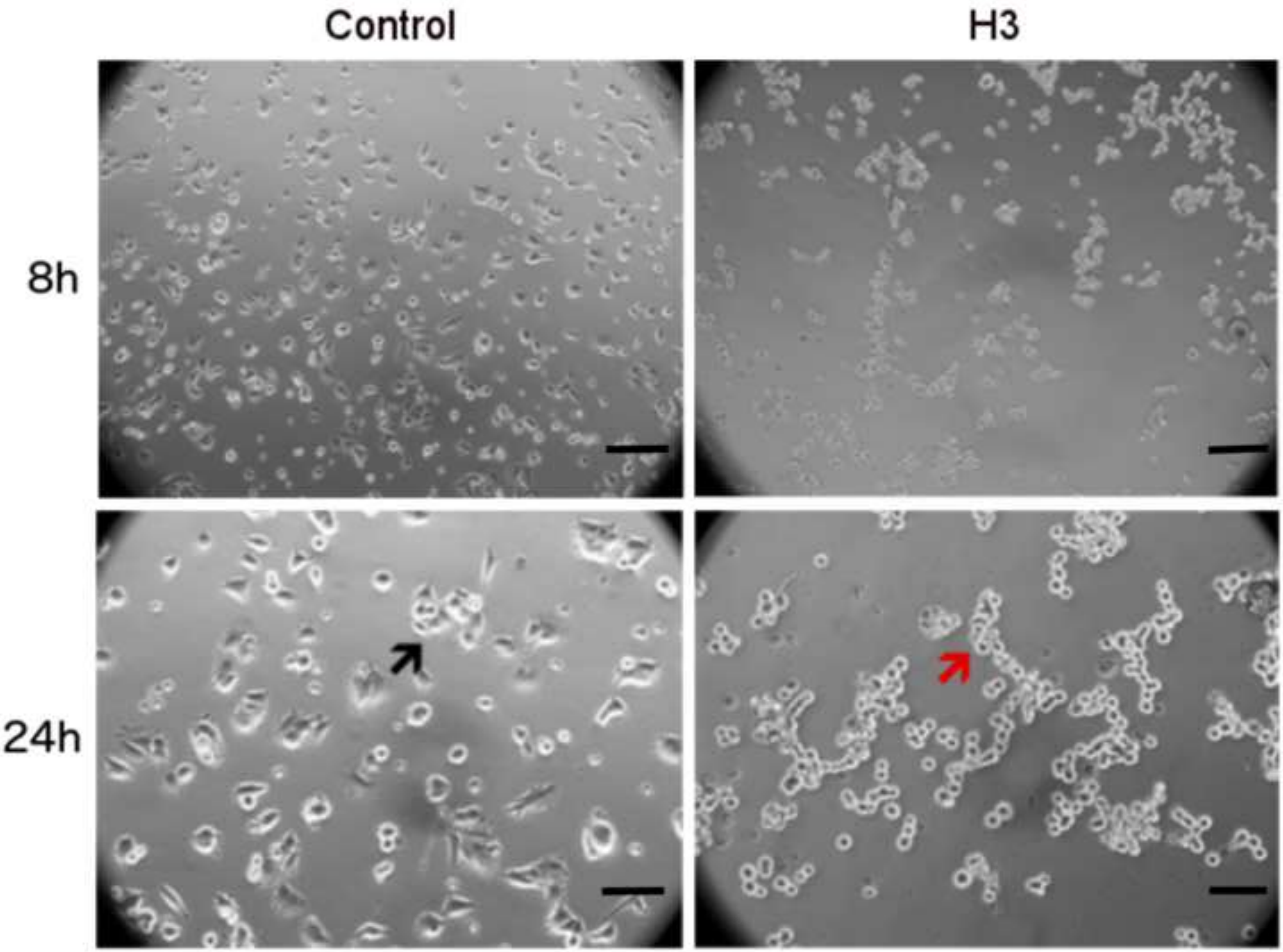

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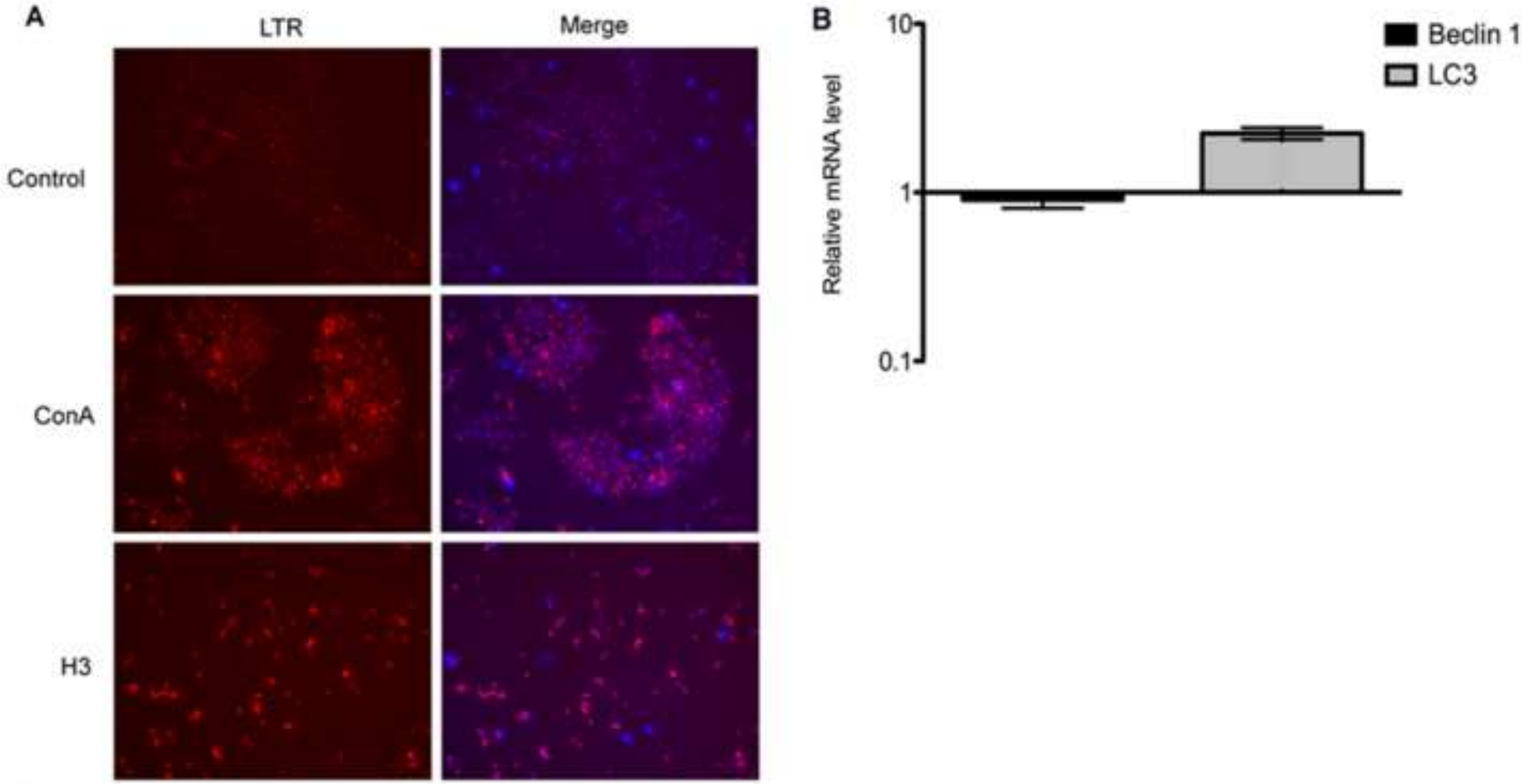

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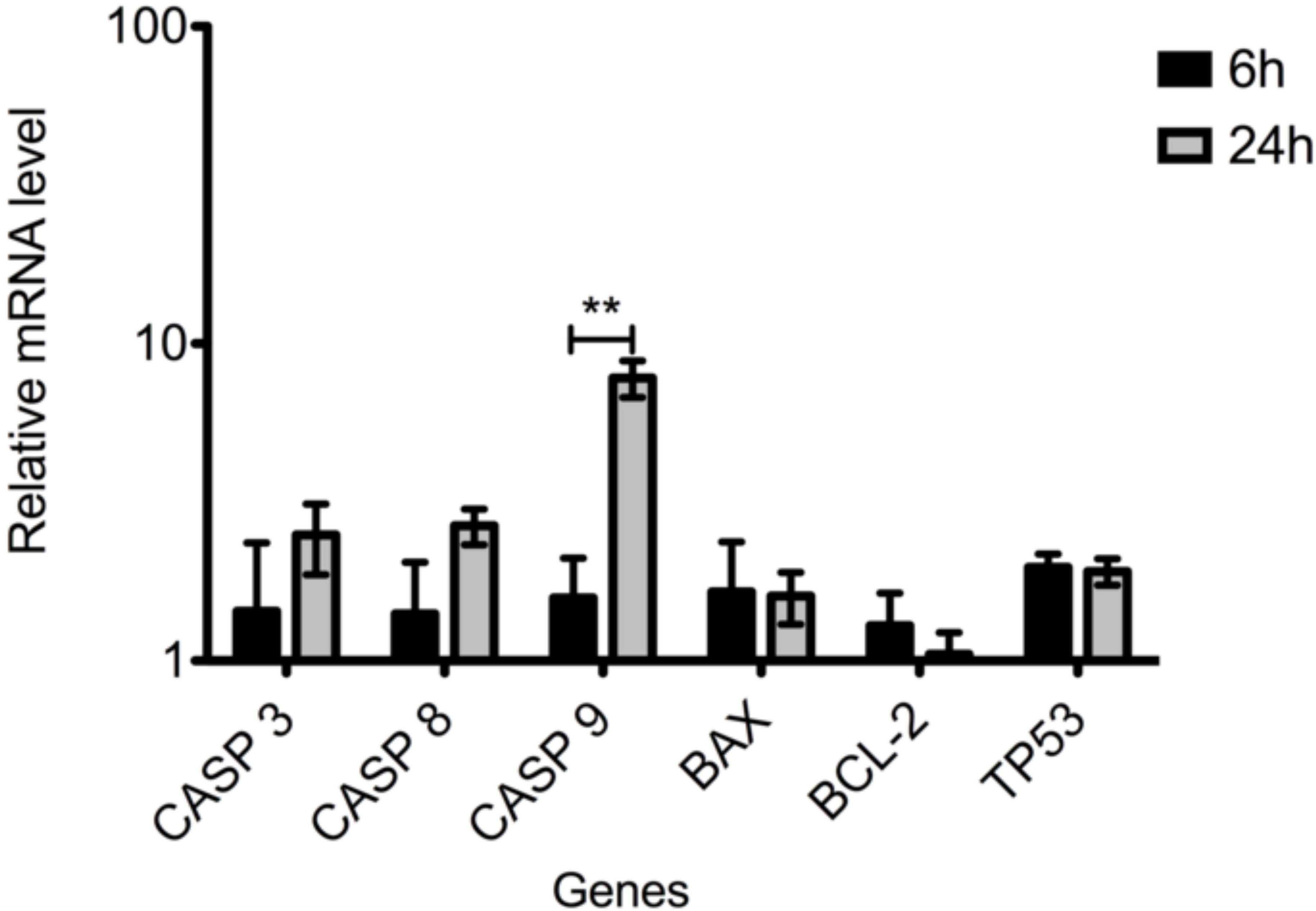

Conflicts of Interest Statement

Manuscript title: **Halilectin-3, a lectin from the marine sponge *Haliclona caerulea*, induces apoptosis and autophagy in human breast cancer MCF7 cells through a caspase-9 and LC3 pathway**

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Capítulo VI

PERSPECTIVAS

VI. Perspectivas

Diante do exposto, pode-se constatar que plantas e invertebrados marinhos são significativas fontes de recursos medicamentosos para o tratamento de várias patologias. Metabólitos secundários de plantas, ainda são pouco explorados no que diz respeito a sua aplicação no tratamento de feridas de pele. No presente trabalho, foi demonstrado que um triterpeno isolado de folhas de *Combretum leprosum* (CLF-1) promove a reepitelização de feridas agudas *in vivo* em um período de 12 dias. O potencial cicatrizante foi confirmado através de um estudo complementar *in vitro*, onde uma ferida artificial do tipo *scratch* foi totalmente restaurada através do tratamento com o CLF-1. Sugere-se que o tratamento com CLF-1, induz a cicatrização da lesão artificial pelo aumento da expressão de TGF-β e diminuição na expressão de TNF-α, acelerando a fase proliferativa da cicatrização. Nesse sentido, estudos mais aprofundados, como a elucidação do mecanismo de ação na cicatrização in vivo e o potencial de CLF-1 em feridas crônicas como úlceras de pressão ou diabéticas, necessitam ser realizados com o objetivo de gerar conhecimento sobre o composto utilizado visando o deposito de patentes e inserção junto a indústrias farmacêuticas para a produção de fármacos baseados no CLF-1.

Em outra vertente, o câncer, como um dos principais problemas de saúde pública, necessita urgentemente de novas alternativas medicamentosas que possam ser utilizadas para diagnóstico e/ou tratamento da população acometida por essa patologia. Lectinas emergem como moléculas promissoras no diagnóstico/tratamento do câncer. O estudo do efeito da lectina isolada da esponja marinha *Haliclona caerulea* (H3) sobre células do adenocarcinoma de mama humano (MCF7), sugere uma forte tendência para morte celular através de eventos de apoptose e autofagia. Em adição, H3 interfere na adesão das células tumorais, sugerindo efeito anti-metastático. O desenvolvimento de terapias antitumorais baseadas em lectinas, são importantes para o melhor entendimento do envolvimento dessas biomoléculas sobre o processo apoptótico. Algumas lectinas vegetais vem sendo utilizadas em testes clínicos e pré-clínicos como ferramentas terapêuticas e/ou diagnósticas. Nesse sentido, o presente trabalho abre novas perspectivas sobre a exploração de lectinas de poríferos como potenciais agentes antitumorais. Outros estudos são necessários para o melhor entendimento do mecanismo de ação de H3, bem como seu efeito sobre diferentes linhas celulares de câncer.

ANEXOS

Artigos e Comunicações
(2012 – 2016)

11th International Meeting of the Portuguese Carbohydrate Group

6th Iberian Carbohydrate Meeting

Polytechnic Institute of Viseu

Viseu, 2015

Scientific Program

Sunday, September 6

15:00-17:00 Registration

17:00-18:00 Opening Session

Chairperson: *Jesús Jiménez Barbero*

18:00-18:45 **PL1:** *Serge PEREZ*, CNRS Grenoble, France
Building, seeing and playing with complex glycans

19h30 **Welcome Cocktail** – Sponsor by Glycom A/S

Monday, September 7

Chairperson: *Joachim Thiem and M. Isabel Ismael*

9:00-9:45 **PL2:** *Cristina NATIVI*, University of Florence, Italy
The cycloaddition way to synthetic immunostimulants

9:45-10:00 **OC1:** *Caroline LIGEOUR*, Institut Pasteur, France
Antibody recognition of synthetic S*higella flexneri* 3a polysaccharides: implications for the development of a glycoconjugate vaccine

10:00-10:15 **OC2:** *François MORVAN*, Institut des Biomolécules Max Mousseron, Montpellier
Innovative strategy to discover glycoclusters against *Pseudomonas aeruginosa* using a glycoarray

10:15-10:45 **IL1:** *Knud JENSEN*, University of Copenhagen, Denmark
Chemoselective carbohydrate chemistry for chemical biology and nanobioscience

10:45-11:15 **Coffee-break**

Chairperson: *Mário Monteiro*

11:15-12:00 **PL3:** *Anne IMBERTY*, CERMAV-CNRS, France
Lectins from bacteria and fungi: therapeutical targets and research tools

12:00-12:30 **IL2:** *José Alexandre FERREIRA*, Portuguese Institute of Oncology, Portugal
Changes in protein glycosylation: biological implications for bladder cancer progression and dissemination

12:30-12:45 **OC3:** *Luiz Gonzaga* do NASCIMENTO-NETO, NOVA University of Lisbon, Portugal
Antitumor and anti-adhesion effect of a lectin isolated from the marine sponge *Haliclona caerulia* on the human breast cancer cell line MCF-7

ANTITUMOR AND ANTI-ADHESION EFFECT OF A LECTIN ISOLATED FROM THE MARINE SPONGE *Haliclona caerulia* ON THE HUMAN BREAST CANCER CELL LINE MCF-7

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Breast cancer is the most common malignancy in women, and a major cause of deaths by cancer. The most effective means to reduce mortalities are early detection and better treatment strategies . An ideal strategy for cancer treatment is the specific induction of death in tumor cells, sparing normal cells. The search for tumor-specific biomolecules with this ability has attracted considerable interest in recent years. Lectins are proteins that possess, at least one non-catalitc domain that reversibly binds to a mono- or oligosaccharide. Among various biological activities, some have the potential to bind specifically to glycans expressed by cancer cells. Indeed, these biomolecules have been used in diagnostic to discriminate benign from malignant tumors, as well as to detect the type and the degree of cancer cells glycosylation that is directly related to process of metastasis. Some lectins from natural sources have attracted interest due to their ability to induce cellular apoptosis and to suppresses tumor growth *in vivo*. We have recently identified a lectin, H3, isolated from the marine sponge *Haliclona caerulia* with specificity for N-acetyl-galactosamine. However the potential of H3 to exert cytotoxicity against cancer is still unknown. The present study aims to evaluate the antitumor potential of H3 on the human breast cancer cell line MCF-7. The isolation and purification of the H3 lectin was performed as previously described [1]. The effect of H3 on cell growth and viability was assessed by MTS assay after 48h of treatment with different lectin concentrations. The specificity of H3 against cancer cells was determined by evaluating in parallel non-malignant cells, as fibroblasts. The induction of apoptosis and alterations in the cell cycle were investigated by flow cytometry. The influence of H3 in the adhesion ability of MCF-7 cells was evaluated by microscopy. H3 effectively reduced the viability of MCF-7 cells in a dose dependent manner (IC_{50}: 100μg/ml). Interestingly, this lectin was ineffective against fibroblasts except at 500μg/ml, which reduced cellular viability in 50.75 ± 1.56%. Cell cycle analysis of MCF-7 showed that 86% of the cell population was in sub-G1 phase after 48h treatment with 100μg/ml of H3 while untreated cells, only have 34% of the cell population in this phase, suggesting that H3 induces DNA fragmentation, i.e. apoptosis of MCF-7 cells. Further flow cytometry analysis with apoptosis markers (Anexin V/7-AAD) confirmed the pro-apoptotic effect of H3, as 100μg/ml of this lectin lead to 59% apoptotic cells, while only 29.86% of untreated cells and 9.67% of cells treated with heat denatured-H3 are apoptotic. Lastly, we observed that MCF-7 cells cultivated in the presence of 100μg/ml of H3, were unable to adhere to the culture plate, after 24 hours, and therefore had impaired proliferation, compared to MCF-7 cells cultivated without H3. In conclusion, our results suggest that the H3 lectin can be considered a new natural biomolecule with potential in the cancer treatment. Further studies are still ongoing to clarify the mechanisms underlying the anticancer activity of this marine invertebrate lectin.

