UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA E FISIOLOGIA

THAMARA FIGUEIREDO PROCÓPIO VASCONCELOS

PURIFICAÇÃO, CARACTERIZAÇÃO E AVALIAÇÃO DE ATIVIDADES BIOLÓGICAS DE LECTINA DE FOLHAS DE *Calliandra surinamensis* Benth. (FABACEAE)

Recife
2018

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Tese apresentada para o cumprimento parcial das exigências para obtenção do título de Doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

Orientador: Prof. Dr. Thiago Henrique Napoleão

Coorientadora: Profª. Dra. Patrícia Maria Guedes Paiva

Recife

2018

Catalogação na fonte
Elaine C. Barroso (CRB4/1728)

Vasconcelos, Thamara Figueiredo Procópio

Purificação, caracterização e avaliação de atividades biológicas de lectina de folhas de *Calliandra surinamensis* Benth. (Fabaceae) / Thamara Figueiredo Procópio Vasconcelos- 2018.

104 folhas: il., fig., tab.
Orientador: Thiago Henrique Napoleão
Coorientadora: Patrícia Maria Guedes Paiva
Tese (doutorado) – Universidade Federal de Pernambuco.
Centro de Biociências. Programa de Pós-Graduação em Bioquímica e Fisiologia. Recife, 2018.
Inclui referências

1. Lectinas 2. Fabaceae 3. Atividade antimicrobiana I. Napoleão, Thiago Henrique (orient.) II. Paiva, Patrícia Maria Guedes (coorient.) III. Título

572.6 CDD (22.ed.) UFPE/CB-2018-438

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Aprovada em: 31/ 07 / 2018

BANCA EXAMINADORA:

_____

Prof. Dr. Thiago Henrique Napoleão (Orientador)
Universidade Federal de Pernambuco

_____

Prof. Dr. Emmanuel Viana Pontual (Examinador Interno)
Universidade Federal Rural de Pernambuco

_____

Profa. Dra. Ana Lúcia Figueiredo Porto (Examinadora Externa)
Universidade Federal Rural de Pernambuco

_____

Profa. Dra. Lidiane Pereira de Albuquerque (Examinadora Externa)
Universidade Federal do Piauí

_____

Dra. Maiara Celine de Moura (Examinadora Externa)
Universidade Federal de Pernambuco

Dedico esse trabalho a Deus, à minha família e aos meus amigos/irmãos que sempre me deram apoio incondicional.

AGRADECIMENTOS

Agradeço de coração a todos aqueles que contribuíram para que eu realizasse esse trabalho. Não foram poucas as pessoas que estiveram ao meu lado ao longo desses últimos anos e cada uma delas contribuiu de uma forma particular para o meu crescimento pessoal e profissional.

Agradeço a Deus, que iluminou o meu caminho durante esta caminhada, pela graça da vida, saúde e a oportunidade de conviver e aprender com todas as pessoas que fazem parte da minha vida, sobretudo a minha família e amigos.

Ao meu querido orientador, o Professor Doutor Thiago Henrique Napoleão, por sua incansável dedicação e brilhante orientação ao longo de todos esses anos da minha vida acadêmica na UFPE. Agradeço também pelo prazer de sua sincera amizade, companheirismo, cumplicidade e paciência. Os meus mais sinceros agradecimentos por me permitr estar ao seu lado em tantos momentos e me ensinar através da sua simplicidade e sincera humildade o quanto o ser humano pode ser maravilhoso. Continue sendo sempre essa pessoa iluminada que faz a diferença na vida de todos que o conhece. Amo você.

A toda a minha família, que vem crescendo nesses últimos anos, em especial aos meus pais (Nilma e Nelson) por me darem a vida e dedicarem as suas vidas para que eu pudesse ser feliz, pelo infinito apoio, amor, dedicação, carinho, respeito e proteção. Não existem palavras para descrever o quanto vocês são importante na minha vida. Aos meus irmãos Thiago e Thaiane pela amizade, amor e apoio por todos os momentos de dificuldades que passamos juntos e que nos fizeram entender o quanto nos amamos mesmo com as nossas diferenças, mas sempre juntos pelo coração. Aos meus novos irmãos, os meus cunhados (Adso e Flaviane), que me acolhem com tanto carinho e cada dia tenho mais respeito e amor por vocês. Aos meus afilhados (Letícia e Victor) que transformaram a minha vida, me alegrando e me enchendo de amor todos os dias da minha vida desde de o momento em que descobri que vocês estavam chegando, um amor que cresce a cada dia de uma forma inexplicável. Um amor que não cabe dentro de mim.

Aos meus novos irmãos Thiago, Nataly, Maiara, Leyde, Lidi e Mano: não existem palavras nesse mundo que podem expressar o quanto vocês são fundamentais na minha vida. Vocês me acolhareram de uma forma tão bonita que me fez ter certeza que os anjos existem, sim! Vocês são os melhores anjos do mundo. Não consigo imaginar a minha vida sem que vocês estejam presentes. Obigada por me acolherem de forma tão generosa na vida e na família de vocês. Vocês nem imaginam o quanto vocês fizeram e fazem a diferança em minha

vida e o quanto me deram força para seguir em frente, sem vocês a vida não teria cor. Deus não poderia ter escolhido melhores anjos para a minha vida.

A Radeval por sempre acreditar em meu potencial e me incentivar a seguir em frente, minha sincera gratidão.

A todos os meus queridos amigos que fazem ou fizeram parte do Laboratório de Bioquímica de Proteínas (BIOPROT) que tornaram os dias de trabalho mais leves. Deixo a todos meu agradecimento, pelo carinho, respeito, alegrias, tristezas e dores compartilhadas durante todos esses anos.

A todos os integrantes do Departamento de Bioquímica da UFPE: professores, funcionários e colegas.

À Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) pela concessão de bolsa de doutorado.

Ao apoio financeiro concedido pela FACEPE, pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Enfim, a todos que direta ou indiretamente contribuíram na minha formação pessoal e acadêmica. Obrigada por tudo!

Deus não vem em pessoa para abençoar.
Ele usa os que estão aqui dispostos a cumprir essa missão.
Todos nós podemos ser anjos. (Chico Xavier)

RESUMO

Lectinas são proteínas que se ligam especificamente a carboidratos ou glicoconjugados. *Calliandra surinamensis* é um arbusto tropical perene utilizado amplamente como espécie ornamental. A presente tese teve como objetivos purificar e caracterizar uma lectina (CasuL) a partir dos foliólulos de *C. surinamensis*, bem como investigá-la quanto às atividades citotóxica para células cancerosas, antimicrobiana, antibiofilme e imunomoduladora. Proteínas dos foliólulos foram extraídas com NaCl 0,15 M e, em seguida, precipitadas com sulfato de amônio (saturação de 60%). A fração de proteínas precipitadas foi aplicada em coluna de gel filtração (Sephadex G-75). Foram determinados o ponto isoelétrico (pI), a massa molecular nativa, a composição em subunidades e similaridades da estrutura primária com outras proteínas vegetais. Estabilidade da atividade hemaglutinante (AH) e da estrutura terciária de CasuL frente a variações de pH e temperatura também foi analisada. A citoxicidade de CasuL para células mononucleares de sangue periférico humano (PBMCs) e células de leucemia (K562) e câncer de mama (T47D) foi determinada. Atividade antibacteriana foi avaliada contra patógenos humanos e causadores de mastite bovina e caprina. Efeitos fungistático e fungicida de CasuL para espécies de *Candida* foram avaliados. CasuL foi avaliada quanto à toxicidade e atividade imunomoduladora sobre esplenócitos de camundongos. CasuL foi isolada com elevado fator de purificação (112), sendo uma proteína acídica (pI 5,82) e oligomérica com massa molecular nativa de 48 kDa. CasuL apresentou similaridade (15%) com a sequência de uma proteína de *Sorghum bicolor*. A lectina não perdeu sua AH e não sofreu desenovelamento quando aquecida; por outro lado, alterações conformacionais foram detectadas quando CasuL foi incubada em pH alcalino, com perda da AH. CasuL não foi citotóxica para células normais (PBMCs), mas foi ativa contra células K562 e T47D (CI_{50} de 67,04 e 58,75 µg/mL, respectivamente). Para isolados patogênicos ao homem de *Staphylococcus saprophyticcus* e *Staphylococcus aureus* (susceptível e resistente à oxacilina), CasuL (6,25–800 µg/mL) apresentou ação bacteriostática e reduziu a formação de biofilme. A lectina inibiu o crescimento de dois isolados de mastite bovina (Sa e SSp6PD) e dois de mastite caprina (Ssp01 e Ssp5D), com concentração mínima inibitória (CMI) entre 3,75 e 15 µg/mL. Casul não causou danos estruturais, tampouco reduziu a viabilidade celular. Efeito sinérgico entre CasuL e tetraciclina foi detectado para os isolados Sa e Ssp6PD, bem como entre a lectina e ampicilina sobre o isolado Ssp01. Foi detectada ação antibiofilme sobre os isolados de mastite Sa e Ssp5D. A combinação CasuL-tetraciclina inibiu a formação de biofilme por Sa, Ssp6PD e a combinação CasuL-ampicilina inibiu a formação de biofilme por

Ssp01. CasuL inibiu o crescimento (CMI: 125 µg/mL) e causou morte (CMI: 250 µg/mL) de células de *Candida krusei*, danificando a parede celular. CasuL (3,12–50 µg/mL) não induziu apoptose ou necrose dos esplenócitos e estimulou a proliferação celular após 48 h a 12,5 µg/mL. CasuL promoveu um aumento na produção das citocinas IL-2 e TNF-α. Em conclusão, os folíolulos de *C. surinamensis* contêm uma lectina termoestável com amplo potencial biotecnológico devido à toxicidade para células cancerosas e atividades imunomoduladora, antibacteriana e antifúngica.

Palavras-chave: Espanador-de-índio. Atividade antimicrobiana. Atividade imunomoduladora. Células cancerígenas. Atividade antibiofilme.

ABSTRACT

Lectins are proteins that bind specifically to carbohydrates or glycoconjugates. *Calliandra surinamensis* is a tropical perennial shrub widely used as an ornamental species. The aim of the present thesis was to purify a lectin (CasuL) from the *C. surinamensis* pinnulae, to characterize its structural and physicochemical properties, as well as to investigate cytotoxicity for cancer cells as well as antimicrobial, antibiofilm and immunomodulatory activities. Pinnulae proteins were extracted with 0.15 M NaCl and then precipitated with ammonium sulfate (60% saturation). The fraction of precipitated proteins was loaded onto a gel filtration column (Sephadex G-75). The isoelectric point (pI), native molecular mass, subunit composition and similarities of the primary structure with other plant proteins were determined. Stability of the hemagglutinating activity (HA) and the tertiary structure of CasuL against pH and temperature variations was also analyzed. CasuL cytotoxicity to human peripheral blood mononuclear cells (PBMCs) and leukemia (K562) and breast cancer (T47D) cells was determined. Antibacterial activity was evaluated against human pathogens and bovine and caprine mastitis isolates. Fungistatic and fungicidal effects of CasuL for *Candida* species were evaluated. CasuL was evaluated for toxicity and immunomodulatory activity on mice splenocytes. CasuL was isolated with a high purification factor (112), being an acidic (pI 5.82) and oligomeric protein with native molecular mass of 48 kDa. CasuL showed similarity (15%) to the sequence of a *Sorghum bicolor* protein. The lectin did not lose its HA and did not undergo unfolding when heated; on the other hand, conformational changes were detected when CasuL was incubated at alkaline pH, which were reflected in the loss of AH. CasuL was not cytotoxic to normal cells (PBMCs) but was active against K562 and T47D cells (IC_{50} of 67.04 and 58.75 μg/mL, respectively). For human pathogenic isolates of *Staphylococcus saprophyticcus* and *Staphylococcus aureus* (susceptible and resistant to oxacillin), CasuL (6.25-800 μg/mL) presented bacteriostatic action and was able to reduce biofilm formation. The lectin inhibited the growth of two isolates of bovine mastitis (Sa and SSp6PD) and two of caprine mastitis (Ssp01 and Ssp5D), with minimal inhibitory concentration (MIC) between 3.75 and 15 μg/mL. CasuL did not cause structural damage nor reduce cell viability. Antibiofilm activity on mastitis isolates Sa and Ssp5D was detected at inhibitory and subinhibitory concentrations. The CasuL-tetracycline combination inhibited biofilm formation by Sa, and Ssp6PD, and the CasuL-ampicillin combination inhibited biofilm formation by Ssp01. CasuL inhibited growth (MIC: 125 μg/mL) and caused death (MFC: 250 μg/mL) of *Candida krusei* cells, damaging the cell wall. CasuL (3.12-50

μg/mL) did not induce apoptosis or necrosis of splenocytes and stimulated cell proliferation after 48 h at 12.5 μg/mL. CasuL promoted an increase in the production of IL-2 and TNF-α cytokines. In conclusion, the *C. surinamensis* pinnulae contain a thermostable lectin with broad biotechnological potential due to its toxicity to cancer cells as well as immunomodulatory, antibacterial and antifungal activities.

Keywords: Pompon-du-marin. Antimicrobial activity. Immunomodulatory activity. Cancer cells. Antibiofilm activity.

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SUMÁRIO

1. INTRODUÇÃO

Lectinas são proteínas que se ligam de forma específica e não-covalente a carboidratos livres ou a glicoconjugados, sendo capazes de induzir respostas celulares e apresentando diversas atividades biológicas (COELHO et al., 2017). Essas proteínas estão envolvidas nos mecanismos de defesa de plantas e várias delas têm sido isoladas de plantas da família Fabaceae (Leguminosae), apresentando diferentes propriedades biológicas, tais como efeito vasorrelaxante (OSTERNE et al., 2014), ação imunomodulatória (MELO et al., 2010; BATISTA et al., 2017), atividades antitumoral (LACERDA et al., 2017), antifúngica (SILVA et al., 2014, WU et al., 2016), antibacteriana (CARVALHO et al., 2015), antibiofilme (CAVALCANTE et al., 2011), inseticida (SHANMUGAVEL et al., 2016) e antiprotozoária (AMINOU et al., 2016).