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Research Article

Antimicrobial Effect of the Triterpene 3β,6β,16β-Trihydroxylup-20(29)-ene on Planktonic Cells and Biofilms from Gram Positive and Gram Negative Bacteria

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This study evaluated the antimicrobial effect of 3β,6β,16β-trihydroxylup-20(29)-ene (CLF1), a triterpene isolated from *Combretum leprosum* Mart., in inhibiting the planktonic growth and biofilms of Gram positive bacteria *Streptococcus mutans* and *S. mitis*. The antimicrobial activity was assessed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The antibiofilm potential was determined by quantifying total biomass and enumerating biofilm-entrapped viable bacteria. In addition, the acute toxicity of CLF1 on *Artemia* sp. nauplii was also determined. The results showed that CLF1 was able in inhibiting the growth of *S. mutans* and *S. mitis* with MIC and MBC of 7.8 μg/mL and 15.6 μg/mL, respectively. CLF1 was highly effective on biofilms of both bacteria. Only 7.8 μg/mL CLF1 was enough to inhibit by 97% and 90% biomass production of *S. mutans* and *S. mitis*, respectively. On the other hand, such effects were not evident on Gram negative *Pseudomonas aeruginosa* and *Klebsiella oxytoca*. The toxicity tests showed that the LC_{50} of CLF1 was 98.19 μg/mL. Therefore, CLF1 isolated from *C. leprosum* may constitute an important natural agent for the development of new therapies for caries and other infectious diseases caused by *S. mutans* and *S. mitis*.

1. Introduction

The resistance to available antimicrobials is currently a public health concern in the world. Several types of infectious diseases have become difficult to treat and expensive to cure mainly due to the low efficiency of antimicrobials for current bacteria [1, 2]. Moreover, the socioeconomic impact caused by infectious diseases is quite significant as shown by the high amount of financial resources spent on the clinical treatment of patients [3, 4]. Taking this into consideration, the ability of microorganisms to grow as biofilms and the increased rate of microbial resistance to conventional antibiotics contribute to the expanding epidemiology of infectious diseases [5].

Biofilms are complex microbial communities that establish themselves on a wide variety of surfaces and are generally associated with an extracellular matrix consisting of different polymers [6, 7]. Biofilms provide protection against the action of antibiotics and supplies a barrier to prevent or reduce the penetration of antimicrobial agents through the matrix [8]. The eradication of microorganisms that live as biofilms becomes extremely difficult or even impossible [9, 10]. Therefore, the search for new molecules able to inhibit biofilm formation, either by inhibiting microbial growth or by inhibiting their adhesion to a substrate, has intensified in recent years.

The shrub *Combretum leprosum* Mart. is popularly known in Brazil as "mufumbo" or "cipoaba" and is commonly found in the northeast. In folk medicine, it is widely used as an expectorant, antimicrobial, and antihemorrhagic agent [11].

Although scarce, research on the biological activities of *C. leprosum* points to similar actions to those observed in other species of the same genus. Nunes and coworkers [11] provided evidences about gastroprotective and antiulcerogenic roles of the ethanolic extract (EE) from the bark of *C. leprosum*, which were attributed to the increase in mucus production and inhibition of gastric acid secretion in rats.

In the present study, we evaluated the antimicrobial potential of the triterpene 3β,6β,16β-trihydroxylup20(29)-ene (CLF1) isolated from leaves of *C. leprosum* Mart. on the Gram positive bacteria *Streptococcus mutans* and *S. mitis* and on the Gram negative bacteria *Pseudomonas aeruginosa* and *Klebsiella oxytoca*.

2. Materials and Methods

2.1. Strains and Culture Conditions. The Gram positive strains *Streptococcus mutans* UA159 (ATCC 700610) and *S. mitis* (ATCC 903) and the Gram negative strains *Pseudomonas aeruginosa* (ATCC 10145) and *Klebsiella oxytoca* (ATCC 13182) were kindly provided by the Oswaldo Cruz Institute (FIOCRUZ, Rio de Janeiro, Brazil). The growth of all strains started from a stock culture maintained at −80°C in BHI broth (Brain Heart Infusion, Himedia, Mumbai, India) with 20% glycerol. Each microorganism was inoculated in 10 mL of fresh sterile BHI broth and incubated for 24 hours at 37°C with or without 10% CO_2 for Gram positive and Gram negative, respectively. After the initial activation, the culture was renewed by transferring 100 μL of inoculum into 10 mL of new sterile BHI broth and grown under the same conditions as previously reported.

2.2. Plant Material. Fresh leaves of *C. leprosum* were collected in June 2009 in Salgado dos Machados district, located 15 km from the city of Sobral, Ceará, Brazil. The plant authentication was performed by Professor Elnatan Bezerra de Souza, a plant taxonomist from Acaraú Valley State University (Sobral, Brazil), and a voucher specimen (N° 4573) has been deposited at the herbarium Francisco José de Abreu Matos (Sobral, Brazil).

2.3. Sample Preparation. Fresh leaves (2.5 kg) of *C. leprosum* were powdered and extracted at room temperature with an EtOH/H_2O solution (8 : 2 v/v). After 15 days, the resulting material was subjected to a simple filtration, followed by evaporation of the solvents under reduced pressure. An aliquot of the obtained solution was then lyophilized, resulting in a crude ethanol extract (EECL). The EECL was suspended in MeOH/H_2O 3 : 1 and partitioned with CH_2Cl_2, EtOAc, and *n*-BuOH. The CH_2Cl_2 extract was evaporated under reduced pressure, yielding a residue (15.5 g) that was fractioned over a Si gel and eluted with hexane/EtOAc (8 : 2, 6 : 4, 2 : 8), EtOAc, and MeOH. The hexane/EtOAc (6 : 4) fraction (4.19 g) was chromatographed over a Si gel and eluted with hexane/EtOAc (in a gradient of 9.5 : 0.5 to 0.5 : 9.5), EtOAc, and MeOH, resulting in 130 fractions which, after thin layer chromatography (TLC), were combined into 10 fractions (F_1–F_{10}). The F7 fraction (528 mg, resulting from elution with hexane/AcOEt 6 : 4) was rechromatographed using the same solvent system to purify CLF1 (54 mg).

2.4. Structural Analysis by Infrared Spectroscopy and Nuclear Magnetic Resonance. IR spectra were recorded using a Perkin-Elmer 1000 spectrophotometer. ^1H and ^{13}C NMR were recorded on a Bruker Avance DPX-500 (500 MHz for ^1H and 125 MHz for ^{13}C); chemical shifts are given in ppm (δ_C and δ_H) relative to $CDCl_3$ (7.27) and (77.23).

2.5. Sample Preparation for Antimicrobial and Toxicity Assays. CLF1 was first solubilized in 99.9% dimethyl sulfoxide (DMSO), and then the concentration was adjusted to 62.5 μg/mL in sterile distilled water to a final concentration of 8% DMSO and serially diluted to obtain concentrations ranging from 31.25 to 3.9 μg/mL. The ethanolic extract of *C. leprosum* (EECL) was equally evaluated regarding its antimicrobial potential. Briefly, EECL was first solubilized in 99.9% DMSO, and then the concentration was adjusted to 500 μg/mL in sterile distilled water to a final concentration of 5% DMSO and submitted to twofold serial dilutions to obtain concentrations ranging from 250 to 31.25 μg/mL. Chlorhexidine at 125 and 31.25 μg/mL and distilled water with 8% DMSO were used as positive and negative controls, respectively.

2.6. Antibacterial Activity. The antibacterial effects of CLF1 and EECL were evaluated through a microdilution test in 96-well "round-bottom" polystyrene plates. First, the wells were filled with 100 μL of the bacterial suspension at 2×10^7 CFU/mL. Then, 100 μL of CLF1 or EECL was added at different concentrations as previously described, obtaining a final volume of 200 μL per well. The plates were incubated for 24 hours at 37°C with or without 10% CO_2 for Gram positive and Gram negative, respectively.

The minimum inhibitory concentration (MIC) for each microorganism was determined to be the lowest CLF1 or EECL concentration that showed a complete inhibition of visible bacterial growth. To determine the minimum bactericidal concentration (MBC), 10 μL from each well, in which no visible growth was detected, was inoculated in Petri dishes containing fresh BHI agar and incubated at 37°C for 24 hours. MBC was considered to be the lowest concentration of compounds able to completely inhibit microbial growth on the plates.

2.7. Antibiofilm Activity

2.7.1. Biomass Quantification. Sterile 96-well "flat-bottom" plates were prepared using a procedure similar to that used in the antimicrobial activity tests, with the same concentration of cells. All plates were incubated on a horizontal shaker (120 rpm/min) at 37°C for 24 hours for biofilm development in the presence of CLF1 or EECL. Then, the supernatant of each well was removed and biofilms washed three times with 200 μL/well sterile water to remove cells weakly adhered. The attached biofilm mass was quantified using crystal violet (CV) staining [12]. Briefly, the plates containing biofilms were air dried for 30 minutes, and 200 μL of 99% methanol was transferred to each well and incubated for 15 minutes to fix the adhered cells. Then, the methanol was removed followed by addition of 200 μL/well of 0.1% CV (Gram-staining set for microscopy; Merck) and the plates were incubated for 5 minutes. After the staining step, the washing process was repeated with sterile water, and the plates were left to dry at room temperature. In order to solubilize the dye bounded to the biomass, 200 μL of 33% (v/v) acetic acid (Merck) was added to each well and the plates were agitated for 15 minutes on a horizontal shaker. The CV solutions were transferred to a new 96-well plate and the optical density of the content was measured using a plate reader (BioTrak II-Amersham Biosciences) at 590 nm.

2.7.2. Biofilm Cell Enumeration. In order to determine the effect of CLF1 on the viability of biofilm-entrapped cells, biofilms' suspension was prepared according the same conditions as previously described. After 24 hours of incubation at 37°C, the supernatant was discarded and the plates were washed with sterile water to remove the weakly adhered cells. Then, 200 μL of sterile water was added to each well and the plates were subjected to ultrasonic bath for 15 minutes to remove the biofilm-entrapped cells. The volume corresponding to five wells of each condition was then collected and pooled in a sterile 1.5 mL tube. The suspensions were serially diluted, plated on BHI agar, and incubated at 37°C for 24 hours. The number of colony-forming units (CFU) was determined and expressed as CFU/mL.

2.8. Acute Toxicity on Artemia sp.

2.8.1. Nauplii Harvesting. To obtain viable nauplii, 30 mg of cysts was weighed and hydrated for 1 hour in distilled water under constant aeration. After hydration, the cysts were treated with 50% sodium hypochlorite under constant agitation, until the color of the cysts changed from brown to orange. In addition to acting in the corium layer by facilitating the nauplii outbreak, sodium hypochlorite also promotes cleansing of the cysts. When the cysts reached the orange color, they were immediately washed with distilled water to remove the sodium hypochlorite. The cysts were then transferred to a conical bottom tube containing filtered seawater under constant aeration and after 48 hours, the nauplii were ready to use in toxicity tests [13].

2.8.2. Toxicity Test. CLF1 was solubilized in seawater plus DMSO 8% to obtain a stock solution of 125 μg/mL. All tests were performed using 5 different concentrations of CLF1 (125, 62.5, 31.25, 15.6, and 7.8 μg/mL). A group with seawater with 8% DMSO was used to verify whether DMSO was toxic to the nauplii. Seawater alone was used as the negative control. All tests were carried out in triplicate and repeated three times independently. The assay was performed in 24-well polystyrene plates with 10 nauplii per well and the mortality was determined after 24 and 48 hours by analysis of the number of nauplii devoid of motility. To determine the LC_{50}, the data were calculated and analyzed using the TSK (Trimmed Spearman-Karber) statistical program. Up to 10% mortality was considered acceptable in the negative control [13].

2.9. Statistical Analysis. Statistical analysis was performed by GraphPad Prism version 3.00 for Microsoft Windows. The method used was one-way ANOVA with Bonferroni *post hoc* test. The data were recorded in triplicate from at least three separate experiments and graphs are presented as mean ± standard deviation. The data were considered significant when $P < 0.001$.

3. Results

3.1. Purification and Structural Analysis of CLF1. CLF1 was purified as a white solid. Its infrared spectrum (IR) showed a broadband that indicated the presence of hydroxyl groups (3463 cm^{-1}), as well as absorptions at 1643 and 883 cm^{-1} corresponding to a 1,1-disubstituted double bond moiety. This moiety was confirmed by chemical shifts of ^{13}C [δ_C 150.1 (C-20) and 110 (C-29)] and ^{1}H [δ_H 4.75 (br s, H-29a) and δ_H 4.64 (br s, H-29b) NMR. The presence of three hydroxyl groups were supported by a chemical shift at δ_C 76.9, 69.0, and 79.3, which, in the HSQC spectrum, showed correlations with the hydrogens at δ 4.52 (br s; H-16), 3.58 (dd, $J = 11.2$ and 4.4; H-6), and 3.12 (t, $J = 6.7$; H-3, resp.). Comparison of the chemical shifts with the literature data [14] revealed that CLF1 is the triterpene of the lupane skeleton named 3β,6β,16β-trihydroxylup-20(29)-ene, which was previously isolated by Facundo and coworkers [15] (Figure 1).

3.2. MIC and MBC. Both EECL and CLF1 inhibited the growth of *Streptococcus mutans* and *S. mitis*. However, CLF1 presented lower MIC and MBC values when compared to EECL (Table 1). On the other hand, the same effect was not seen on Gram negative *Pseudomonas aeruginosa* and *Klebsiella oxytoca* (data not shown).

When compared to EECL, CLF1 inhibited the growth of *Streptococcus mutans* more than 16-fold (Table 1).

3.3. Antibiofilm Activity. Regarding antibiofilm activity, the results showed that CLF1 was effective only on biofilms of Gram positive strains. At a concentration of 7.8 μg/mL, CLF1 inhibited the biomass of *S. mutans* and *S. mitis* by 97% and 90%, respectively. For EECL at 125 μg/mL the inhibition was approximately 97.3% for *S. mutans* and 44% for *S. mitis*.

Table 1: Values of MIC, MBC, and biomass inhibition of *Streptococcus mutans* and *S. mitis* treated with ethanolic extract (EECL) and 3β,6β,16β-trihydroxylup-20(29)-ene (CLF1) from *Combretum leprosum*.

Microorganism	MIC (μg/mL)		MBC (μg/mL)		Biomass inhibition (%)	
	EECL	CLF1	EECL	CLF1	EECL	CLF1
Streptococcus mutans	125	7.8	250	15.6	97.3	97
Streptococcus mitis	62.5	7.8	125	15.6	44	90

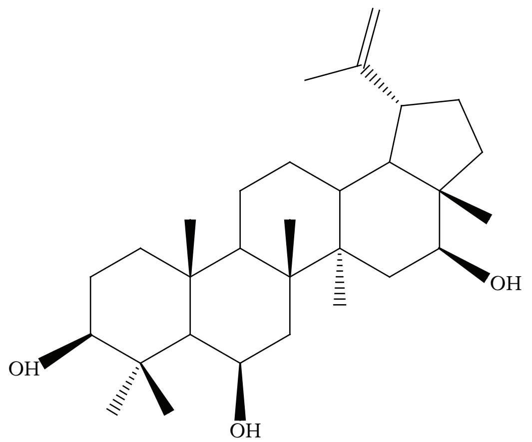

Figure 1: Structure of 3β,6β,16β-trihydroxylup-20(29)-ene extracted from *Combretum leprosum*.

According to these findings, the CLF1 efficiency at 7.8 μg/mL is quite similar to that of chlorhexidine at 31.25 μg/mL for both bacteria (Figure 2).

In order to evaluate the effect of CLF1 on biofilm-entrapped cells, *S. mutans* and *S. mitis* biofilms were grown in the presence of CLF1 at concentrations ranging from 7.8 to 0.95 μg/mL. The results showed that CLF1 at 7.8 μg/mL significantly decreased the viability of bacterial cells (Figure 3).

3.4. Toxicity Analysis. Analysis of acute toxicity with *Artemia* nauplii was carried out using 5 different concentrations of CLF1. In order to determine the LC_{50}, the number of *nauplii* deprived of motility was evaluated after 24 hours of coincubation. The results showed that CLF1 presented a LC_{50} of 98.19 μg/mL, which is significantly higher than the concentration that kills bacteria in antimicrobial assays.

4. Discussion

The search for new molecules with remarkable antimicrobial activities has become an important area of study. Moreover, natural products are a rich source for discovery of new antimicrobial agents [16]. According to Newman and Cragg [17], studies involving medicinal plants with antimicrobial activities have great potential mainly due to the necessity of plants to produce natural products that protect them against infections.

Pathogenic microorganisms that form biofilms are the focus of intense research due to their involvement in a large number of chronic infections and their role in the colonization of medical instruments, such as cardiovascular devices [18]. These findings indicate an increasing need for more effective antimicrobial agents with lower costs [19].

Studies about the phytochemical and biological characteristics of *C. leprosum* can be readily found in the literature. Among them, a study by Facundo and coworkers in 1993 established the structures of three triterpenoids belonging to the lupane series [3β,6β,16β-trihydroxylup-20(29)-ene], oleanane (arjunolic acid) and cycloartenol (mollic acid), and two flavonoids derived from the quercetin (3-*O*-methylquercetin and 3-*O*-α-L-rhamnopyranosylquercetin) [15].

Triterpenes belong to class of terpenoids, which are chemically characterized by the presence of six isoprene units with a total of 30 carbon atoms. Such molecules are responsible for different biological activities found in plant extracts and essential oils [20].

The bioactive metabolite 3β,6β,16β-trihydroxylup-20(29)-ene (CLF1) was isolated from the dichloromethane fraction of ethanolic extract of *C. leprosum* leaves (EECL). This compound appears as a white solid with an average yield of 0.02%. As previously reported by Facundo and coworkers [15], this molecule was first isolated from the hexane and ethanolic extracts of leaves and roots of *C. leprosum* and identified as a triterpene of the lupane class, with a double bond between carbons 20 and 29.

Recently, Horinouchi and colleagues [21] provided important evidences about the antiproliferative and anti-inflammatory role of the ethanolic extract of flowers from *C. leprosum*. According to the authors, the extract must be considered as a new potential tool for the treatment of several skin inflammatory diseases, since it reversed the skin inflammatory and hyperproliferative process in a very significant manner.