Lectinas vegetais têm apresentado notáveis propriedades anticancerígenas *in vivo* e *in vitro* e, em estudos com humanos, demonstraram que podem funcionar como terapia alternativa contra o câncer (MEJIA & PRISECARU, 2005; LIU et al., 2010; YAU et al., 2015). Estratégias para biodisponibilização de lectinas antitumorais têm sido investigadas (ANDRADE et al., 2004; BONIFÁCIO et al., 2014).

A atividade antimicrobiana de lectinas também tem sido descrita e geralmente está relacionada com a capacidade dessas proteínas de interagir com carboidratos presentes na superfície celular dos microrganismos. Lectinas com ação antibacteriana podem interagir com lipopolissacarídeos e peptideoglicanos presentes na parede celular de bactérias Gram-positivas e Gram-negativas, interferindo na viabilidade celular, bem como bloqueando sítios de interação com células do hospedeiro, prevenindo a infecção (IORDACHE et al., 2015; FANG et al., 2016; SILVA et al., 2016). As lectinas antifúngicas podem interagir com quitina, celulose, glucanas e manoproteínas presentes na parede celular, interferindo no crescimento fúngico, na homeostasia celular e na germinação de esporos (PAIVA et al., 2010; SANTOS et al., 2012).

A atividade antibiofilme de lectinas pode ocorrer por diversos mecanismos, tais como prevenção da adesão inicial das células bacterianas a superfícies e na alteração da expressão de genes associados com virulência e formação de biofilme (MOURA et al., 2017). A inibição da formação de biofilme em bactérias Gram-negativas por lectinas pode resultar de interações entre lectinas e lipopolissacarídeos, afetando assim a adesão. Ainda, lectinas também podem intergir com os sinais do *quorum sensing*, enzimas e polissacarídeos envolvidos na formação

do biofilme (CAVALCANTE et al., 2013; HASAN et al., 2014). Além de inibir a formação de biofilme, lectinas também são capazes de erradicar biofilmes preexistenstes (ISLAM et al., 2009; KLEIN et al., 2015).

Lectinas com ação imunomoduladora podem exercer diferentes efeitos em células do sistema imune. Tais efeitos podem ser desencadeados pela interação das lectinas com porções glicídicas de proteínas da superfície de células imunológicas, levando à alteração na produção de citocinas e óxido nítrico (KATRLÍK et al., 2010; GAO et al., 2013; SUNG et al., 2013), bem como na proliferação e na ativação de macrófagos (DIAS-NETIPANYJ et al., 2016), mastócitos (BARBOSA-LORENZI et al., 2016) e linfócitos (MELO et al., 2011).

Calliandra surinamensis (Fabaceae) é um arbusto tropical perene, de baixa ramificação, utilizado amplamente como espécie ornamental em todo o Brasil, mas pouco estudado quanto às suas propriedades biológicas e potencial biotecnológico. Plantas ornamentais geralmente apresentam de moderada a forte resistência contra patógenos e insetos e, por isso, podem ser potenciais fontes de moléculas bioativas (Patriota et al., 2016). Sendo as lectinas compostos que participam da defesa das plantas, foi estabelecida a hipótese de que folhas de *C. surinamensis* podem ser fontes dessas proteínas.

Nesse contexto, esta tese descreve a purificação, a caracterização e a avaliação de atividades biológicas de uma lectina obtida a partir dos foliólulos das folhas de *C. surinamensis*, denominada CasuL (do inglês *C. surinamensis lectin*). CasuL foi avaliada quanto à citoxicidade para células humanas normais e cancerígenas, quanto às suas propriedades antibacteriana e antibiofilme sobre patógenos humanos e sobre causadores de mastite em bovinos e caprinos, atividade antifúngica contra espécies de *Candida* e atividade imunomoduladora sobre esplenócitos murinos.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. O GÊNERO *Calliandra*

O gênero *Calliandra* pertence à família Fabaceae, que é uma das maiores do reino vegetal, possuindo distribuição cosmopolita, com cerca de 18 mil espécies distribuídas dentro de 650 gêneros. No Brasil, têm sido descritas cerca de 1.500 espécies desta família (EMBRAPA, 2016). A qual é subdividida em três subfamílias: Faboideae (Papilionoideae), Caesalpinioideae e Mimosoideae. A subfamília Mimosoideae compreende quatro tribos (Acacieae, Ingeae, Mimoseae e Mimozygantheae), 78 gêneros e aproximadamente 3.270 espécies (LEWIS et al., 2005). Em Ingeae são reconhecidos dois grupos: um com folhas pinadas, representado pelo gênero *Inga*, e o outro com folhas bipinadas, constituído pelos demais gêneros, incluindo *Calliandra* (ROMERO, 2005).

O gênero *Calliandra* inclui leguminosas utilizadas de diversas formas pela população. As folhas e os ramos de espécies deste gênero são fontes de substâncias, tais como compostos fenólicos, com ação contra nematóides intestinais de ruminantes (*Calliandra calotyrsus*) (WABO et al, 2011), com propriedades analgésica, anticonvulsiva e antiulcerogênica (*Calliandra porturicensis*) (AGUWA & LAWAL, 1988; AKAH & NWAIWU, 1988) e também utilizadas contra malária e leishmaniose (*Calliandra pulcherrima*) (MILLIKEN, 1997).

Calliandra surinamensis, conhecida popularmente como “esponjinha rosa” ou “espanador de índio”, é um arbusto tropical perene (Figura 1A) cujas folhas são bipinadas (Figuras 1B, 1C) (com folíolos compostos por 6 a 8 pares de foliólulos) e unijugadas, não sensitivos, subcoriáceos, discolares e glabros (SOUSA et al., 2009). Essa planta é amplamente utilizada como espécie ornamental, mas pouco estudada quanto às suas propriedades biológicas e potencial biotecnológico.

C. surinamensis é usada na medicina popular para o tratamento de tosses, feridas e inflamações (OMAR et al., 2016). Extrato metanólico da casca apresentou atividade antimicrobiana, (IRABOR et al., 2007) e o extrato metanólico das folhas e suas frações apresentaram efeitos antimicrobianos e ação trombolítica moderada (SIKDER et al., 2012). Miricitrina, lupeol, ácido ferúlico e flavonoides glicosídeos foram identificados em casca, folhas e flores de *C. surinamensis*, mas nenhuma atividade biológica tem sido atribuída a essas moléculas (ALZAHRANI et al., 2016; OMAR et al., 2016).

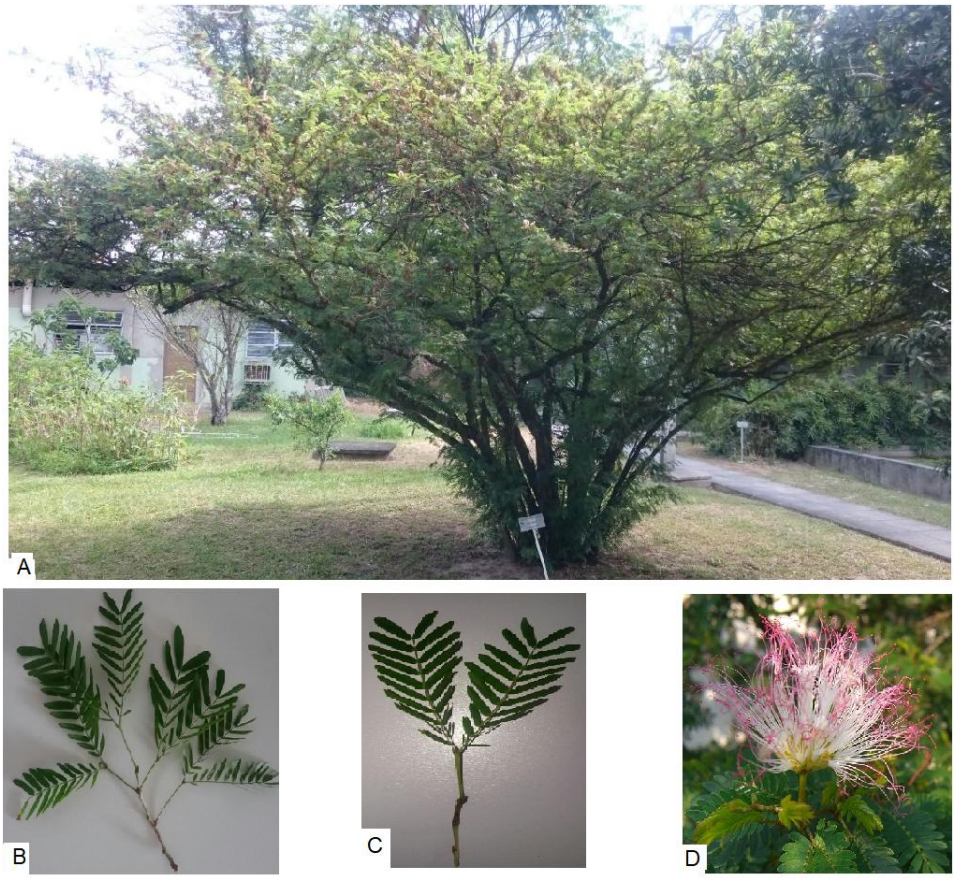

Figura 1: *Calliandra surinamensis*. (A) Visão geral da planta. (B) Folhas. (C) Folha bipinada, com folíolos unijugados, cada um contendo vários pares de foliólulos.(D) Inflorescência. Fonte: A, B e C: Thamara Procópio (2016), D: Philipp Weigell (2008)

2.2. COMPOSTOS ENVOLVIDOS NA DEFESA DAS PLANTAS

As plantas estão naturalmente expostas a diversos tipos de condições adversas, tais como oscilações das condições abióticas e ataque de diferentes tipos de patógenos (vírus, bactérias, fungos, insetos). Consequentemente, elas desenvolveram ao longo de sua evolução mecanismos físicos, químicos e biológicos capazes de estabelecer uma resposta eficiente para sua proteção (MUTHAMILARASAN & PRASAD, 2013; WIRTHMUELLER et al., 2013).

Os mecanismos de defesa das plantas podem ser divididos em constitutiva e induzida. A defesa constitutiva está presente de modo contínuo e representada por estruturas morfológicas (tricomas, cutículas, epiderme espessada, abundância de cristais e fibras nas folhas) e compostos químicos que dificultam o acesso de agentes danosos às plantas. Já a defesa induzida ocorre em resposta à exposição a uma determinada condição ambiental ou ao

ataque por patógeno, herbívoro ou predador, provocando, assim, alguma mudança na morfologia ou fisiologia da planta (KARBAN & BALDWIN, 1997; SÃO-JOÃO & RAGA, 2016).

A defesa química pode ser mediada por metabólitos primários e secundários. Os metabólitos primários (carboidratos, lipídeos, proteínas, ácidos nucleicos) estão presentes em todas as plantas e promovem os processos básicos de sobrevivência, atuando como intermediários nas vias catabólicas e anabólicas, além de constituírem ponto de partida para a produção dos metabólitos secundários. Os metabólitos secundários (Figura 2) incluem compostos nitrogenados (alcaloides, por exemplo), terpenoides e compostos fenólicos (taninos, flavonoides, ligninas, derivados cinâmicos), e apresentam funções como defesa contra herbívoros e microorganismos, proteção contra raios ultravioleta, atração de animais polinizadores ou dispersantes de sementes, dentre outras funções (SANDES & DI BLASI, 2000; TAIZ & ZEIGER, 2009).

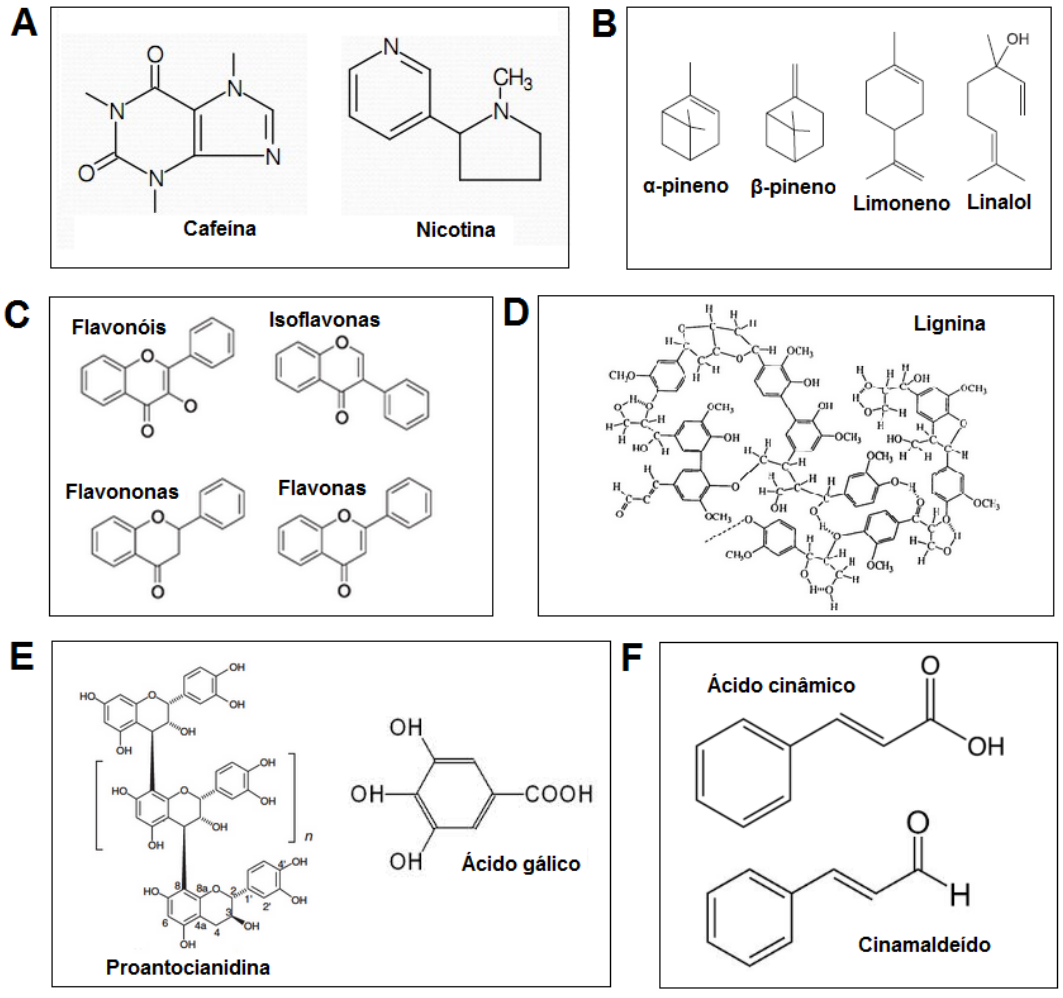

Figura 2: Metabólitos secundários produzidos por plantas pertencentes às classes dos alcaloides (A), terpenoides (B), flavonoides (C), ligninas (D), taninos (E) e compostos cinâmicos (F).