In the present work, the antimicrobial activity of CLF1 was assessed on Gram positive bacteria *Streptococcus mutans* and *S. mitis*. MIC and MBC values for both bacteria are 7.8 and 15.6 μg/mL, respectively. No effects were seen on the growth of *Pseudomonas aeruginosa* and *Klebsiella oxytoca*, both Gram negative bacteria. Such MIC value is lower than that found by a casbane diterpene isolated from barks of *Croton nepetaefolius*, which wasevaluated on oral streptococci, including *S. mutans* and *S. mitis* [22]. Furthermore, several triterpenes isolated from different species from the genus *Miconia* presented antibacterial activity against oral streptococci with MIC ranging from 30 to 200 μg/mL [23].

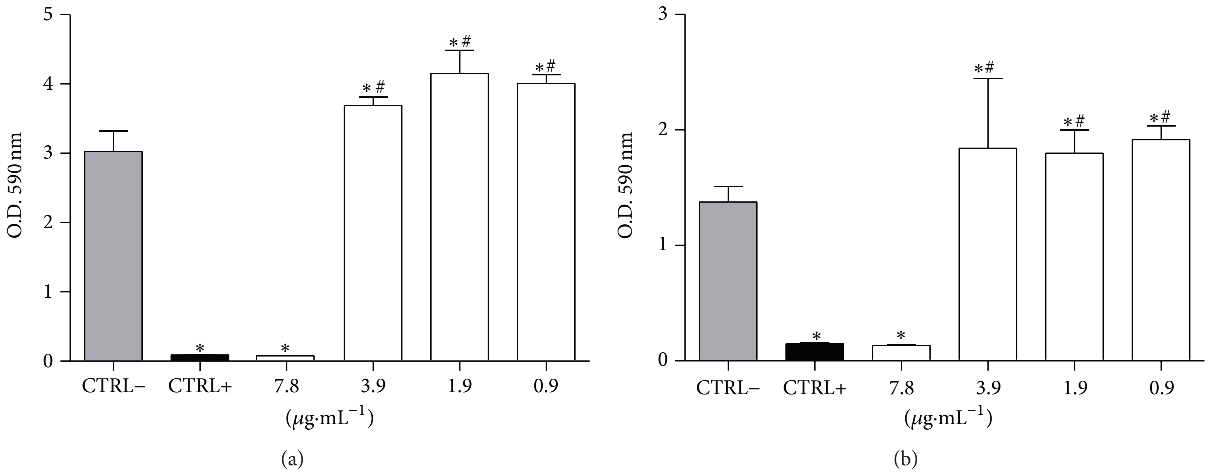

Figure 2: Effects of CLF1 on (a) *Streptococcus mutans* and (b) *S. mitis* biofilm mass. Biofilms were grown for 24 hours in the presence of CLF1 at different concentrations. The negative control was performed with 8% DMSO and the positive control with chlorhexidine at 31.25 μg/mL, and both are diluted in ultrapure water. Error bars represent standard deviation and statistical P value (represented by $*$ or #) indicates concentrations that are significantly different from negative and positive controls, respectively. $^{*}P < 0.001$; $^{\#}P < 0.001$.

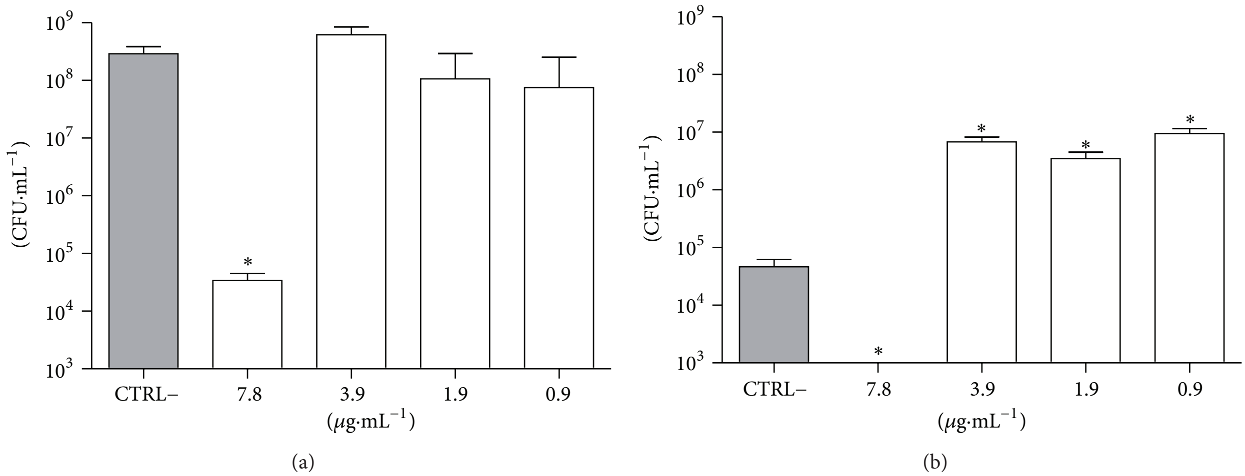

Figure 3: Effect of CLF1 on viability of biofilm-entrapped cells from (a) *Streptococcus mutans* and (b) *S. mitis*. Biofilms were grown for 24 hours in the presence of CLF1 at different concentrations. The negative control was performed with 8% DMSO and the positive control with chlorhexidine at 31.25 μg/mL, and both are diluted in ultrapure water. Error bars represent standard deviation and statistical P value (represented by $*$ or #) indicates concentrations that are significantly different from negative and positive controls, respectively. $^{*}P < 0.001$; $^{\#}P < 0.001$.

S. mutans is a Gram positive bacterium highly acidogenic and aciduric, and several clinical and laboratory studies point to the involvement of this species as the primary pathogen in human dental caries [24]. Moreover, according to Gross and coworkers [25], *S. mutans* was the most abundant species observed in samples from children with caries.

Some studies have shown that Gram positive bacteria are more susceptible to the action of plant extracts that contain flavonoids and triterpenes [26]. Regassa and Araya [27] tested different species of the genus *Combretum* and elucidated the antimicrobial potential of *Combretum molle* ethanolic extract against *Staphylococcus aureus* and *S. agalactiae*, both Gram positive bacteria.

The effect of CLF1 on biofilm development was similar to that found on planktonic growth. CLF1 at 7.8 μg/mL inhibited biofilms' mass by 97% and 90% for *S. mutans* and *S. mitis*, respectively, and reduced the number of biofilm-entrapped cells. The effect of CLF1 on the inhibition of biofilm formation is likely associated with a decrease in cellular viability since the concentrations inhibiting the growth of biofilms and planktonic cells are similar. Sá and coworkers [22] found a diterpene with inhibitory properties on *Streptococcus mutans* biofilm-entrapped cells. Such diterpene inhibited *S. mutans* biofilms in 94.28%. In addition, Raja and coworkers [28] showed that a pentacyclic triterpenoid (acetyl-11-keto-b-boswellic acid) inhibited the biofilm formation and reduced preformed biofilms from *S. mutans* and *Actinomyces viscosus* at concentrations ranging from 16 to 128 μg/mL. Nevertheless, other chemical structures based on terpenoids have been described in the literature as molecules possessing antimicrobial activities [29–32].

Studies from 1987 to 2004 involving secondary metabolites suggest that the antibacterial activity exerted by these compounds may be associated with mechanisms such as damage to the plasma membrane (perforation and/or reduction in membrane fluidity) [33], inhibition of nucleic acid synthesis (inhibition of topoisomerases), and inhibition of the energetic metabolism (caused by inhibition of reductase and NADH cytochrome-c) [34].

Katerere and coworkers [35] reported that some pentacyclic triterpenes isolated from *Combretum* and *Terminalia* genera proved to be ineffective against Gram negative bacteria. In such study, CLF1 showed no significant effects on the growth of *Escherichia coli*.

Thus, based on the mechanisms of action of the compounds cited above, and considering the results of our study, the antimicrobial activity of CLF1 may be related to its hydrophobicity. This feature probably allows a nonspecific interaction with cell membrane phospholipids of Gram positive bacteria. The presence of an outer membrane composed by phospholipids, lipoproteins, and, particularly, lipopolysaccharides (LPS) in Gram negative probably blocks the interaction of the molecule with the plasma membrane [36, 37].

Finally, a limiting factor in the prospection of new molecules with antimicrobial potential is the toxicity exhibited by several compounds. In the present study, the LC_{50} of CLF1 on *Artemia* nauplii is higher than its MIC. Thus, the results are very promising and CLF1 must be considered as a new antimicrobial agent against infections by *S. mutans* and *S. mitis*, including their associated biofilms. On the other hand, CLF1 is not indicated for infections caused by Gram negative bacteria, since only a small interference was seen on *P. aeruginosa* growth.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors contributed and approved this paper.

Acknowledgments

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Article

Characterization of Isoforms of the Lectin Isolated from the Red Algae *Bryothamnion seaforthii* and Its Pro-Healing Effect

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Abstract: Lectins are a structurally heterogeneous group of proteins that have specific binding sites for carbohydrates and glycoconjugates. Because of their biotechnological potential, lectins are widely used in biomedical research. The present study aimed to evaluate the healing potential of the lectin isolated from the marine red alga *Bryothamnion seaforthii* (BSL). The lectin was purified using ion exchange chromatography with DEAE cellulose and characterized using tandem mass spectrometry. For healing tests, skin wounds were induced in the dorsal thoracic region of mice. These animals were randomly divided into three groups and subjected to topical treatment for 12 days with BSL, bovine serum albumin and 150 mM NaCl. To evaluate the potential of each treatment, the animals were anesthetized and sacrificed on days 2, 7 and 12, respectively. The parameters evaluated included the wound area, the proportion of wound closure and the histological diagnosis. The wound closure was more effective with BSL (Postoperative Day 7 and 12) than controls. The luminal epithelium was completely restructured; the presence of collagen in the dermis and the strongly active presence of young skin annexes demonstrate the potential of treatment with BSL compared with controls. Our findings suggest that BSL has pro-healing properties and can be a potential medical process in the treatment of acute wounds.

Keywords: lectins; algal proteins; wound healing

1. Introduction

Cutaneous wounds are the result of a disruption of skin integrity and represent a major problem in public health and the integration of resources in many countries [1,2]. The healing process is a complex arrangement, but well-ordered phases overlap in which highly specialized cells interact with the extracellular matrix to result in growth and tissue repair [3,4].

The holistic understanding of the processes involved in wound healing has expanded our knowledge of cicatrization, which defines the set of directions for therapeutic decisions in nature and the development of products that provide solutions to the problem [5].

A delayed healing process is considered to be a complex therapeutic problem in modern medicine, especially in light of the knowledge that patients with diabetes and other physiological disorders still require differentiated approaches [1,6]. In this context, the pharmaceutical industry has appealed increasingly to new sources of drugs of natural origin [7].

Lectins are proteins that have the ability to bind to carbohydrates and glycoconjugates without altering their structure [8]. These biomolecules serve as a promising alternative in the treatment of skin wounds due to their potential for promoting healing [9–12]. To understand the different biological activities related to lectins it is necessary to have knowledge about characterization and structure studies. However, in marked contrast to the lectins from higher land plants, marine algal lectins have been isolated and characterized at a much slower pace. Moreover, to date, biochemical and structural information regarding algal lectins is scarce; hence, the functional and phylogenetic classification of these lectins remains obscure [13]. Moreover, lectins from marine algae appear to have enormous potential for use in biochemistry and biomedical sciences. Despite the small number of published studies, these proteins are known to exert several biological activities, including the induction of neutrophil migration *in vitro* and *in vivo* and anti-nociceptive and anti-inflammatory effects [14–17].

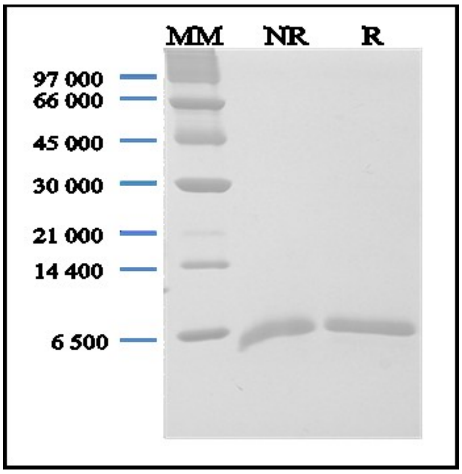
Thus, the aim of the present work was to characterize the lectin isoforms from the marine red alga *Bryothamnion seaforthii* using mass spectrometry and to investigate the healing potential of topical administration of the lectin on surgically induced skin wounds in a murine model.

2. Results and Discussion

2.1. Amino Acid Sequence of BSL Isoforms

The pure lectin active fraction was obtained by ion-exchange chromatography as described by Ainouz and co-worker. The hemagglutinating activity of the lectin was inhibited only by the glycoproteins fetuin, avidin and porcine mucin [18]. The purified BSL is observed in SDS-PAGE as a broad band with an apparent molecular mass of 9 kDa in the presence and absence of 2-mercaptoethanol (Figure 1).

Figure 1. SDS-PAGE of purified BSL. Lane MM: a marker kit including phosphorylase B (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsinogen (21 kDa), lactalbumin (14.4 kDa) and aprotinin (6.5 kDa). Lane NR: non-reduced BSL. Lane R: reduced BSL.



We speculate that the broad band pattern could be due to the presence of isomeric forms of BSL with different molecular mass. To confirm the isoforms, the purified BSL was investigated by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The mass spectrometric analysis revealed that the purified BSL contained a mixture of five isoforms, namely: BSL1, BSL2, BSL3, BSL4 and BSL5 at *m/z* 8898.1 ± 2, 8960.8 ± 2, 9012.8 ± 2, 9065.8 ± 2 and 9096.1 ± 2, respectively (Figure 2).

The existence of lectin isoforms was also observed in the lectins isolated from *Bryothamnion triquetrum* [19], *Eucheuma serra* [20] and *Hypnea japonica* [21]. The MALDI-TOF masses of intact BSL did not change upon incubation of the lectin with iodoacetamide under non-reducing conditions, indicating that BSL does not possess free sulfhydryl groups (data not shown). In contrast, a MALDI-TOF-MS analysis of denatured, reduced and carbamidomethylated BSL (CA-BSL) exhibited five major ions at *m/z* 9133.1 ± 2, 9194.1 ± 2, 9245.3 ± 2, 9296.5 ± 2 and 9328.7 ± 2, indicating the presence of four cysteine residues involved in two intrachain disulfide bonds (Figure 3).

Figure 2. MALDI-TOF-MS analysis of BSL under denaturing conditions. The BSL isoforms exhibit major ion peaks at *m/z* 9065.8 ± 2 and 8960.8 ± 2 and three ion peaks with minor intensity at *m/z* 8899.1 ± 2, 9012.8 ± 2 and 9096.1 ± 2.

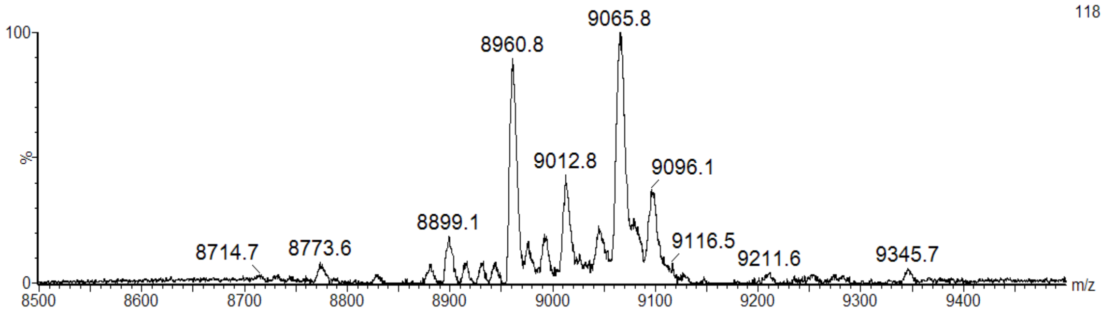
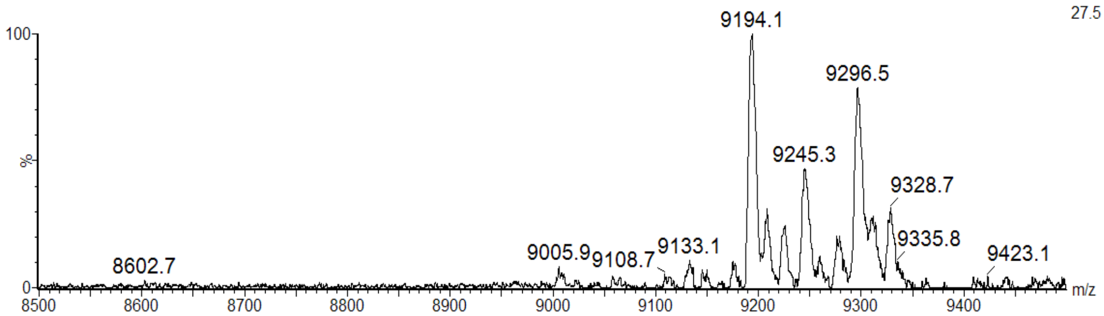


Figure 3. MALDI-TOF-MS analysis of CA-BSL under denaturing and reducing conditions in the presence of iodoacetamide. The BSL samples treated with dithiothreitol (DTT) and iodoacetamide exhibited increased masses at 232 Daltons (4 × 58.00), suggesting the presence of four cysteine residues engaged in the formation of two intrachain disulfide bonds.



These data were confirmed by an analysis of reduced and ethylpyridylated BSL in which the mass increment was 436 Daltons to each native BSL isoform (data not shown). Similar results were obtained for the *Bryothamnion triquetrum* lectin (BTL) and the *Hypnea japonica* lectin (HJA) [19,21]. HML and HCA, lectins isolated from the marine red alga *Hypnea musciformis* and *Hypnea cervicornis*, respectively, have masses of 9 kDa and contain 14 half-cystines each in their amino acid sequences. Furthermore, they are composed of two similar polypeptide chains linked by S–S bonds [13]. The presence of cysteinyl residues in BSL lectins may contribute to their extreme thermostability [22].

The complete amino acid sequences of BSL2, BSL3 and BSL4 were determined using tandem mass spectrometry (MS/MS) by overlapping the sequences of the peptides generated by digestion of CA-BSL with trypsin and chymotrypsin (Figure 4, Table 1). Sequence heterogeneity was observed at positions 8 (S/P), 23 (T/P), 27 (V/P), 31 (D/S), 47 (S/H), 49 (P/L) and 57 (A/V).