Tradicionalmente, os metabólitos secundários são os mais estudados na busca por novos compostos bioativos. No entanto, o estudo da defesa das plantas identificou a importância de receptores celulares e proteínas de defesa (metabólitos primários) que reconhecem patógenos invasores e desencadeiam respostas celulares para proteger a planta ainda na etapa inicial da infecção (LANNOO & VAN DAMME, 2014). Também existem proteínas que atuam reduzindo os níveis de herbivoria e predação aos órgãos vegetais (BODE & HUBER, 1992; PINTO et al., 2011).

Os receptores de reconhecimento de patógenos (PRPs) são exemplos da participação de proteínas no sistema imune inato de plantas. Os PRPs reconhecem dois tipos de moléculas: as que estão associadas a danos celulares causados pela entrada de invasores (DAMPs: *damage associated molecular patterns*) como, por exemplo, fragmentos de celulose, monômeros de cutina e defensinas; e as que estão presentes em estruturas microbianas (PAMPs/MAMPs: *pathogen/microbial associated molecular patterns*), por exemplo lipopolissacarídeos de bactérias Gram-negativas, peptideoglicanos de bactérias Gram-positivas, flagelos, quitina e determinadas proteínas (LANNOO & VAN DAMME, 2014).

Outra classe de proteínas envolvidas na defesa das plantas são as lectinas, proteínas que se ligam especificamente a carboidratos livres ou glicoconjugados, sem promoverem alterações na estrutura covalente dos ligantes. As lectinas de plantas são capazes de reconhecer organismos invasores ou danos na parede celular das plantas e, consequentemente, desencadear respostas eficientes ao ataque, tais como ativação celular e imobilização de patógenos (COSTA et al., 2010; LANNOO & VAN DAMME, 2014; MOURA et al., 2015). Lectinas também podem ser ingeridas por herbívoros e predadores, diminuindo a assimilação de nutrientes, retardando o crescimento e desenvolvimento, bem como induzindo a morte por inanição (MARGIS et al., 2008; PINTO et al., 2011; PAIVA et al., 2013). Dessa forma as plantas tornam-se menos atrativas para hebívoros e predadores.

Muitos dos PRPs carregam um domínio lectínico para o reconhecimento de glicanos presentes em microorganismos e em derivados de danos da parede celular. Os PRPs e outros receptores celulares podem ou não estar ligados a proteínas quinases, ativando diversos eventos de sinalização intracelulares, como produção de espécies reativas de hidrogênio, visando impedir o crescimento microbiano. Além dessa percepção inicial, as plantas possuem uma segunda barreira de proteção, conhecida como imunidade desencadeada por efetores (*effector-triggered immunity*, ETI) que atua dentro da célula, ativando genes de resistência

específica a produzir proteínas de defesa, dentre elas lectinas (WIRTHMUELLER et al., 2013, LANNOO & VAN DAMME, 2014).

2.3. LECTINAS: CARACTERÍSTICAS GERAIS, PURIFICAÇÃO E CARACTERIZAÇÃO

O início do estudo sobre lectinas foi atribuído a Peter Hermann Stillmark (1888), que observou a aglutinação de células por extratos vegetais. Ele identificou a ricina, uma proteína hipotética presente no extrato tóxico de *Ricinus comunis*, que era capaz de aglutinar hemácias de diferentes animais. Dessa forma, além de sua capacidade aglutinante, as lectinas foram inicialmente conhecidas por sua toxicidade, porém, Landsteiner e Raubitschek (1907) descobriram lectinas de leguminosas não tóxicas provenientes de *Phaseolus vulgaris, Pisum sativum, Lens culinaris* e *Vicia sativa*. A partir de então outras lectinas foram identificadas. O termo lectina (derivado do latim *legere*, que significa "selecionado") foi proposto na década de 40 e até hoje os termos aglutinina e hemaglutinina são usados como sinônimos de lectina (VAN DAMME et al., 2008).

Lectinas estão amplamente distribuídas na natureza e vêm sendo isoladas de bactérias (VAINAUSKAS et al., 2016), fungos (WANG et al., 2018), plantas (SILVA et al., 2018) e animais (SREERAMULU et al., 2018). Em plantas, lectinas vêm sendo isoladas nos mais variados tecidos, tais como flores, folhas, sementes, frutos e raízes (DIAS et al., 2015). As lectinas possuem, pelo menos, um domínio não catalítico capaz de reconhecer e ligar seletiva e reversivelmente a carboidratos livres ou conjugados. A seletividade de lectinas a determinados carboidratos é determinada por sua sequência proteica e por sua conformação. Resíduos aromáticos, por exemplo, podem estar envolvidos na especificidade da lectina a monossacarídeos e oligossacarídeos (COLLINS et al., 2004; HAN et al., 2005; OHTSUBO et al., 2005; MACEDO et al., 2015). O sítio de ligação a carboidratos pode conter íons metálicos (por exemplo, Mn^{2+} e Ca^{2+}), que são requeridos para a interação com carboidratos.

A detecção de lectinas que possuem pelo menos 2 sítios de ligação a carboidratos é realizada através de ensaio de hemaglutinação, o qual explora a capacidade de interação entre a lectina e os carboidratos da superfície de eritrócitos: quando a lectina consegue interagir com esses carboidratos, ocorre a formação de uma rede de hemaglutinação (Figura 3A), caso contrário tais eritrócitos ficarão depositados no fundo da placa (KENNEDY et al., 1995). No entanto, alguns compostos (como taninos, lipidios e íons bivalentes) podem promover dispersão dos eritrócitos, criando uma falsa impressão de hemaglutinação. Dessa forma, para

confirmar que a aglutinação é promovida por lectinas, é realizado um teste de inibição em que são adicionados carboidratos livres ou glicoproteínas que interagem com as moléculas de lectina e, assim, impedem que a mesma se ligue à superfície dos eritrócitos e forme a rede de aglutinação (Figura 3B). O resultado para ambos os testes é de fácil visualização em laboratório.

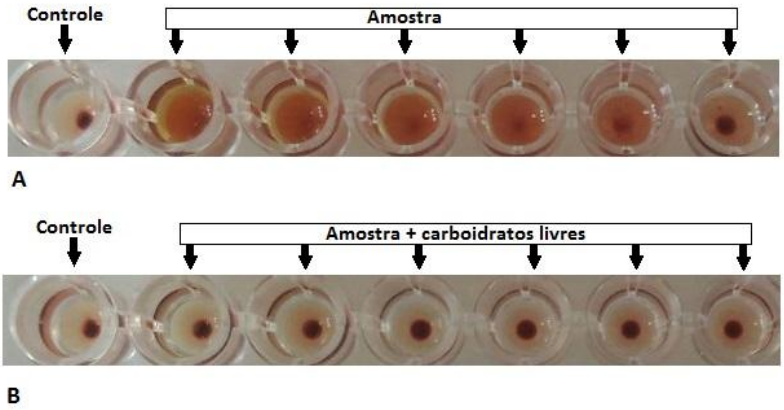

Figura 3: Detecção de lectinas através dos ensaios de atividade hemaglutinante (A) e inibição da atividade hemaglutinante (B). Fonte: Elaborada pelo autor

O ensaio de hemaglutinação é de fundamental importância para o monitoramento da atividade da lectina ao longo do processo de purificação, bem como possibilita a caracterização da especificidade de ligação a carboidratos através do ensaio de inibição, e a determinação da estabilidade em diferentes condições fisico-químicas, como variação de pH e temperatura, e na presença de íons e de outras substâncias capazes de alterar a estrutura e/ou atividade da lectina.

Lectinas podem ser classificadas nos seguintes grupos, conforme o número e característica dos domínios que possuem: *merolectinas*, quando possuem apenas um domínio de ligação a carboidratos e dessa forma não apresentam atividade aglutinante; *hololectinas*, que apresentam dois ou mais domínios ligadores de carboidratos; *quimerolectinas*, que possuem um domínio que liga a carboidratos e outro que possui uma atividade biológica diversa; e *superlectinas*, que possuem dois ou mais domínios que reconhecem estruturas diferentes de carboidratos (VAN DAMME et al., 1998). Outra classificação para lectinas se baseia na estrutura e sequência dos domínios que reconhecem carboidratos: (1) aglutininas homólogas a *Agaricus bisporus*, (2) amarantinas, (3) homólogas de quitinase classe V, (4) aglutinina de *Euonymus europaeus*, (5) aglutinina de *Galanthus nivalis*, (6) proteínas com domínio heveínico, (7) jacalinas, (8) lectinas de leguminosas, (9) lectinas com domínio Lys

M, (10) aglutinina de *Nicotiana tabacum* e (11) família ricina-B (MACEDO et al., 2015). Contudo, muitas das lectinas conhecidas não necessariamente se enquandram em uma dessas 11 classes.

As lectinas de plantas podem ser produzidas constitutivamente ou apenas em resposta ao estresse ambiental ou ataque de patógenos (lectinas induzíveis). Normalmente, as lectinas produzidas constitutivamente são armazenadas em vacúolos ou no espaço extracelular e estão presentes, principalmente, em tecidos de armazenamento e sementes; já as induzidas são encontradas no núcleo e no citoplasma (PEUMANS &VAN DAMME, 1995; LANNOO & VAN DAMME, 2010). Estudos analisando mudanças na expressão gênica de trigo (*Triticum aestivum*) durante infecção pela mosca *Mayetiola destructor* identificaram três genes de lectinas provavelmente envolvidos na defesa da planta, uma vez que a expressão desses genes aumentaram após a infestação quando comparado com plantas não infestadas (WILLIAMS et al., 2002; PUTHOFF, et al., 2005; MACEDO et al., 2015).

As estratégias para o isolamento de lectinas se utilizam das etapas gerais para purificação de proteínas. Ao serem extraídas de seu meio biológico, as proteínas ficam expostas a diversas condições ambientais que são diferentes daquelas do meio em que se encontravam originalmente, o que podem comprometer sua estrutura e função. Sendo assim, é necessário, ao longo de todo o processo de purificação, certificar-se de que a proteína isolada está mantendo sua atividade biológica. Ainda, o processo deve resultar em um aumento da atividade específica (relação entre a atividade da proteína de interesse e o total de proteínas presentes na amostra), o que indica que as moléculas da proteína em estudo estão sendo concentradas, enquanto proteínas que não são desejadas estão sendo eliminadas (PERALTA, 2003; SANTOS et al., 2013). No caso de lectinas, a atividade hemaglutinante é utilizada para monitorar a presença dessas proteínas durante o processo de purificação.

O primeiro passo para purificação de lectinas consiste em extrair a proteína do tecido. Para isso é necessário levar em consideração quais são as condições em que a proteína é mais facilmente extraída do tecido, mas que também permanece mais estável. Fatores importantes a serem considerados são pH, temperatura, necessidade de uso de detergentes ou agentes desnaturantes (proteínas de membrana), presença de íons metálicos, entre outros (SINGH et al., 2018; WANG et al., 2018; SREERAMULU et al., 2018).

Após a extração, podem ser empregadas etapas de pré-purificação com a finalidade de concentrar a proteína de interesse e eliminar as demais. As técnicas de pré-purificação podem explorar diferenças de solubilidade, de pH ótimo e de estabilidade frente a variações de

temperatura. Uma das técnicas mais utilizadas é a precipitação com sulfato de amônio, uma vez que este sal é altamente hidrofílico, pode ser facilmente removido por diálise e as proteínas precipitadas por ele mantêm sua conformação nativa (PERALTA, 2003; PAIVA et al., 2011; NELSON & COX, 2014).

Técnicas cromatográficas são constituídas por duas fases (estacionária e móvel) e consistem em um método físico-químico de separação dos componentes de uma mistura, que se separam devido à migração/interação diferencial conforme a passagem da fase móvel através da fase estacionária (Figura 4A). A fase estacionária determina o tipo de cromatografia, e as mais usadas na purificação de proteínas sãs as matrizes de exclusão molecular, troca iônica ou afinidade (PERALTA, 2003; STRYER, 2004; NELSON e COX, 2014).