The isolectins from the marine red alga *Bryothamnion seaforthii* (BSL) are polypeptides composed of 91 amino acid residues including four half-cystines. Methionine and glutamine were not detected in their sequences. The molecules contained a remarkably high content of Ser (13, 12 and 11 in BSL2, BSL3 and BSL4, respectively), Gly (18 in all) and Val (10 in all), indicating that these three amino acids constitute approximately 44% of the total amino acid residues. The isotope-averaged molecular masses calculated for the BSL2, BSL3 and BSL4 isoforms were 8960.28, 9010.309 and 9064.32, respectively, which is in excellent agreement with the experimentally determined masses. Four half-cystines were found in BSL, and no free sulfhydryl groups were identified (Figure 3). The ion at *m/z* 2289.9487 from the pepsin digestion of BSL corresponds to the peptides ^{1}ADPVCGSPSGY^{11} and ^{53}WVGKASEGGCANF^{65} linked by a disulfide bond between cysteines 5 and 62. The reduction and alkylation of this peptide results in the dissociation of the disulfide bond and the formation of two ions at *m/z* 1430.5880 and 1070.4320 that correspond to the ethylpyridylated peptides WVGKASEGGCANF and ADPVCGSPSGY, respectively. These data suggest that two intrachain disulfide bonds, Cys^{5}–Cys^{62} and Cys^{12}–Cys^{90}, are present in BSL.

Figure 4. Complete amino acid sequence of BSL 2, BSL 3 and BSL 4. The tryptic and chymotryptic peptides are represented by T and Q, respectively. The underlined residues represent micro heterogeneity of the isoform sequences.

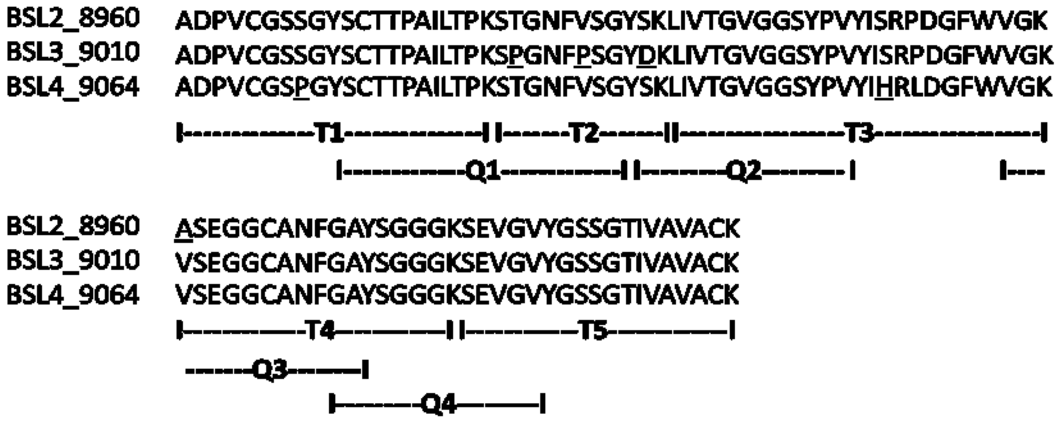


Table 1. Sequenced peptides and their respective molecular masses.

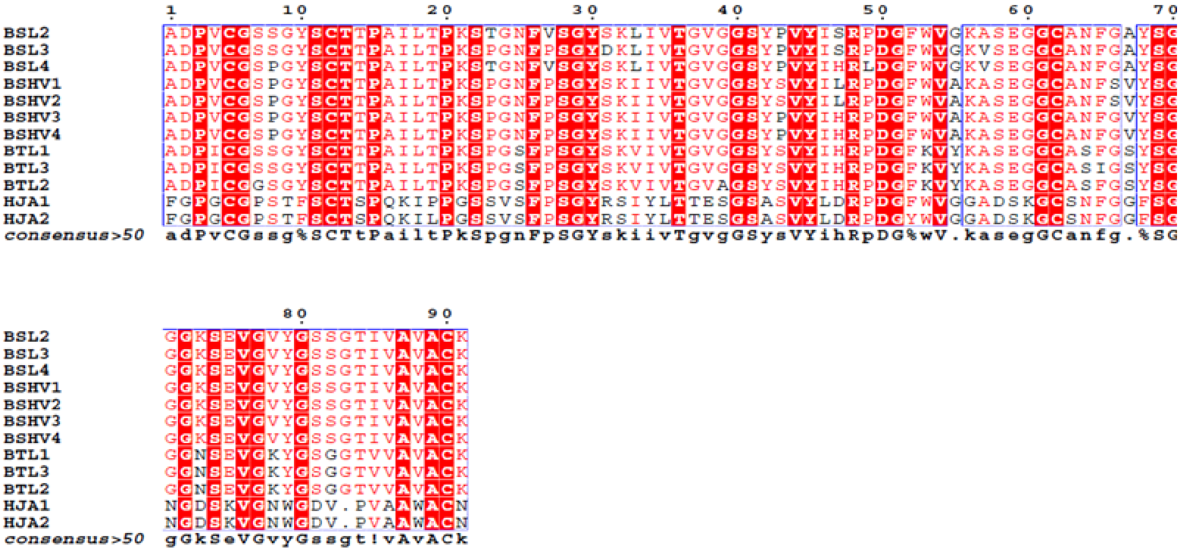
Peptide	Sequence **	Calculated mass	Experimental mass	Δ Mass (Da)
T1.1 *	ADPVCGSPGYSCTTPAILTPK	2192.99	2192.90	0.09
T1.2 *	ADPVCGSSGYSCTTPAILTPK	2182.88	2182.88	0.00
T2.1	SPGNFPSGYDK	1167.52	1167.43	0.09
T2.2	STGNFVSGYSK	1145.53	1145.50	0.03
T3.1	LIVTGVGGSYPVYISRPDGFWVGK	2566.36	2566.30	0.06
T3.2	LIVTGVGGSYPVYIHRLDGFWVGK	2632.41	2632.19	0.22
T4.1 *	ASEGGCANFGAYSGGGK	1589.68	1589.64	0.04
T4.2 *	VSEGGCANFGAYSGGGK	1617.64	1617.67	−0.03
T5 *	SEVGVYGSSGTIVAVACK	1783.82	1783.87	−0.05
Q1 *	SCTTPAILTP KSPGNFPSGY	2094. 99	2094. 93	0.06
Q2	SKIIVTGVGGSYPVY	1538.83	1538.74	0.09
Q3 *	VGKASEGGCANFGAY	1487.64	1487.60	0.04
Q4	GAYSGGGKSEVGVY	1329.62	1329.55	0.07

* Peptides with carbamidomethylated cysteine; ** All peptides were sequenced using tandem mass spectrometry. The CID spectra were analyzed manually.

The alignment of the BSL sequence against the BLAST database reveals 48% identity and 64% similarity between BSL and HJA, a lectin isolated from marine red alga *Hypnea japonica*. Compared with the *B. triquetrum* lectin (BTL), BSL presents only seven exclusive positions, resulting in

83% identity. Relative to BSH, a hemagglutinin isolated from *B. seaforthii* collected on the Venezuela coast [22], BSL diverges in five exclusive positions beyond the heterogeneities among their own isoforms: 43 (P/S), 47 (S/L), 55 (G/A), 66 (G/S) and 67 (A/V) (Figure 5).

Figure 5. Alignment of the family of lectins similar to BSL. BSL2 to BSL4 are isolectins from *Bryothamnion seaforthii*, BSHV1 to BSHV4 are isolectins from *B. seaforthii* from the Venezuela coast, BTL1 to BTL3 are isolectins from *B. triquetrum*, and HJA1 to HJA2 are isolectins from *Hypnea japonica*.



In recent years, a large number of lectins have been isolated from marine red alga [23–25]. These lectins generally have a low molecular weight, an isoelectric point in the range of pH 4–6, no affinity for simple sugars, no requirement for divalent cations for their biological activities and, in some cases, the presence of repeated domains [24]. Although some of these proteins exhibit potential biological activities [26–30], only a few have actually been studied at the structural level. BTL was the first marine algal lectin to have its primary structure determined [19].

In the same year, Hori *et al.* [21] reported the primary structure of HJA, which shares sequence similarity with BTL and constitutes the first marine red alga lectin family. Based on the identity between HML and HCA and the differences in the amino acid sequences compared with BTL/HJA, Nagano *et al.* [13] suggested that HCA/HML constitute a new lectin family. Conversely, the monomeric lectins isolated from *Eucheuma serra*, *E. amakusaensis*, and *E. cottonii* [20] have masses of approximately 28 kDa, exist as monomers, and share *N*-terminal sequence similarity with the complete amino acid sequence of isolectin 2 from *Eucheuma serra* (ESA-2) [31].

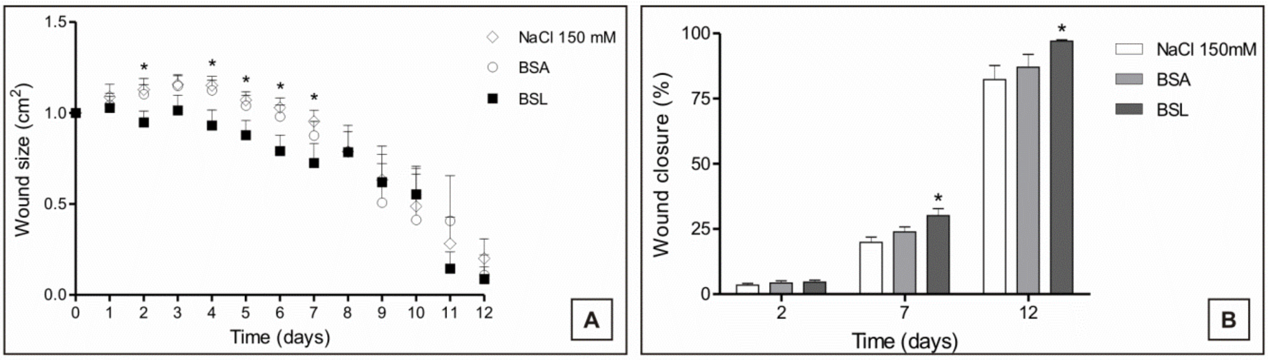
Additionally, the primary structure of ESA-2 contains repeated domains. These data suggest that lectins from the genus Eucheuma can be grouped in a third family of marine red alga lectins. In the present study, five isoforms of BSL, namely BSL1, BSL2, BSL3, BSL4 and BSL5, were identified, and the amino acid sequence of three (BSL2, BSL3, and BSL4) were determined from an MS/MS analysis of tryptic and chymotryptic peptides. BSL exhibited four cysteines in its primary structure that are involved in two intrachain disulfide bonds: Cys^{5}–Cys^{12} and Cys^{62}–Cys^{90}. However, in BTL, one of these cysteines can interact with a cysteine present in another polypeptide chain to eventually form a dimer [19]. In HJA, the disulfide bonds are exclusively involved in intrachain bonds with the pattern Cys^{5}–Cys^{62} and Cys^{12}–Cys^{89} [21]. The data obtained with BSL suggest that the disulfide bonds are also exclusively intrachain with the same pattern observed in HJA. Although the pattern of disulfide bonds between HJA and BSL is different from that presented by BTL, given the similarity between these lectins and the presence of isoforms among other similar biochemical characteristics, the data strongly suggest that BSL is a novel member of the first marine red alga lectin family that includes the isoforms of HJA, BTL and BSH.

2.2. Evaluation of Healing Potential

It was observed that the treatment of wounds with the lectin isolated from the marine red alga *Bryothamnion seaforthii* (BSL) induced a greater inflammatory process, which is observed during clinical evaluation and by well-evidenced phlogistic signs, such as edema and hyperemia in the first days following treatment, when compared with controls. Such signs are indicative of a pro-inflammatory effect by the lectin tested (data not shown).

The measurement of the areas of the experimental lesions was performed daily to observe the evolution of healing in the experimental groups. During the inflammatory and proliferative phase, it could be observed that treatment with BSL induced a decrease in the areas of the lesions of the experimental animals in this group (Figure 6).

Figure 6. (**A**) Evolution of the areas; and (**B**) proportion of experimental closure of skin lesions in mice treated topically with 150 mM NaCl, bovine serum albumin (BSA) and *Bryothamnion seaforthii* lectin (BSL); * Significant in relation to the 150 mM NaCl group, $p < 0.05$.



The percentage of wound closure was evaluated from the exposed areas of each lesion according to the time. BSL treatment induced rapid and more effective healing compared with the controls (BSA and 150 mM NaCl) in which a higher proportion of closure in the animals treated with the lectin was observed on POD (postoperative day) 2, 7 and 12 (Figure 6). Thus, the greater effectiveness of treatment with BSL can be observed in the proliferative and remodeling phases of the healing process when compared to the controls.

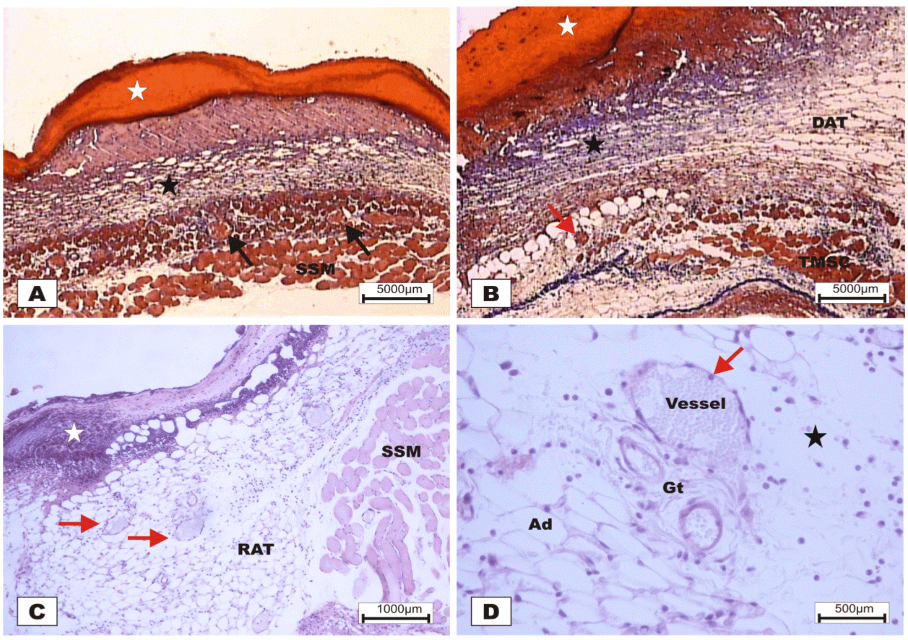
The BSL treatment induced an increased closing in the areas of the lesions in the group treated with BSL from POD 1 to 12, and this was possibly influenced by the contraction process of the wounds during healing.

2.3. Histopathological Assessment

For the histopathological evaluation of the injured tissue, samples were collected from each group on POD 2, 7 and 12 ($n = 4$ for all groups).

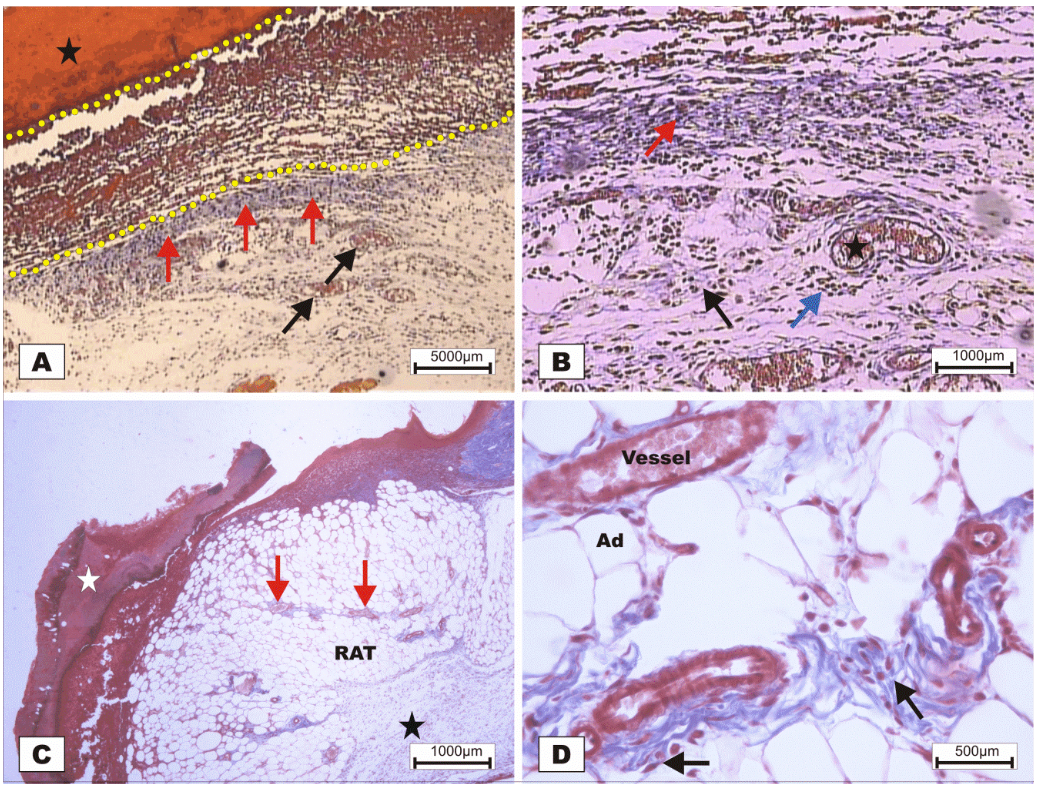
The study revealed that the wounds treated with BSA and BSL exhibited scabs sealing the epithelium opening on POD 2 (Figure 7). During the histopathological diagnosis, wounds treated with BSL showed intense inflammatory exudates in the reactional adipose tissue, with a mild inflammatory infiltrate in the reticular dermis in addition to the presence of granulation tissue in the reactional adipose tissue (Figure 7C,D). In wounds treated with BSA, there were intense inflammatory exudates, collagenolysis, and edema of the reticular dermis with the degenerative adipose tissue showing the beginning of a poor healing (Figure 7A,B).

Figure 7. Photomicrographs of surgically induced skin wounds in mice treated with a topical administration of BSA and BSL in POD 2 (Stain: hematoxylin-eosin and Masson's trichrome). (**A**) (BSA). Note the presence of a scab (white star) covering the wound and intense inflammatory exudate. Below the exudate, a collagenolysis area and edema of the reticular dermis are observed (black star). Immediately below the dermis is the subcutaneous muscle layer with congested vessels (arrows). A 4× objective was used; (**B**) (BSA). Note the presence of a thicker crust (white star) covering the wound bed and a less-intense inflammatory exudate. Below the exudate, a collagenolysis area, edema of the reticular dermis and intense inflammatory infiltrate are observed (black star) with degeneration fatty tissue (DAT). Note the muscle fibers spaced by muscle tissue and fat cells with congested vessels near the collagenolysis area (red arrow). A 4× objective was used; (**C**) (BSL). A thick scab covers the wound bed (white star). Note the congested vessels of the granulation tissue (arrow) in the reactional adipose tissue (RAT). A 10× objective was used; (**D**) (BSL). Detail from the previous photo showing vessels of the granulation tissue in areas of intense inflammatory exudate (star). Observe the possible diapedesis of the neutrophil PMNs into the inflamed area (red arrow). A 40× objective was used.