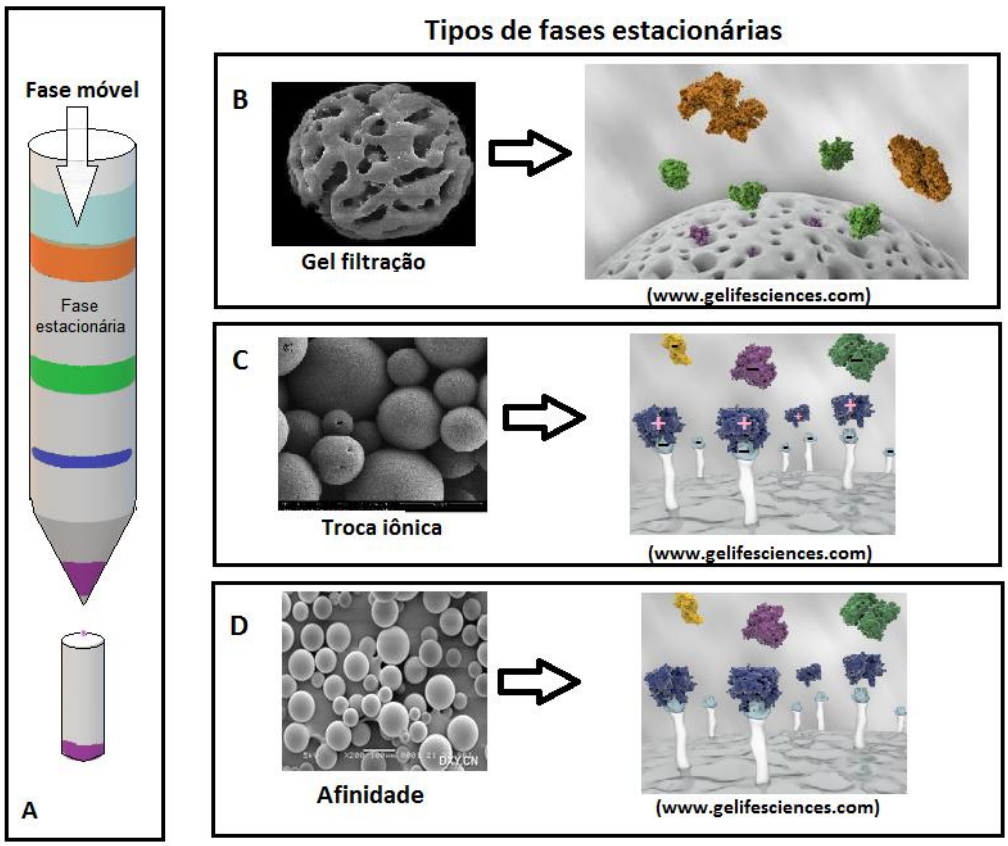

Figura 4: Métodos cromatográficos. (A) Esquema geral das duas fases que constituem uma cromatografia. Representação das matrizes usadas como fases estacionárias para purificação de lectinas por cromatografia de gel filtração (B), troca iônica (C) e afinidade (D). Fontes: B: Tiainen et al., 2007 e www.gelifesciences.com; C: Staby et al., 2005 e www.gelifesciences.com; D: www.bonabio.com e www.gelifesciences.com.

A cromatografia de exclusão molecular (ou gel filtração) separa as moléculas conforme o tamanho e forma. A fase estacionária é formada por uma matriz composta por microesferas porosas (Figura 4B). Moléculas menores penetram nos poros das microesferas tendo o seu percurso retardado enquanto as maiores não são capazes de penetrar nos poros e são excluídas rapidamente da coluna (SANTOS et al., 2013; NELSON e COX, 2014). Exemplos de matrizes usadas nesse processo são a Sephadex, a Sepharose e o Bio-Gel (PERALTA, 2003; PROCÓPIO et al., 2016).

A cromatografia de troca iônica separa as proteínas de acordo com a sua carga elétrica líquida, a qual varia conforme o pH do meio. A carga líquida de proteínas é positiva em valores de pH abaixo do seu ponto isoelétrico (PI) e negativa em valores de pH acima do PI. A fase estacionária é constituída por uma matriz carregada (positiva ou negativamente) que retarda a passagem de proteínas com carga oposta (Figura 4C). A eluição das proteínas adsorvidas pode ser realizada através de alterações no pH ou da força iônica da fase móvel. São exemplos de matrizes de troca iônica DEAE-celulose e CM-celulose (PERALTA, 2003; NELSON & COX, 2014).

A cromatografia por afinidade separa as proteínas conforme a ligação específica entre a proteína e a matriz (fase estacionária) (Figura 4D). Dessa forma, a proteína de interesse fica retida na coluna enquanto as outras são rapidamente eliminadas. Após essa eliminação, a proteína ligada à matriz pode ser eluída por mudança no pH ou na molaridade da fase móvel, bem como por irrigação da coluna com solução do ligante específico na forma livre. No caso de lectinas, as matrizes de afinidade podem conter monossacarídeos ou glicoconjugados imobilizados, bem como podem consistir em suportes polissacarídicos (PERALTA, 2003; COELHO et al., 2012; SANTOS et al., 2013; NELSON & COX, 2014).

A caracterização de lectinas consiste no uso de diferentes técnicas experimentais que visam identificar características da proteína relacionadas a grau de pureza, constituição de aminoácidos, conformação, constituição em subunidades, especificidade de ligação, condições em que mantêm sua atividade, dentre outras características.

A eletroforese em gel de poliacrilamida é um método analítico que consiste na migração, em gel de poliacrilamida, de partículas carregadas sob influência de um campo elétrico. Esse gel pode ter malhas com tamanhos diferentes, que variam de acordo com a concentração de acrilamida e bis-acrilamida. Por meio da separação das proteínas por entre as malhas desse gel e sua posterior visualização utilizando técnicas de coloração, é possível verificar o grau de pureza da amostra, bem como o ponto isoelétrico e a massa molecular da

proteína (PERALTA, 2003; PAIVA et al., 2011). Existem diversos tipos de eletroforese: para proteínas ácidicas ou básicas (verifica proteínas em condição nativa e são usadas para avaliação de homogeneidade), em condições desnaturantes (verifica o peso molecular da amostra e sua composição em subunidades), em condições redutoras (verifica presença de subunidades unidas por pontes dissulfeto) e eletroforese bidimensional (permite a análise do ponto isoelétrico e massa molecular da proteína, possibilitando a detecção de isoformas[1]) (PERALTA, 2003; SANTOS et al., 2013).

O agente desnaturante mais utilizado em eletroforese é o dodecilsulfato de sódio (SDS). O SDS se liga às porções hidrofóbicas da proteína fazendo com que esta permaneça em uma conformação estendida estável e adiciona carga negativa a toda a proteína anulando a sua carga líquida original. Assim, a velocidade da migração depende apenas do tamanho do complexo proteína-SDS: quanto menor for esse complexo, mais rápida é a migração. A presença do SDS separa ainda subunidades que estavam unidas por ligações não-covalentes. Para separa as subunidades unidas por pontes dissulfeto é necessário utilizar um agente redutor, como β-mercaptoetanol ou ditiotreitol (PERALTA, 2003; PAIVA et al., 2011).

A eletroforese bidimensional é formada por duas etapas: a primeira corresponde à focalização isoelétrica, na qual as proteínas migram em um meio com um gradiente de pH até o pH correspondente ao seu ponto isoelétrico. Após a migração nessa etapa, a amostra é colocada em um gel de poliacrilamida, que corresponde à segunda etapa, sendo separada quanto a sua massa molecular (PERALTA, 2003; SANTOS et al., 2013).

A fluorimetria é uma técnica que permite medir a luz emitida por uma substância em consequência de absorção de luz. Quando uma molécula em seu estado basal absorve luz, seus elétrons adquirem uma energia cinética que poderá ser capaz de mover esses elétrons a um estado excitado com um nível de energia maior. Ao retornar ao seu estado basal esses elétrons liberam energia na forma de calor ou de luz. A emissão de luz é chamada de fluorescência e possui sempre um comprimento de onda maior que a luz de excitação, pois parte da energia é perdida na forma de calor (LOPES, 2009).

Essa técnica tem sido muito utilizada para investigar alterações na estrutura terciária de proteínas (LAKOWICZ et al., 2006; MESQUITA, 2010). Esse estudo pode se basear na fluorescência intrínseca das proteínas, a partir da análise dos aminoácidos aromáticos presentes na cadeia polipeptídica, os quais podem ficar mais ou menos expostos ao meio aquoso, alterando o seu padrão de emissão de fluorescência, conforme as condições físico-

[1]Isoformas: Formas distintas de uma proteína que são produzidas a partir de genes diferentes ou por processos alternativos, ou seja, pelo processamento alternativo de um mesmo gene.

químicas em que a proteína se encontra. Mas as análises também podem ser efetuadas analisando a fluorescência extrínseca de um marcador ou sonda que se liga de forma não covalente à cadeia polipeptídica (COSTA, 2008).