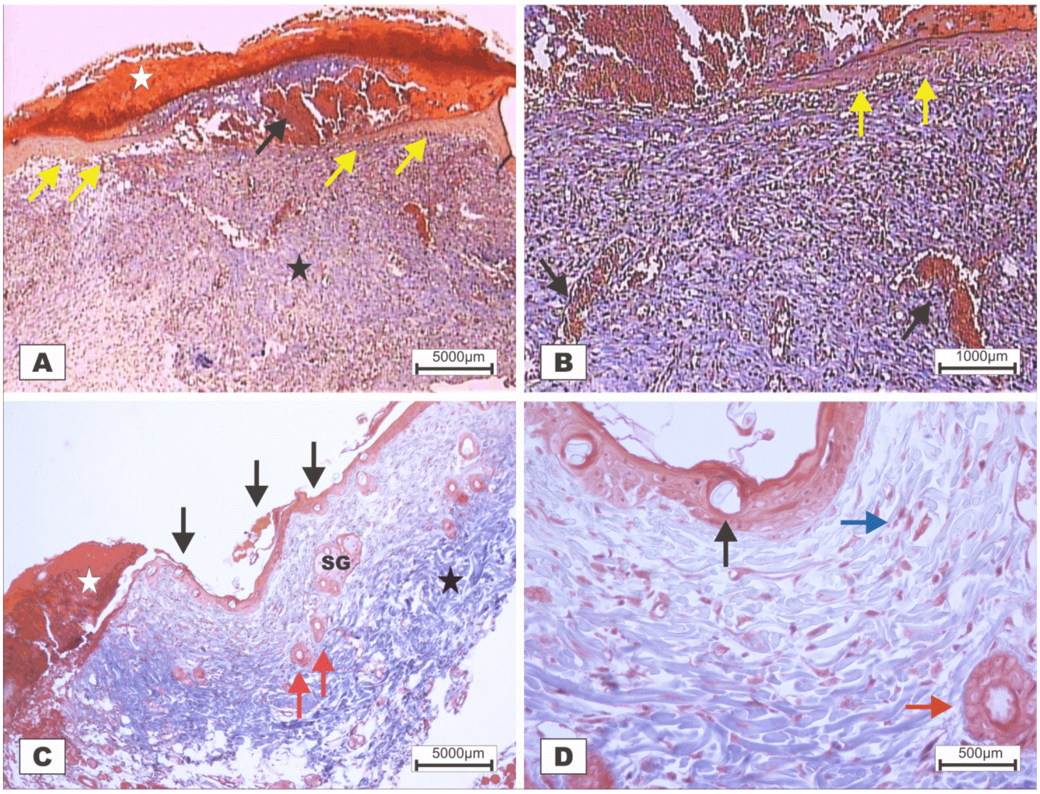
On POD 7, densification of collagen fibers in the reticular dermis, which is primarily surrounded by the vessels of the granulation tissue in adipose tissue with the presence of discrete reactional inflammatory exudates, was observed (Figure 8C,D). In the group treated with BSA (Figure 8A,B), intense inflammatory exudates and early fibroblastic proliferation with moderate collagen synthesis were still observed, demonstrating a less evolved healing process when compared with the wounds treated with BSL.

Figure 8. Photomicrographs of surgically induced skin wounds in mice treated with a topical administration of BSA and BSL in POD 7 (Stain: Masson's trichrome). (**A**) (BSA). Note the presence of a scab (star) covering the wound bed and intense inflammatory exudate (delimited by dashed yellow lines). Below the exudate, observe the area of granulation tissue with congested vessels (arrow) and intense fibroblastic proliferation. Immediately beneath the inflammatory exudate, note the bluish band resulting from the synthesis of collagen (red arrows). A 4× objective was used; (**B**) (BSA). Details of the granulation tissue. Note the fibroblastic proliferation (arrow) and mild inflammatory infiltrate around the vessels (blue arrow). Note the collagen above the range of the granulation tissue (red arrow). Vessels of the granulation tissue are observed (star). A 10× objective was used; (**C**) (BSL). Overview of the injured area. Note the presence of a crust sealing the epithelial opening (white star). Vessels of the granulation tissue in the reactional adipose tissue (RAT) surrounded by thickening collagen fibers (arrows) resulted from intense fibroblastic synthesis. An area with a mild inflammatory exudate is observed (black star). A 10× objective was used; (**D**) (BSL). Detail from the previous photo showing the area of granulation tissue vessels surrounded by active collagen. Note the active fibroblasts in the area (arrows). A 40× objective was used.



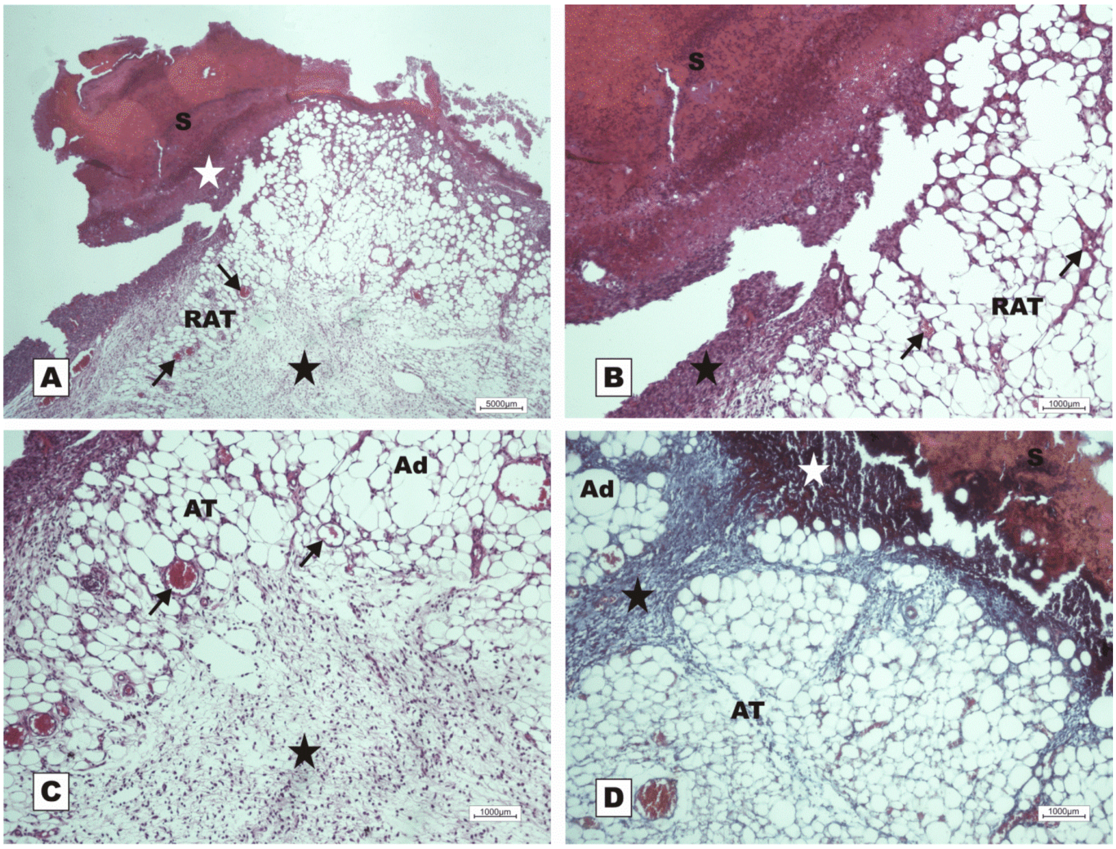
At POD 12, the BSL-treated animals experienced restructuring of the epithelium covering with little production of keratin, formation of cutaneous immature annexes, and active collagen in the region of the reticular dermis with presence of fibroblastic activity (Figure 9C,D). In the lesions treated with BSA over the same period, an epithelial scab sealing the epithelium opening, a mild inflammatory infiltrate beneath the crust and progression of the epithelial lining were observed (Figure 9A,B).

Figure 9. Photomicrographs of surgically induced skin wounds in mice treated with a topical administration of BSA and BSL in POD 12 (Stain: Masson's trichrome). (**A**) (BSA). Presence of a scab (white star) and an inflammatory exudate beneath the scab (arrow). Note the progression of the luminal epithelium (yellow arrows) below the crust and inflammatory exudate. Dermis with less collagen deposition and the presence of active granulation tissue (black star) in a regression process is observed. A 4× objective was used; (**B**) (BSA). Details of the previous photo. Observe the granulation tissue characterized by the presence of vessels (black arrows) and young collagen in the extracellular matrix with inflammatory infiltrate. Note the projection of the epithelium beneath the crust (yellow arrows). A 10× objective was used; (**C**) (BSL). Note also the newly formed epithelium coated with little production of keratin (black arrows). The presence of a fragment of crust on the edge of the injured area (white star), the formation of skin appendages as sebaceous glands (SG), and the remaining vessels of the granulation tissue can be observed (red arrows). Note the active collagen in the reticular dermis (black star). A 4× objective was used; (**D**) (BSL). Details of the previous photo showing the presence of cutaneous annexes in the area of the newly formed epithelium (black arrow), fibroblasts in the papillary dermis area (blue arrow) and characteristic neoangiogenesis vessels in the granulation tissue (red arrow). A 40× objective was used.



In the control group treated with 150 mM NaCl, there was a delay in wound healing in which, at POD 7, acute inflammation and granulation tissue at the beginning of proliferation were observed (Figure 10).

Figure 10. Photomicrographs of surgically induced skin wounds in mice treated with a topical administration of 150 mM NaCl in POD 7 (Stain: hematoxylin-eosin and Masson's trichrome). (**A**) (NaCl). Open wound with a thick scab and dystrophic calcification (white star) sealing the epithelial opening (S). Observe the beginning of granulation tissue formation originating from the reactional adipose tissue (RAT), and note the presence of congested vessels (arrows) and the area of inflammation (star). A 4× objective was used; (**B**) (NaCl). Details showing the congested vessels in fat tissue (arrows) and the inflammatory infiltrate beneath the crust (star). A 10× objective was used; (**C**) (NaCl). Details of the area of acute inflammation in the degenerative adipose tissue (star) and the granulation tissue vessels above the inflamed area (arrows). A 10× objective was used; (**D**) (NaCl). Observe the inflammatory exudate beneath the crust (white star). There is poor collagen deposition and poor formation of granulation tissue in the dermis (black star) between the areas of adipose tissue. A 10× objective was used.



Wound healing is characterized as a complex process that results in contraction and wound closure, as well as restoration of the epithelium as a functional coating [32]. Thus, the repair of skin wounds is accompanied by an ordered sequence of biological events starting with the closure of the wound and advancing to the remodeling of damaged tissue [33].

The use of natural products to improve and accelerate the healing process has been widely envisioned because delayed skin healing, such as a problem in tissue repair in individuals suffering from diabetes or leprosy, is a major economic implication in medicine. This economic factor is a result of large drug spending over a long period that patients who suffer from chronic wounds or ulcers spend in hospitals [1,6,34]. Thus, agents that decrease the time of the tissue repair process are desired to contribute to a faster and more effective healing process [35].

Lectins are a structurally heterogeneous group of proteins of non-immune origin that have the characteristic to bind carbohydrates reversibly with high selectivity [36]. Lectins are very versatile proteins and have many direct applications in biological and biomedical research [37].

The field of application of lectins in the treatment of wounds is unexplored. However, important studies have shown that these biomolecules have healing potential in the treatment of acute wounds [11,12].

The biological application of lectins from marine algae deserves special interest because these lectins are lower molecular weight proteins than plant lectins. Thus, it is expected that these marine algae lectins are much less antigenic than others [38]. Additionally, lectins from marine algae seem to express their biological activities in a stronger manner than lectins from other sources. Furthermore, they possess great molecular stability due to several disulfide bridges and show high specificity for complex carbohydrates and glycoconjugates, especially for mucins [13,21,39].

The lectin isolated from the marine red alga *Bryothamnion seaforthii* has been shown to have several biological activities, as described in the literature [14,27,40,41], and may be considered to be an important tool for biomedical applications.

Our findings demonstrate the potential pro-healing activity of the BSL, which was responsible for acceleration in the healing of skin wounds treated with this lectin. Thus, the histopathological findings showed that the lectin used in this study exerts its activity on the migration of polymorphonuclear cells to the injured site, confirming the data already published [11] that suggest that the lectin of *Artocarpus integrifolia* (KM+) induced a more effective healing of the corneal epithelium via a mechanism that also involves the migration of neutrophils.

It is possible that BSL is also able to act on the activation and proliferation of fibroblasts because most of the lectin-treated animals exhibited strongly active collagen in the dermis. However, future experiments are needed to better characterize the action of the BSL on fibroblasts.

3. Experimental Section

3.1. Algal Collection and Lectin Purification

Specimens of the marine red alga *B. seaforthii* were collected at the northeast coast of Brazil (Pacheco beach, Ceará). The material was cleansed from epiphytes and stored at −20 °C until it was used. The purification of BSL was performed according to the method of Ainouz *et al.* [18]. The frozen algae were thawed, rinsed with distilled water, ground to a fine powder under liquid nitrogen, extracted with three volumes of 20 mM sodium phosphate, pH 7.0, containing 150 mM NaCl for 4 h while stirring, filtered through nylon tissue and centrifuged at 7000× *g* for 30 min at 4 °C. The supernatant was acidified and maintained at 4 °C for 4 h. The precipitates were removed by centrifugation at 15000× *g* for 20 min at 4 °C, and the supernatant was adjusted to pH 7.0 and subjected to fractionated precipitation with 60% ammonium sulfate saturation. The precipitated proteins were pelleted by centrifugation, resuspended in a small volume of 20 mM phosphate buffer (PB) pH 7.0, and applied to a DEAE-cellulose column. The column was equilibrated and washed with PB, and this was followed by elution with 1 M NaCl in the same buffer. Fractions of 2 mL were manually collected at a flow rate of 1 $mL \cdot min^{-1}$ and analyzed using a spectrophotometer to measure the absorbance at 280 nm and tested for hemagglutinating activity. The unadsorbed fractions were rechromatographed on the same column, pooled, dialyzed against water and lyophilized until further use.

3.2. Protein Analytical Methods

The protein purity and homogeneity were achieved using SDS-PAGE [42]. The molecular mass was determined using MALDI-TOF mass spectrometry with a SYNAPT HDMS instrument operating at a 20 kV accelerating voltage in the reflector V-mode. Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) saturated in 50% acetonitrile and 0.1% trifluoracetic acid (TFA) was used as sample matrix.

3.3. Determination of Sulfhydryl Groups and Disulfide Bonds

For the quantization of free cysteine residues in BSL, the lectin was solubilized in 10 mM ammonium bicarbonate, incubated with either 5 mM iodoacetamide or 5 mM 4-vinylpyridine for 1 h at room temperature and subjected to MALDI-TOF-MS. The number of free cysteine residues (N_{SH}) was determined using the following equation:

$$N_{SH} = (M_{IA} - M_{NAT})/57.05 \text{ or } (M_{VP} - M_{NAT}/104.14)$$

M_{IA} is the mass of the non-reduced protein in the presence of iodoacetamide, M_{VP} is the mass of the non-reduced protein in the presence of 4-vinylpyridine, M_{NAT} is the mass of the native protein, 57.05 is the mass increment due to the carbamidomethylation of one thiol group and 104.14 is the mass increment due to the ethylpyridylation of one thiol group.

For the quantization of the disulfide bonds, BSL was incubated with 1 mM DTT followed by the addition of five-fold molar excess of 4-vinylpyridine or iodoacetamide as alkylating agents. The samples were incubated for 1 h at room temperature before being subjected to MALDI-TOF-MS analysis. The number of total cysteine residues (N_{Cys}) was derived using the following equation:

$$N_{Cys} = [(M_{CM} - M_{IA})/58.05] + N_{SH} \text{ or } N_{Cys} = [(M_{P} - M_{VP})/105.04] + N_{SH}$$

M_{CM} is the mass of the reduced and carbamidomethylated protein, and 58.05 is the mass increment due to the carbamidomethylation of a cysteine residue. MP is the mass of the reduced and ethylpyridylated protein, and 105.04 is the mass increment due to the ethylpyridylation of a thiol group, which was involved in the formation of a disulfide bond prior to reduction.

Lastly, the number of disulfide bonds ($N_{S–S}$) was calculated as follows:

$$N_{S–S} = (N_{Cys} - N_{SH})/2$$

All mass values are in Daltons.

To determine the positions of disulfide bonds, native BSL was digested with pepsin in 10 mM HCl containing 1.5 M guanidine hydrochloride at 37 °C for 18 h. The peptides produced were separated using HPLC with a Sephasil C18 column at flow rate of 1 $mL \cdot min^{-1}$. The 1 mL fractions were collected and analyzed using MALDI-TOF-MS. The peptides containing disulfide bonds were reduced and alkylated to confirm the position of the cysteines involved in the S–S bonds.

3.4. Enzymatic Digestion and Mass Spectrometry Analysis

The Coomassie stained protein bands of BSL were excised from the SDS-PAGE gel and subjected to tryptic and α-chymotryptic digestion overnight at 37 °C at an enzyme: substrate ratio of 1:50 (w/w). Briefly, the excised gel pieces were washed with 100 mM ammonium bicarbonate (NH_4HCO_3) and 50% acetonitrile until the gel pieces appeared colorless. The gel pieces were then dehydrated in 100% acetonitrile. Reduction of protein was performed in 10 mM DTT containing 50 mM ammonium bicarbonate at 56 °C for 45 min. Alkylation of protein was performed with 55 mM iodoacetamide in 50 mM ammonium bicarbonate at room temperature for 1 h. The gel pieces were washed three times with 100 mM ammonium bicarbonate in 50% acetonitrile, dehydrated in 100% acetonitrile and dried in a speed vac. A trypsin plus α-chymotrypsin solution was prepared in 50 mM NH_4HCO_3, and 20 µL of this solution was added to each tube and stored at 4 °C for absorption analysis. When needed, more NH_4HCO_3 was added until that the gel was completely immersed.

The digestion was stopped by the addition of 2% formic acid. The gel pieces were washed four times with 5% formic acid in 50% acetonitrile. The supernatants were then collected and transferred to fresh tubes, pooled, dried in a speed vac and resolubilized in 20 µL of 0.1% formic acid. Finally, 2 µL of the peptide solution was chromatographed on a C-18 (0.075 × 100 mm) nano column coupled to a nanoACQUITY system. The column was equilibrated with 0.1% formic acid and eluted with a 10% to 85% acetonitrile gradient containing 0.1% formic acid. The eluates were directly infused in the electrospray source of a mass spectrometer instrument (Synapt HDMS). The mass spectrometer operated in positive mode with a source temperature of 363 K and a capillary voltage of 3.0 kV. The LC-MS/MS was performed according to DDA (data dependent acquisition). The selected precursor ions were fragmented by collision-induced dissociation (CID). The CID spectra were interpreted manually.

3.5. Bioinformatics Analysis

The amino acid sequence similarity searches were performed against a non-redundant protein databank using BLAST. The alignments were performed using ESPript 2.2 [43].

3.6. Induction and Treatment of Skin Wounds

The *in vivo* study employed 84 ten-week-old male Swiss albino mice (*Mus musculus*) weighing 35.0 ± 5.0 g that were supplied by the laboratory animal facility of the Federal University of Ceará (BIOCEN/UFC). During the experimental procedures, the animals were kept in individual cages in a controlled environment (circadian cycle, 25 ± 2 °C, 55 ± 10% humidity, food and water *ad libitum*) at the School of Medicine, Sobral (UFC).

Prior to the surgical procedure, the animals were randomly distributed into three groups (n = 12, each group with 4 animals) according to the topical treatment administered: G-I (200 µg/mL BSL); G-II (200 µg/mL BSA); and G-III (150 mM NaCl).

The mice were then anesthetized using a subcutaneous administration of 2% xylazine with 10% ketamine hydrochloride (10 mg/kg and 115 mg/kg, respectively) [44], and this was followed by trichotomy and antisepsis of the dorsal thoracic region with povidone-iodine and a sterile saline solution (150 mM NaCl). After marking the skin with a sterile mold (1.00 cm^2), circular aseptic skin wounds were created by incision with a scalpel (#15) followed by resection of the subcutaneous tissue with fine dissection tweezers.

Immediately after the surgical procedure, the wounds were topically treated during 12 days with a single dose per day containing 100 µL of the respective substance (BSL, BSA or NaCl) as previously mentioned.

The experimental protocol was approved by the Ethics Committee of Ceara State University (UECE) under entry #11042434-4, and all animals were treated according to the recommendations of the Brazilian College of Animal Experimentation (COBEA) and the Guide for the Care and Use of Laboratory Animals of the US Department of Health and Human Services (NIH publication No. 85–23, revised 1985).

3.7. Evaluation of Healing Potential

The wounds were measured daily for 12 days to evaluate the healing potential of each treatment. The wound area was expressed as the mean ± standard deviation, as previously reported [45].

The animals were anesthetized prior to the collection of histopathological material and subsequently euthanized [44]. Samples (4 per group) of injured tissue were collected on the 2nd, 7th and 12th postoperative day (POD), fixed in 10% formaldehyde (v/v) buffered in 0.01 M PBS (pH 7.2), prepared in 5 mm cuts for routine histological analysis and stained with hematoxylin-eosin (HE) and Masson's trichrome. The histopathological assessment included the following parameters: the presence of scabs, re-epithelialization, collagen deposition, neovascularization and exudate.

The analysis was performed under a Leica light microscope (model DM 500) at 4, 10 and 40× magnification.

3.8. Statistical Assessment

Differences between the wound area and closure percentage were tested with the Mann-Whitney test. The data were processed using the statistics software GraphPad Prism v.3.00 for Windows®. The values are given as the mean ± standard deviation. The level of statistical significance was set at $p < 0.05$.

4. Conclusions

Our findings demonstrate the presence of isoforms with biochemical characteristics similar to other lectins from marine algae. These results placed BSL in the group of marine algae that possess isoforms. Treatment with BSL (native lectin) in induced skin wounds in mice can stimulate the healing process, possibly by inducing immune system cells in the inflammatory phase, resulting in the modulation of growth factors and cytokines, as well as stimulating collagen synthesis by fibroblasts and their differentiation into myofibroblasts for traction and wound contraction, which promotes faster closure of the lesion.

However, further studies are required to confirm the role of the lectin used on cells and the growth factors involved in the healing process, as well as possible mechanism of action of the lectins.

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Review

Antimicrobial peptide control of pathogenic microorganisms of the oral cavity: A review of the literature

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ABSTRACT

Antimicrobial peptides, molecules produced in many different organisms, have high biocidal activity against several microorganisms. However, several questions about these molecules remain unclear. Therefore, this report details a systematic survey of the literature on the use of antimicrobial peptides against oral pathogens and indicates which peptides and microorganisms are most extensively studied. Articles were located using the PubMed and Science Direct databases with the following inclusion criteria: publication date between 2002 and 2011; keywords "biofilm OR biological film OR biological layer OR bacterial growth" AND "peptide" AND "oral cavity OR mouth OR buccal mucosa OR oral mucosa OR mouth mucosa"; and abstract in English. A total of 73 articles were selected after refinement of the data. An increase in publications focusing on the use of antimicrobial peptides against oral microorganisms was observed. In addition, the peptides produced by cells of the oral mucosa (defensins, LL-37 and histatins) as well as *Streptococcus mutans* (among cariogenic bacteria) and *Porphyromonas gingivalis* (among periodontal bacteria) were the most studied subjects. It was concluded that the use of antimicrobial peptides as a tool for microbial control is of increasing importance, likely due to its widespread use, mechanism of action, and low rates of bacterial resistance.

Contents

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1. Introduction

Dental caries and periodontal disease are considered the most prevalent bacterial infections diseases in humans because they together affect 60–90% of the world population [50].

With respect to carious lesions, epidemiological data vary according to the population. In the United States, approximately 90% of all teenagers or young adults have or have had carious lesions [11]. In Australia, 60% of adolescents over the age of 20 have one or multiple sites of tooth demineralization [30].

Periodontal diseases are considered the main causes of tooth loss, which occurs in 15–25% of American adults [5].

The main etiological factor of such oral lesions is the colonization of pathogenic microorganism on the oral surfaces [35]. Such microorganisms vary depending on the lesion studied, development time, and host factors. However, the ability of these colonizers to grow in biofilms is one of the most important virulence factors and, correspondingly, one of their most extensively studied properties [34].

Biofilms are complex microbial communities established on a wide variety of surfaces. They are generally associated with an extracellular matrix consisting of several types of biopolymers [1].

Growth within biofilms grants protection from antibiotics due to the reduced penetration of various antimicrobial agents into its inner structure [14]. Microorganisms in biofilms are thus extremely difficult or impossible to eradicate [2,64].

Therefore, the search for new molecules that inhibit the growth of planktonic microbes and/or the development of such microbes into biofilms has been intensified in recent years.

In this context, antimicrobial peptides (AMPs) have been the focus of several studies in the field of Oral Microbiology. AMPs are a heterogeneous group of molecules produced by various tissues and cell lines in a range of species of invertebrates, plants and animals. As components of the innate immune response of multicellular organisms, AMPs, which possess biocidal activity against several microorganisms, significantly reduce infections. These molecules have been shown to be promising agents in controlling microbial growth due to its low concentration and selective antimicrobial activity, as well as low rates of microbial resistance induction [3,60].

The objective of this study was to carry out a systematic survey of the literature on the use of antimicrobial peptides against oral pathogens, focusing on dental caries and periodontal disease, and indicate which peptides and microorganisms are the most extensively studied, with the ultimate goal of tracing a profile of scientific publications in this area.

2. Materials and methods

2.1. Sample delimitation

The following international journal databases were reviewed: Science Direct and PubMed. Selection of these databases was based on the breadth of journals covered by each and our goal to provide an overview of the scientific research devoted to this topic over the long timeframe under analysis [56].

The following inclusion criteria were considered during the review: (a) articles published between January 2002 and December 2011; (b) the presence of the MeSH keywords “biofilm OR biological film OR biological layer OR bacterial growth” AND “peptide” AND “oral cavity OR mouth OR buccal mucosa OR oral mucosa OR mouth mucosa”, entered into the advanced search form; and (c) the availability of an abstract in English. A total of 280 articles were found.

For comparison, we also conducted a second survey of publications on agents with antimicrobial activity. This survey was conducted using the same inclusion criteria described above but replaced the keywords “peptide” AND “antimicrobial agent”.

2.2. Sample selection and classification

All the abstracts found were read. Review articles and those that did not fit the scope of the survey were excluded, resulting in a total of 73 publications related to AMPs and 388,727 articles related to the larger area of antimicrobial agents. These abstracts were read and classified for subsequent quantitative analysis. In cases where the abstract was too short to allow for classification, the full article was read. Each article was classified according to the following attributes: title of article, year of publication (subcategories [biennium]: first period, 2002–2003; second period, 2004–2005; third period, 2006–2007; fourth period, 2008–2009; fifth period, 2010–2011), journal name, language of publication, number and types of AMPs assessed, and number and species of microorganism analyzed in the study. The origin of the AMPs as well as the model of antimicrobial assay (planktonic and/or biofilm) were verified.

2.3. Statistical analysis

Data were entered into Microsoft Excel 2007 spreadsheets and systematically transferred to the Statistical Package for the Social Sciences (SPSS) version 17.0 for statistical analysis. Frequency distribution calculations were used to assess general sample characteristics, to investigate possible spelling errors in the raw data, and to obtain an overview of the use of AMPs against oral microorganisms.

3. Results

3.1. Study distribution by time period

Analysis of the distribution of publications over the different bienniums showed homogeneity during the first four periods. However, despite slight increases in earlier years, the fifth period (2010–2011) showed a large increase in the number of publications on the use of antimicrobial peptides to control pathogenic microorganisms. The publications from this period comprise 43.8% (32 articles) of all publications analyzed in this study (Table 1).

Studies of the larger field of antimicrobial agents also increased slightly in earlier years. In contrast with the increase in publications seen above, papers in the larger field decreased in the final biennium (Table 2).

Table 1
Distribution of publications related to AMP from 2002 to 2011, per biennium.

	Absolute value (n)	Relative value (%)	Cumulative relative frequency (%)
2002–2003	9	12.4	12.4
2004–2005	9	12.4	24.8
2006–2007	11	15	39.8
2008–2009	12	16.4	56.2
2010–2011	32	43.8	100
Total	108	100	

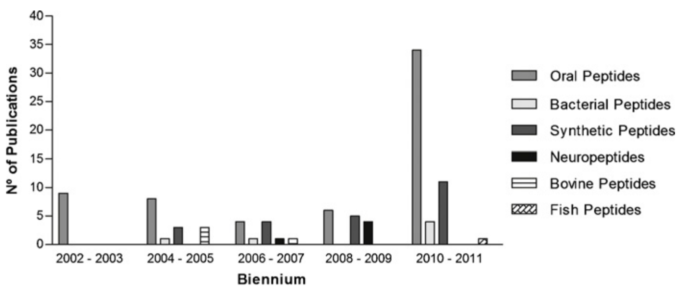

Fig. 1. Distribution of publications in accordance with the source of AMPs studied, per biennium.

Fig. 1 shows the same distribution of studies, however, divided according to the origin of the analyzed AMP. A predominance of publications in all bienniums were the AMPs produced by cells of the oral mucosa. These studies, as well as those on synthetic AMPs, showed significant growth over the years, especially during the final period (three articles in the second period, four in the third, five in the fourth, and 11 in the fifth) (Fig. 1).

3.2. Language of publication

Most of the reviewed articles were written in English (63 articles, 86.3%), followed by German (3, 4.1%), Japanese (3, 4.1%), French (3, 4.1%) and Chinese (1, 1.4%).

3.3. Number of species studied and susceptibility assay used

From a total of 73 studies, 43 (58.9%) analyzed only one species, seven (9.6%) analyzed two, and 23 (31.5%) analyzed three or more species. Furthermore, the assay used for testing susceptibility to AMPs should be noted; 52 articles (71.3%) performed analyzes on microorganisms in their planktonic form, 18 (24.6%) used monospecies microorganisms in biofilms, and only three articles (4.1%) performed tests on multi-species biofilms.

3.4. AMPs and microorganisms studied

The list for the analyzed AMPs and microorganisms, as well as their absolute and relative frequencies, is noted in Tables 3 and 4.

Table 2
Distribution of publications in the larger area from 2002 to 2011, per biennium.

	Absolute value (n)	Relative value (%)	Cumulative relative frequency (%)
2002–2003	65.202	16.77	16.77
2004–2005	73.091	18.81	35.58
2006–2007	79.967	20.57	56.15
2008–2009	86.811	22.33	78.48
2010–2011	83.656	21.52	100
Total	388.727	100	

Table 3
AMP distribution according to their classification by origin.[a]

	Absolute value (n)	Relative value (%)	Cumulative relative frequency (%)
Oral peptide			
α-Defensin 1	6	6	6
α-Defensin 2	2	2	8
α-Defensin 3	1	1	9
β-Defensin 1	5	5	14
β-Defensin 2	9	9	23
β-Defensin 3	8	8	31
β-Defensin 4	1	1	32
Cathelicidin LL-37	12	12	44
Histatin 3	1	1	45
Histatin 5	13	13	58
Lactoferrin	5	5	63
Neuropeptides			
Neurokinin A	2	2	65
Neuropeptide Y	3	3	68
Bacterial peptides			
Nisin	5	5	73
Fish peptides			
Pleurocidin	1	1	74
Bovine peptides			
Lactoferrin	3	3	77
Synthetic peptides			
2-1G2	1	1	78
C16G2	1	1	79
Clavanin	1	1	80
CSP	1	1	81
DHVAR5	1	1	82
GL13	1	1	83
GL13-K	1	1	84
GL13-NH2	1	1	85
IgG-SMAP28	2	2	87
IgG-SMAP29	1	1	88
JH8194	1	1	89
JH 8195	1	1	90
JH8944	1	1	91
KSL	1	1	92
KSL-W	1	1	93
M8(KH)-20	1	1	94
PMX000519	1	1	95
PMX30016	1	1	96
PMX70004	1	1	97
SM4	1	1	98
SM8	1	1	99
Subtilosin A	1	1	100
Total	100	100	

[a] More than one AMP was assessed in some articles. Therefore the total number of AMPs analyzed exceeds the total number of studies reviewed.

Among all the AMPs found, oral peptides were the most predominantly studied (63%), followed by synthetic AMPs (23% of studies).

With respect to the microorganisms studied, a homogeneous distribution can be noted when evaluating the absolute values. However, when analyzing the relative frequencies, we find a concentration of publications focused on the microorganisms involved in the process of dental caries (52.5%) and periodontal disease (28.8%). Moreover, the main bacteria related to their respective infectious processes were the main targets of research (*Streptococcus mutans*, 36 articles; *Candida albicans*, 21 articles; *Porphyromonas gingivalis*, 13 articles; and *Enterococcus faecalis*, 10 articles).

Finally, in order to better demonstrate the spectrum of scientific publications in this area, comparisons were made between the primary AMP evaluated in a paper and the groups of microorganisms studied in that paper (Table 5). It was found that the group of periodontal bacteria was most associated with the cathelicidin LL-37 peptide (15 studies), and

Table 4
Distribution of microbial species according to their associated pathology.[a]

	Absolute value (n)	Relative value (%)	Cumulative relative frequency (%)
Periodontopathogenic bacteria			
A. actinomycetemcomitans	9	5	5
A. israelii	1	0.5	5.5
A. naeslundii	5	2.8	8.3
A. viscosus	2	1.1	9.4
E. corrodens	1	0.5	9.9
F. nucleatum	5	2.8	12.7
P. gingivalis	13	7.2	19.9
P. intermedia	3	1.7	21.6
P. micros	3	1.7	23.3
P. nigrescens	1	0.5	23.8
T. denticola	3	1.7	25.5
T. forsythia	2	1.1	26.6
V. atypica	4	2.2	28.8
Cariogenic bacteria			
L. acidophilus	6	3.3	32.1
L. casei	7	3.8	35.9
L. fermenti	2	1.1	37
S. gordonii	15	8.4	45.4
S. mitis	7	3.8	49.2
S. mutans	36	19.9	69.1
S. sanguinis	15	8.4	77.5
S. sobrinus	7	3.8	81.3
Endodontic-lesion-related bacteria			
E. faecalis	10	5.5	86.8
Candidiasis-related Yeasts			
C. albicans	21	11.6	98.4
C. dubliniensis	2	1.1	99.5
C. glabrata	1	0.5	100
Total	181	100	

[a] More than one microbial species was assessed in some articles. Therefore, the total number of microorganisms analyzed exceeds the total number of studies reviewed.

the β-defensin 2 AMP was most associated with cariogenic bacteria (9 studies); nisin was associated with microorganisms associated with endodontic infections (3 studies), and histatin 5 was associated with fungi related to candidiasis (12 studies).

4. Discussion

4.1. Oral biofilms development

The concept that microorganisms, especially bacteria, preferably live in communities enclosed in a polysaccharide matrix bound onto surfaces emerged gradually from scientific observations over time. In the last two decades, however, this concept was remarkably strengthened [33]. These microbial communities, called biofilms, are complex structures that give associated microorganisms a survival mode that differs from the one they experience in planktonic form [12,13].

In the oral cavity, many microorganisms are capable of developing in harmony with the host. However, frequent ecological changes occur within the microbial community and result in the development of two major oral diseases: dental caries and periodontal disease [4,58].

The development of oral biofilms is complex and follows a very careful chronology. In general, oral bacteria possess mechanisms for adhesion to solid surfaces coated with saliva film, such as teeth, or a desquamative surface, such as epithelial tissue, or even bacteria already adhered on the surface [33,46]. The adherence of microbial cells to bacteria that are already immobilized on a surface is called coadhesion, while the binding of bacteria or microorganisms in suspension is called coaggregation [34]. Thus, the construction of oral biofilm is initiated by certain bacterial strains that have the ability to adhere to tooth enamel. Such bacteria are called early colonizers, and their main representatives are *Streptococcus oralis*, *Streptococcus sanguinis*, *Streptococcus gordonii*, and *Streptococcus parasanguinis* for predominantly cariogenic biofilms. Bacteria of the genus *Actinomyces* are implicated in periodontopathogenic biofilms. This relationship between early colonizers and the pathological process is due to bacterial selectivity itself in the coadhesion system; however, this strategy does not prevent the cross-holdings of these early colonizers in other diseases [19,34]. After the initial adhesion by the primary colonizers, biofilm development begins with the adhesion of other cells with receptors (selectins and adhesins) that allow selective adhesion to other microorganisms [19]. In the caries process, *S. mutans* and *Streptococcus sobrinus* deserve particular attention, while *Fusobacterium nucleatum* and *P. gingivalis* have a strong impact on the periodontopathogenic process [33,34]. Given the crucial importance of these microorganisms in these pathogenic processes, many studies are focused on the discovery of new methods of bacterial control (Table 4).

Although they are not the original colonizers of cariogenic biofilm, *S. mutans* and *S. sobrinus* are the main components responsible for tooth demineralization due to their ability to produce acid, which promotes the tooth demineralization process, and survive in an acidic environment [6].

Periodontal bacteria lack the acidogenic potential of cariogenic bacteria but are able to form more resistant biofilms and secrete lytic enzymes that degrade periodontal tissue [17,59]. Moreover, the dominant characteristic of periodontopathogenic bacteria is their ability to survive in anaerobic or facultative aerobic conditions [61].

Table 5
AMP distribution in accordance with the group of microorganisms examined in the review articles.[a]

		Analyzed AMP α-Defensin 1	β-Defensin 2	Cathelicidin LL-37	Histatin 5	Human Lactoferrin	Neuropeptide Y	Nisin	IgG-SMAP28	Total
Periodontopathogenic bacteria	N	2	9	15	1	2	1	2	9	41
	%	1.8	7.8	13.1	0.9	1.8	0.9	1.8	7.8	36
Cariogenic bacteria	N	9	4	6	3	7	8	8	2	47
	%	7.8	3.5	5.2	2.6	6.1	7	7	1.8	41.2
Endodontic-lesion-related bacteria	N	0	0	2	0	1	1	3	0	7
	%	0	0	1.8	0	0.9	0.9	2.6	0	6.1
Candidiasis-related Yeasts	N	1	0	2	12	1	2	1	0	19
	%	0.9	0	1.8	10.5	0.9	1.8	0.9	0	16.7
Total	N	12	13	25	16	11	12	14	11	114
	%	10.5	11.4	22	14.1	9.6	10.5	12.3	9.6	100

[a] More than one microorganism and/or AMP was studied in some articles; therefore, the total number of publications exceeds the total number reviewed.

When studying the topic "*Number of species studied and susceptibility assay used*", it was noted that more than half of the studies used only a single organism. Furthermore, the primary method used was to assess the susceptibility of microorganisms in their planktonic state. This state is not characteristic of the oral cavity, in which the biofilm mode of survival predominates.

However, studies addressing monospecies biofilms or bacteria in a planktonic state should not be disregarded, given the fact that they are fully able to demonstrate the antimicrobial potential of a given compound, as showed in studies by [9,10].

4.2. A new class of antimicrobial agents: the AMPs

Between 1920 and 1950, several compounds with antimicrobial properties were isolated from secretions of various living beings, including arachnids, amphibians, plants, and humans [22,57]. These compounds showed biological activity in small concentrations, exhibiting a peculiar selectivity for both Gram-positive and Gram-negative bacteria. These were designated as antimicrobial peptides and reported to kill or retard the growth of invading microorganisms, thereby contributing to the natural mechanism of adaptive immunity of the host [44].

In recent years, the study of AMPs as a new class of antimicrobial therapeutic agents has attracted considerable interest in many research centers [45,62]. This concern is justified by the rapid increase in the incidence of pathogens resistant to conventional drugs, which represents an important public health problem around the world [20,63].

In Table 1, the significant growth in the number of studies involving AMPs is evident, and those involving oral microorganisms were slightly evaluated in the first eight years of the studied period. However, in the last biennium, a significant leap in the number of publications was observed. It can be suggested that the advancements made in the techniques of obtaining and purifying molecules, in addition to constant research on these molecules, is the main factor that caused this increase [3,23,32,44].

Unlike the larger field of antimicrobial compounds, research in this subarea grew during the period in question (Table 2). The increase in number of publications noted in this table is slight in the early years, whereas the number of publications decreased during the last two years. Comparison of Tables 1 and 2 shows how researchers are increasingly focused on AMPs relative to other antimicrobial compounds.

The relationship between AMPs and the oral mucosal immune system may be responsible for the results shown in Fig. 1, which highlights the focus on oral cavity AMPs in the most recently published studies. These studies target compounds such as cathelicidin LL-37 (12 items) and histatin-5 (13 items) (Table 3).

4.3. Cathelicidin LL-37

The cathelicidins are proteins with N-terminal regions carrying highly conserved cathelin domains and a C-terminal region that is less well conserved and carries antimicrobial properties. These properties vary among species, which produce peptides with a wide variety of sizes, structures, and sequences [24,66]. Only one cathelicidin has been identified in humans, the cationic antimicrobial peptide of 18 kDa (hCAP18). This peptide is expressed by neutrophils and epithelial tissues, especially in the oral cavity and respiratory tract. When in need, hCAP18 is expressed and cleaved by proteases that generate a 37 amino acid antimicrobial peptide, LL-37 [27]. Several studies have demonstrated the potent antimicrobial activity of this peptide against oral microorganisms [31,41,51], including *Staphylococcus* species [16,52], and its function in chemotaxis and stimulation of monocytes, T cells, neutrophils and mast cells [27].

4.4. Histatins

The histatins comprise a family of low molecular weight cationic proteins and are produced and secreted by the parotid, submandibular, and sublingual glands [21]. Histatins generally exhibit apoptotic activities on various microbial strains, as well as potent antifungal activity [21]. Histatin-5, a peptide composed of 24 amino acids, has the highest level of activity against *C. albicans* among all family members, including activity against species resistant to conventional antifungal agents. It is believed that this peptide exerts its antifungal activity by binding to receptors on the cell membrane and, after internalization, inhibits mitochondrial respiration by forming reactive oxygen species, which damage mitochondrial and cytoplasmic membranes and lead to ATP efflux and cell death [43]. Studies on the activity of histatin-5 have been conducted against biofilms of *C. albicans* because they are common colonizers of dental prostheses and can cause diseases such as candidiasis [49,53].

4.5. Defensins

A third class of peptides, the defensins, is notably highly studied (Table 3). This group of AMPs, composed of more than 380 members, contains conserved motifs of six cysteine residues forming intramolecular disulfide bonds and β-sheets [23]. They are believed to have originated as components of procaryotic immune systems [68], and over time, differentiated into the defensins of plants, arthropods, and higher animals such as birds, reptiles, and mammals. At some point in the evolution of mammals, defensins separated into two subgroups, the α-defensins and β-defensins, which are structurally similar but have distinct activities and production sites [65]. In general, defensins are expressed in response to microorganism-induced stress and are responsible for the migration and activation of cells from the innate immune system [18]. Furthermore, studies show the antimicrobial activity of defensins on oral streptococci through mechanisms of cell rupture and inhibition of lipopolysaccharide production [26,55].

4.6. AMPs mechanisms of action

The mechanisms of action of AMPs are not yet fully understood. However, some theories about how these molecules act on cell membrane and bacterial metabolism have been proposed following studies using fluorescent microscopy, circular dichroism, mass spectrometry, and studies with model membranes [8,29,40,60,67].

Biocide activity models include cell death by both extracellular and intracellular paths. Regarding the models of extracellular interactions, the AMPs are initially attracted to the surface of microorganisms by electrostatic interactions between the anionic or cationic peptide and structures on the cell surface [39]. In the case of Gram-negative bacteria, the main interactions occur between cationic peptides and the negative charges present on the bacterial envelope, principally the anionic phospholipid and the phosphate group of lipopolysaccharide. In Gram-positive bacteria, the primary relationship occurs between anionic AMPs and the cell surface teichoic acid [8]. After the initial attraction, the AMP binds to cell surfaces and initiates the attachment phase. In this phase, the AMP crosses through the outer membrane (lipopolysaccharides in Gram-negative bacteria and teichoic and lipoteichoic acids in Gram-positive bacteria) and interacts directly with the bacterial cytoplasmic membrane [8]. At low peptide/lipid ratios, the AMPs are connected in parallel to the lipid bilayer. As this ratio increases, the peptides begin to orient perpendicularly to the membrane.

When peptide/lipid ratios are high, AMPs will start to penetrate the cell membrane, leading to the formation of transmembrane pores that subsequently cause cell death by loss of fluid and disruption of the cell membrane [40].

Though seemingly simple, different modeling studies have explored the dynamics of death generated by membrane damage. The barrel-stave model suggests that peptides arrange in the membrane in the form of a “barrel”, which allows for the flow of intracellular contents out into the extracellular environment [25]. The carpet model highlights the idea that when AMPs accumulate parallel with the cell surface, they are oriented to penetrate the lipid bilayer and form pores for the entry of more peptides into the cell. The accumulation of these peptides, which are now also on the inner side of the cell membrane, induces the formation of mycelia, which are released from the membrane, and starts its disintegration [41]. Finally, the toroidal-pore model indicates that the insertion of AMP helixes on the cell membrane leads to connection of the lipid monolayers opposed to each other continuously and bilaterally, which causes the formation of pores that, similarly to the first model, allow fluid outflow to the external environment [7].

Although the lysis of the cell membrane leads to bacterial killing, the increase in the number of studies report that there are other methods of cell death is remarkable. Recent research revealed the presence of important intracellular targets for cell death. Examples of such activities are the inhibition of DNA synthesis, RNA and proteins, usually related to pleurocidin, α-defensin 1 and α-defensin 2, and the inhibition of enzymatic activity associated with histatins [32,47,48,54].

Though the membrane damage models vary, they are most likely linked. In 2005, Brogden noted that such mechanisms do not appear independently but are correlated and appear gradually. However, several factors may be closely associated with the effectiveness and specificity of the peptide, such as its size, amino acid sequence, charge, conformation and structure, hydrophobicity and amphipathicity [8].

Table 5 highlights some aspects that deserve attention. Initially, it can be noted that studies related to periodontal and cariogenic bacteria occur frequently and analyze a broad distribution of peptides. This finding is consistent with the epidemiological data discussed earlier, which indicates that, due to their clinical impacts, studies of diseases are more attractive to researchers. The distribution of studies on fungal diseases differs from the above trends, as such publications are frequent and closely related to histatin-5, a peptide that has demonstrated antifungal activity against other yeasts [36–38].

4.7. AMPs limitations and future directions

Despite all the advantages that AMPs demonstrate, including activity at low concentrations and across a wide spectrum, there are some limits to their therapeutic utility. One limitation is that the high cationic activity most AMPs have in non-physiological conditions is significantly reduced in biological fluids such as plasma, serum or saliva. When combined with the high toxicity of some AMPs and their quick renal excretion, it is difficult or impossible to use some AMPs by the parenteral route. Furthermore, the high cost of production of native peptides is, sometimes, an obstacle for therapeutic application of these molecules [3,44].

Attempts to work around these limitations include the design of modified peptides with the same antimicrobial characteristics of the original AMP, but with altered cytotoxic domains, which confers greater biocompatibility with human tissues [15]. In addition, specifically targeted AMPs (STAMPs) have been explored in recent studies. Such molecules are modified to carry peptide to recognition domains specific for a particular group of microorganisms. This feature enables the AMPs to affect specific microbes without changing the whole indigenous microbial population [28,42].

5. Conclusions

After analyzing the results obtained in this bibliographical research and the discussion held earlier, it can be concluded that antimicrobial peptides are valuable tools for the control of microorganisms and biofilms of the oral cavity and that their study has become increasingly common over the last several years.

Although other studies are needed to better understand the mechanisms of AMP action and to reduce the adverse effects of these molecules, methods such as the design of synthetic peptides and STAMPs are become increasingly viable, especially when new developments in protein synthesis and purification are considered.

However, larger studies focused on more organisms and, in particular, the evaluation of AMPs on microorganisms in biofilms multispecies is necessary in order to fully investigate the potential for AMPs used in the oral cavity.

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Article

Lectin from *Canavalia brasiliensis* Seeds (ConBr) Is a Valuable Biotechnological Tool to Stimulate the Growth of *Rhizobium tropici in Vitro*

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Abstract: To study the interactions between a *Rhizobium tropici* strain and lectins isolated from the seeds of *Canavalia ensiformis* (ConA) and *Canavalia brasiliensis* (ConBr), a lectin fluorescence assay was performed. In addition, an experiment was designed to evaluate the effect of the two lectins on bacterial growth. Both lectins were found to bind to *R. tropici* cells, but the interactions were inhibited by D-mannose. Interestingly, only ConBr stimulated bacterial growth in proportion to the concentrations used (15.6–500 μg/mL), and the bacterial growth stimulation was inhibited by D-mannose as well. Structure/Function analyses by bioinformatics were carried out to evaluate the volume and carbohydrate recognition domain (CRD) configuration of ConA and ConBr. The difference of spatial arrangement and volume of CRD may indicate the variation between biological activities of both lectins. The results suggest that ConBr could be a promising tool for studies focusing on the interactions between rhizobia and host plants.

Keywords: lectin; *Canavalia brasiliensis*; *Canavalia ensiformis*; *Rhizobium tropici*; interaction; growth

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1. Introduction

Diazotrophic bacteria capable of forming symbiotic N_2-fixing associations with many plant species of the Leguminosae family are collectively known as rhizobia [1]. The establishment of symbiosis between rhizobia and host plants requires the recognition and exchange of molecular signals [2]. Several signaling molecules are exchanged between plant and bacterium (such as flavonoids, betaines and aldonic acid), thereby regulating the initiation, differentiation and functioning of the nodule [3].

Rhizobium tropici, a major symbiont of the common bean (*Phaseolus vulgaris*) in tropical soils, is abundant in all Brazilian biomes [4]. Certain strains of *R. tropici* are particularly effective and competitive at nitrogen fixation [5,6].

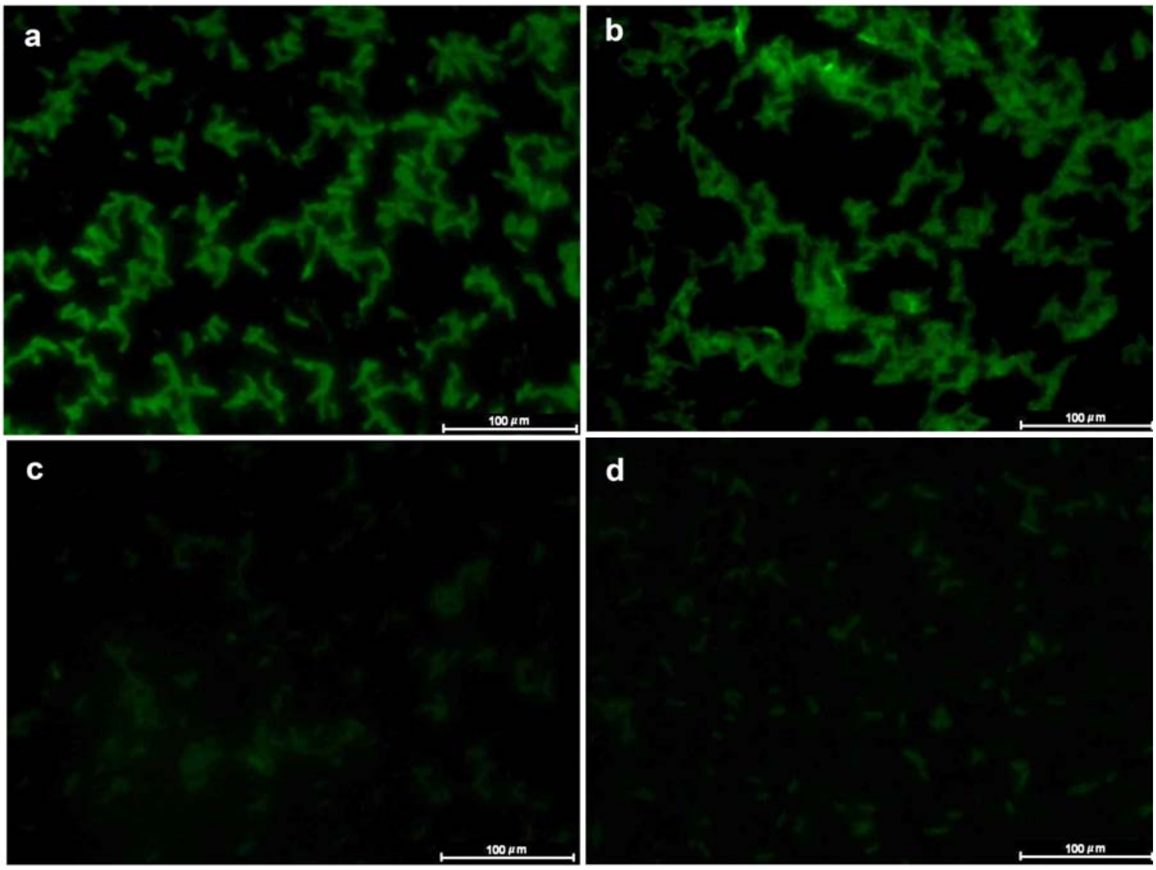
Lectins recognize and bind reversibly to carbohydrate epitopes with high specificity. They have a number of different functions in nature, the most important of which is acting as plant defense proteins and information mediators in biological systems through interactions with glycoproteins, glycolipids and oligosaccharides. Lectins can interact with bacterial polysaccharides, mainly lipopolysaccharides (LPS), to promote bacterial adhesion and improve N_2 fixation [7,8]. Lectins also stimulate cellular respiration, induce bacterial growth and modulate metabolism [9–12]. The lectins ConA and ConBr were isolated from the seeds of two species of the Diocleinae subtribe, *Canavalia ensiformis* and *Canavalia brasiliensis*, respectively. Although lectins isolated from Diocleinae seeds have a high degree of similarity and bind to glucose and mannose [13], they differ in their biological activity [14–16].

The objective of this study was to investigate the interaction between *Rhizobium tropici* CIAT 899 and the homologous lectins ConA and ConBr and to evaluate the effect of these proteins on bacterial growth.

2. Results and Discussion

Using fluorescence assays, this study showed that both ConA and ConBr interacted with *Rhizobium tropici*. The two lectins had similar fluorescence emission profiles (Figure 1a,b). When D-mannose was added to ConA and ConBr solutions, interaction between the lectin and bacterial surface decreased abruptly (Figure 1c,d). No fluorescence was observed when the cells were treated with FITC-BSA or FITC alone. Likewise, no intrinsic fluorescence was detected (data not shown).

Figure 1. Lectin-fluorescence microscopy of interaction between FITC-lectins and *Rhizobium tropici* CIAT 899. (**a**) FITC-ConA; (**b**) FITC-ConBr; (**c**) FITC-ConA plus D-mannose; and (**d**) FITC-ConBr plus D-mannose.



As shown in the Figure 2a, ConBr had a stimulatory effect on the growth of *R. tropici* (500 µg/mL), as compared to NaCl control. This effect was observed for all experimental incubation periods (12, 24, 36 and 48 h). Similarly, ConBr enhanced bacterial growth as compared to the ConBr-D-mannose complex (Figure 2b).

Figure 2. (**a**) Effect of ConBr on the growth of *R. tropici*. (★) $p < 0.01$ related to 0.15 M NaCl; (**b**) Comparison between the effects of ConBr and ConBr complexed with D-mannose on the growth of *R. tropici*. (★) $p < 0.01$ related to ConBr plus D-mannose.

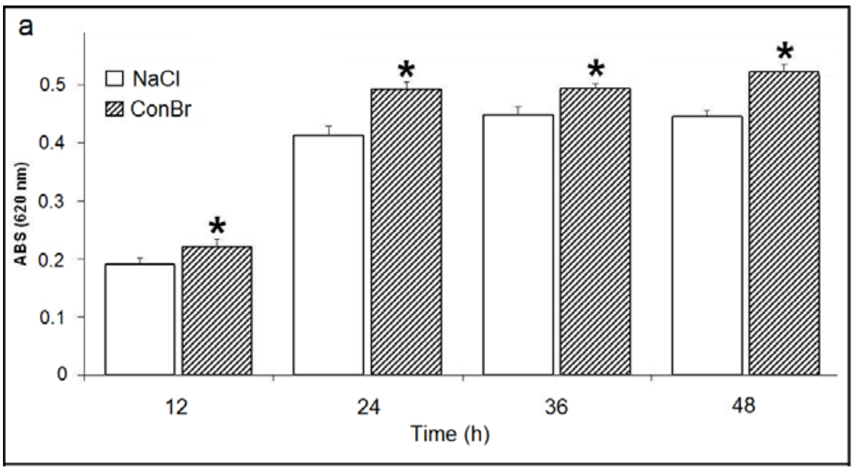
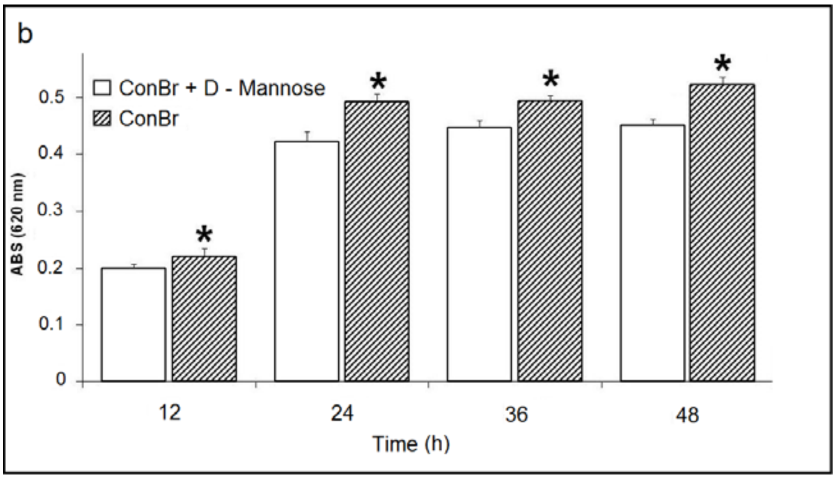
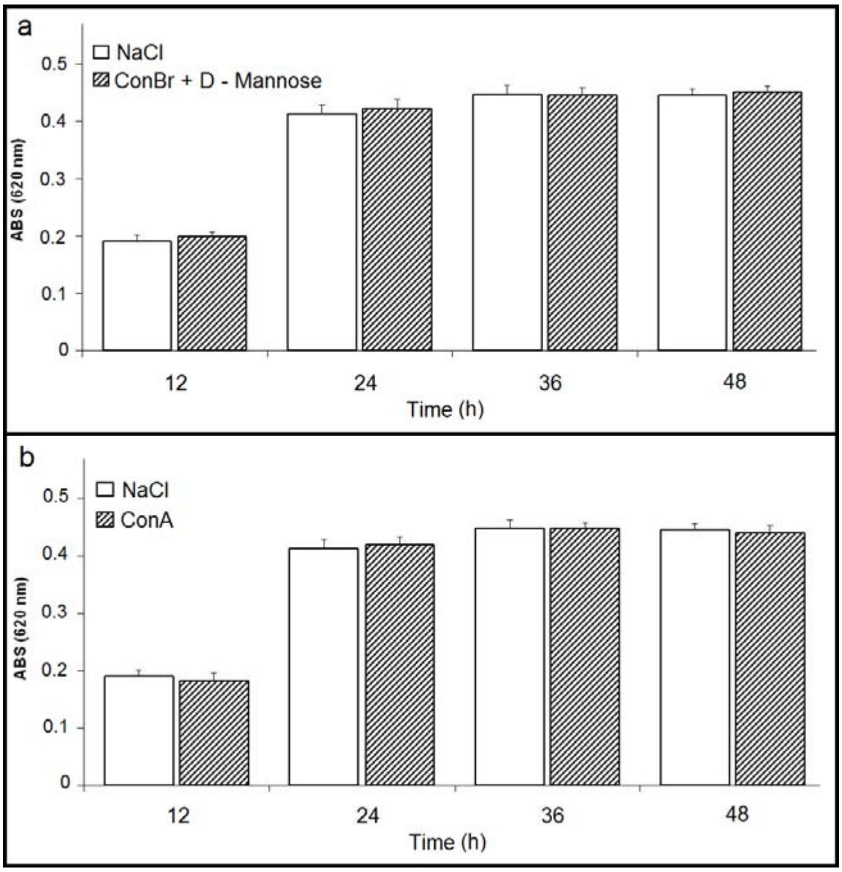


Figure 2. *Cont.*



Nevertheless, when ConBr was inhibited by the addition of D-mannose, no significant change in bacterial growth was reported (Figure 3a). Both ConA (Figure 3b) and ConA complexed with D-mannose had no effects on the growth of *R. tropici* (data not shown), similar to the 0.1 M D-mannose negative control (data not shown).

Figure 3. (**a**) Effect of ConBr complexed with D-mannose on the growth of *R. tropici*; (**b**) Effect of ConA on the growth of *R. tropici*.



Lectins can recognize and bind reversibly to carbohydrates on the cell surface and interact with cell wall polysaccharides and/or glycoconjugates in the cell membrane [17,18]. Carbohydrates are directly involved in many biological processes, both physiological and pathological [18,19]. Strathmann and colleagues [20] showed that ConA isolated from seeds of *Canavalia ensiformis* interacted with carbohydrates present in the extracellular matrix of the Gram-negative bacterium *Pseudomonas aeruginosa*. Likewise, another glucose/mannose-specific lectin isolated from the roots of *Sesbania aculeate* interacted with different *Rhizobium* strains [21].

Lectins are known to interact with nitrogen-fixing bacteria and play an important role in metabolism. For example, wheat germ agglutinin (WGA), is a *N*-acetylglucosamine-binding lectin isolated from wheat germ, stimulated N_2 fixing, ammonia excretion, glutamine synthetase activity and indole acetic acid production in *Azospirillum brasiliensis* [22]. In addition, *Vatairea macrocarpa* lectin (VML) enhanced H^+ efflux in *Rhizobium tropici* [10]. Previously, Martinez and colleagues [11] demonstrated that VML and ConBr stimulated the respiration of *R. tropici* and *Rhizobium etli*.

In the present study, ConBr bound to *Rhizobium tropici* and stimulated bacterial growth probably by modifying its metabolism. However, the effect was neutralized by the presence of its specific sugar D-mannose. These findings were consistent with those published by Bajaj and colleagues [9] who demonstrated that the D-glucose-specific lectin of *Pisum sativum* stimulated the growth of *Rhizobium leguminosarum*, probably by enhancing nutrient transport.

Although ConA also binds to bacteria, it displayed no discernible activity in experiments. Similar results were reported by Araújo-Filho and colleagues [23] for three Diocleinae lectins: *Canavalia ensiformis* (ConA), *Canavalia maritima* (ConM) and *Dioclea guianensis* (Dgui). All lectins were found to bind spores of *Colletotrichum gloeosporioides*, but only Dgui inhibited germination.

Although Diocleinae lectins are similar in many respects, they vary significantly with regard to biological activity [13,24,25]. In this study, the growth of *Rhizobium tropici* was stimulated *in vitro* by the glucose/mannose-binding lectin ConBr, but not the homologous lectin ConA. Differences in biological activity between homologous lectins may be due to small changes in the configuration of important residues in the carbohydrate recognition domain (CRD) [26] or to pH-dependent oligomerization [13].

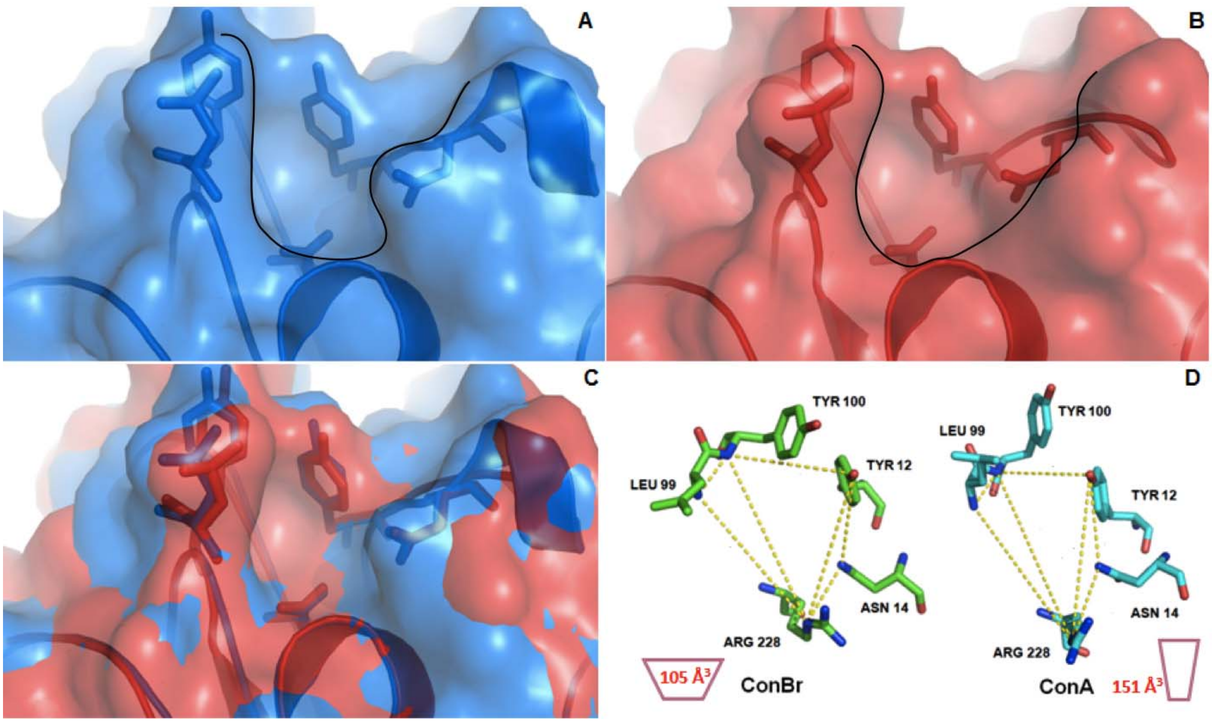
Structural comparisons showed that ConA and ConBr differ by only four residues: Asp58, Ala70, Asp151 and Glu155 in ConA *versus* Gly58, Gly70, Glu151 and Arg155 in ConBr (PDB codes: 1AZD and 1JBC). The CRDs of Diocleinae lectins, such as ConA and ConBr, consist of highly conserved residues (Tyr12, Asn14, Leu99, Tyr100, Asp208 and Arg228). These residues are key elements in the interaction between lectin and carbohydrate [27]. The difference in biological activity between ConA and ConBr has been attributed to a non-conservative substitution (amino acid in position 58), which makes the CRD more open in ConBr [28]. This more open conformation was due to the CRD design that induces a reduction in the volume of the ConBr carbohydrate binding site [29]. To show that highly similar Diocleinae lectins can nevertheless interact differently with carbohydrates, Ramos and colleagues [30] evaluated the fine glycan specificity of Diocleinae lectins for a number of glycoproteins using surface plasmon resonance. ConBr was observed to be much more reactive than ConA or ConM, suggesting that the carbohydrate binding site might be extended by neighboring surface residues [30]. Moreover, ConA displays homogenous behavior above pH 7.0, whereas ConBr presents a mixture of dimers (31%) and tetramers (69%) at pH 8.0 [28]. According to Cavada and colleagues [13], differences in the proportions of tetrameric and dimeric forms may explain the differences in the biological activities of Diocleinae lectins.

Comparative X-ray crystallographic analyses of dimannoside-complexed Diocleinae lectins isolated from *Canavalia maritima* and *Canavalia gladiata* showed that even very similar lectins differ in their interactions with disaccharides. This result suggests that differences in configuration and distance between the residues in the CRD can provide information on the ability of lectins to bind to glycoprotein and glycolipid receptors [31].

ConBr exhibits differences in the distances between specific amino acid residues that compose the primary carbohydrate binding site, thus altering the volume of the site [29]. Previous evidence indicates that these differences might explain the divergences in biological activities. The results reported in this study suggest that the lower volume found in ConBr could enhance the interaction with LPS, which are composed mainly by *O*-polysaccharides in *Rhizobium*.

Figure 4 highlights the differences in CRD design and volume between ConA and ConBr. ConBr has a more open carbohydrate binding site, whereas the site of ConA is narrower and deeper. Bezerra and colleagues [29] used this evidence to rationalize the stronger interactions between the *N*-glycans and both ConA and ConM *versus* the weaker interactions displayed by ConBr [29].

Figure 4. Surface analysis and structure alignment of ConA and ConBr tridimensional structures. (**A**) Carbohydrate binding site of ConA (Blue); (**B**) Carbohydrate binding site of ConBr (Red); (**C**) Structure alignment of ConA and ConBr showing differences in the topology which favors ConBr interactions with LPS; (**D**) Schematic representation of site volume in ConA (right) and ConBr (left). Calculated volume of each site is represented inside the schemes. Black lines in (**A**) and (**B**) delimitate the topology of sites.



3. Experimental

3.1. Microorganism

Rhizobium tropici CIAT 899 was kindly provided by Embrapa Agropecuária Oeste, Dourados, MS, Brazil.

3.2. Lectin Purification

ConA and ConBr were obtained from the Biologically Active Molecules Laboratory (Biomol-Lab), Department of Biochemistry and Molecular Biology, Science Center, Federal University of Ceará, Brazil. The purification steps are described by Moreira and Cavada [32].

3.3. Lectin Complexation with Mannose

In bacterial growth experiments and with FITC-labeled lectins, ConBr and ConA were inhibited with D-mannose. The lectins were incubated with 0.1 M D-mannose at 37 °C for 30 min to induce complex formation. The solution was then filtering through a 0.22 μm Millipore filter and stored for later use.

3.4. FITC-Labeled Proteins

FITC-labeled proteins were prepared with inhibition buffer (0.1 M D-mannose in 0.1 M carbonate-bicarbonate buffer, pH 9.0), conjugation buffer (0.1 M carbonate-bicarbonate buffer, pH 9.0) and washing buffer (phosphate-buffered saline: 0.01 M sodium phosphate buffer, 0.027 M KCl and 0.15 M NaCl, pH 7.4). Initially, ConBr and ConA were dissolved in inhibition buffer and incubated at 37 °C for 1 h. Then, 250 μL fluorescein isothiocyanate (FITC) (500 μg/mL in conjugation buffer) was added drop wise. The solution was incubated for 2 h at room temperature with gentle stirring. Subsequently, unconjugated FITC was separated from FITC-lectin by molecular exclusion chromatography using a pre-equilibrated Sephadex G-25 column, and labeled lectins were eluted with washing buffer. The absorbances of all fractions were determined at 280 nm (protein) and 495 nm (FITC) to verify chromatographic efficiency. Labeled lectins were then dialyzed against 1 M acetic acid for 1 h to remove the inhibitor carbohydrate and extensively dialyzed against distilled water. Except for the inhibition with D-mannose, the same labeling protocol was used to label BSA with FITC.

3.5. Cultivation of Microorganisms

Strain CIAT899 was stock-cultured on yeast-mannitol (YM) [33] broth containing 20% glycerol and stored at −80 °C. The inoculum was then transferred to Petri dishes containing YM agar and incubated at 28 °C for 48 h. Subsequently, a colony was carefully transferred to 10 mL YM broth and incubated at 28 °C for 48 h under constant agitation. Immediately before use, the bacterial suspension was adjusted to 1×10^8 cells/mL using a McFarland scale.

3.6. Lectin Fluorescence Microscopy

Bacterial cells were incubated separately with FITC, FITC-lectins, FITC-lectins in complex with D-mannose and FITC-bovine serum albumin (FITC-BSA) and agitated for 15 min. The cells were then washed 3 times in PBS, heat-fixed on slides and observed under a fluorescence microscope (Eclipse E200/epi-fluorescence, Nikon, Tokyo, Japan) equipped with a digital camera (DS-2Mv, Nikon, Tokyo, Japan). Images were acquired with NIS-Elements version 2.3 software (Nikon, Tokyo, Japan). Cells were observed using 100× oil-immersion objective with 1.5 s of exposure and 1× gain. Bacterial cells without treatment were observed to verify intrinsic fluorescence. The resolution of all acquired images was 5.0 Mpixels.

3.7. Bacterial Growth Assay

Assays were carried out using 96-well polystyrene plates. Following serial dilution from 500 to 15.6 µg/mL, the lectins were mixed with 100 µL bacteria suspension (1×10^8 cells/mL) and incubated at 28 °C for 12 h. A 20 µL aliquot of bacterial suspension was then inoculated into 180 µL YM broth and incubated at 28 °C. The optical density at 620 nm (ABS 620nm) was measured at 12, 24, 36 and 48 h using a microplate reader (BioTrak II-Plate Reader). BSA and 0.15 M NaCl were used as protein and negative control, respectively. Lectin-D-mannose complexes were used in the bacterial growth assays. To prevent carbohydrates from interfering with metabolism, an additional negative control consisting of 0.1 M D-mannose was used. The bacterial growth assays were designed with 9 repetitions per group.

3.8. Statistical Analysis

The normality of the data within each experimental group was verified with the Shapiro-Wilk test, and the groups were compared pair wise with Student's t test. The level of statistical significance was set at $p < 0.01$. All statistical analyses were performed with the software STATISTICA version 10.0 (Statsoft Inc., Tulsa, OK, USA).

3.9. Structure/Function Analysis

For the structural analysis, the crystal structures of ConA (PDB code: 1JBC) and ConBr (PDB code: 3JU9) were visualized using COOT [34] and PyMol [35]. Volume calculations were performed using the Q-SiteFinder program [36].

4. Conclusions

In this study, lectins from *Canavalia brasiliensis* (ConBr) and *Canavalia ensiformis* (ConA) bound to *Rhizobium tropici*, but only ConBr affected bacterial growth. This might be due to an extended and more opened carbohydrate binding site presented by ConBr which favors the interaction with *Rhizobium* LPS. Based on our results, ConBr may be a useful biotechnological tool in studies on the interaction between rhizobia and host plants.

Acknowledgments

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Sample Availability: Contact the authors.