Um exemplo de sonda fluorescente para uso em estudos com proteínas é o bis-ANS (4,4′-bis-1-anilinonaftaleno-8-sulfonato), um composto que não fluoresce ou fluoresce fracamente em água, mas fluoresce fortemente quando em ambiente hidrofóbico; a fluorescência do bis-ANS pode aumentar em até 200 vezes quando ligado a estruturas hidrofóbicas (ROSEN & WEBER, 1969; COSTA, 2008). Esse fenômeno permite acompanhar alterações estruturais durante a desnaturação da proteína, pois a exposição de sítios hidrofóbicos à água resulta em diminuição da fluorescência dessa sonda. Normalmente, os espectros de bis-ANS são obtidos excitando as amostras a 360 nm e o espectro de emissão é monitorado entre 400 e 600nm (COSTA, 2008).

2.4. LECTINAS DE FABACEAE

Fabaceae (leguminosas) é a família de plantas mais explorada no estudo de lectinas, muitas das quais se caracterizam por possuírem alta similaridade estrutural, embora as propriedades biológicas e especificidade a carboidratos sejam distintas (ALMEIDA, 2016). A similaridade destas lectinas está principalmente relacionada às suas estruturas primária e terciária (BRINDA et al., 2005; MORENO, 2008). Quanto à estrutura quaternária, as lectinas de leguminosas raramente são proteínas monoméricas, sendo geralmente encontradas nas formas dimérica ou tetramérica (MORENO, 2008). Contudo, nem todas as lectinas isoladas de vegetais pertencentes a esta família possuem estruturas típicas de lectinas de leguminosas (VACARI, 2010).

Acredita-se que essas proteínas proporcionam proteção aos tecidos vegetais em que estão presentes, pois é conhecida a toxicidade de muitas lectinas de leguminosas para predadores de plantas (CAVADA et al., 1996; CHRISPEELS & RAIKHEL, 1991; SAUVION et al., 2004a, 2004b; BARBOSA, 2013). Um estudo com plantas de tabaco transgênicas que expressavam o gene da lectina de *Pisum sativum* (ervilha) demonstrou um aumento da resistência do tabaco em relação ao verme *Heliothis virescens* (BOULTER et al., 1990). Larvas do inseto *Meligethes aeneus* que se alimentaram de *Brassica napus* geneticamente modificada para expressar a lectina de *Pisum sativum* (PSA) tiveram uma perda no ganho de massa com consequente redução da taxa de crescimento (MELANDER et

al., 2003). Larvas de *Spodoptera exígua* que se alimentaram de folhas do tabaco modificadas pela inserção do gene da lectina SBL (do inglês, *soybean lectin*) tiveram uma perda de peso considerável, retardando o desenvolvimento das larvas e a metamorfose (GOU, et al., 2013)

O estudo de lectinas de leguminosas está principalmente focado em membros da subfamília Papilionioidae, enquanto a investigação sobre lectinas de Caesalpinioideae e Mimosoideae é menos abundante (SILVA et al., 2014). A primeira lectina isolada da subfamília Mimosoideae foi a de sementes de *Parkia speciosa* (SUVACHITTANONTS & PEUTPAIBOON, 1992); em seguida outras lectinas desse gênero foram isoladas tais como as lectinas de sementes de *Parkia platycephala* (CAVADA et al., 1997*)*, *Parkia discolor* (CAVADA, 2000) e *Parkia biglobosa* (SILVA, 2013). Santi-Gadelha et al. (2012) isolaram uma lectina das sementes de *Acacia farnesiana*. Não há registros de lectinas isoladas do gênero *Calliandra*.

O estudo de proteínas bioativas em plantas ornamentais é estimulado pela resistência, de moderada a forte, que essas plantas geralmente apresentam contra patógenos e insetos (PATRIOTA et al., 2016). Sendo as lectinas proteínas que participam da defesa das plantas, foi estabelecida a hipótese de que folhas de *C. surinamensis* podem ser fontes dessas proteínas.

2.5. LECTINAS ANTI-CÂNCER

O câncer ocorre quando determinadas células perdem o controle sobre a divisão celular, ocorrendo uma proliferação anormal formando tumores. Estes podem ser denominados benignos (células permanecem localizadas, prejudicando apenas o órgão em que estão localizadas) ou malignos (as células perdem a capacidade de diferenciação e de aderência, tornando-se capazes de invadir e colonizar os mais diversos tecidos e órgãos, fenômeno conhecido como metástase) (FAHEINA-MARTINS et al., 2012; ARAÚJO, 2015). Ainda, células em tumores malignos podem apresentar resistência à morte apoptótica (ABBAS et al., 2012).

As células cancerígenas são fenotipicamente diferentes das células normais como, por exemplo, no padrão de carboidratos expressos na superfície celular. Essas mudanças podem ser causadas por alteração na atividade de glicosiltransferase e glicosidases e são observadas em glicolipídeos, glicoesfingolipideos e glicoproteínas de membrana (GHAZARIAN et al., 2011). Tal diferença no padrão de glicosilação entre células tumorais e células normais

permite que as lectinas sejam capazes de distinguir entre estas células e células cancerígenas (TOZAWA-ONO et al., 2017).

Lectinas vegetais têm apresentado notáveis propriedades anticancerígenas *in vivo* e *in vitro* e, em estudos de casos humanos, demonstraram que podem funcionar como terapia alternativa contra o câncer (MEJIA & PRISECARU, 2005; LIU et al., 2010; YAU et al., 2015). Estratégias para biodisponibilização de lectinas antitumorais têm sido investigadas. Por exemplo, a lectina recombinante de *Cratylia mollis* (rCramoll) encapsulada em lipossomas reduziu em 75% o crescimento do tumor em camundongos transplantados com células de sarcoma 180 sem provocar toxicidade substancial ao animal (BONIFÁCIO et al., 2014; CUNHA et al., 2016). As lectinas podem ser tóxicas para células cancerígenas via diferentes mecanismos, os quais podem ser indução de apoptose, autofagia ou necrose e inibição do crescimento celular (LEI & CHANG, 2009).

Lectinas de plantas promovem apoptose ou autofagia por modularem importantes vias de sinalização que envolvem proteínas das famílias Bcl-2, caspases, p53, PI3K/Akt, ERK, BNIP3, Ras-Raf e ATG (YAU et al., 2015). Esses mecanismos são geralmente iniciados pela interação com receptores específicos, glicosilados ou não, na membrana das células cancerígenas; após este passo, as lectinas podem ser internalizadas via endocitose, direcionadas a diferentes compartimentos e ativar outras cascatas de sinalização ligadas à morte celular (GABOR et al., 2001; YAU et al., 2015). Lectina isolada do veneno de *Bothrops leucurus* exibiu atividade citotóxica contra linhagens de células tumorais humanas (K562, NCI-292 e Hep-2); a detecção de externalização de fosfatidilserina e de despolarização mitocondrial indicam que a morte celular foi por indução de apoptose (NUNES et al., 2012). A lectina coagulante de *Moringa oleifera* (cMoL) foi citotóxica para as células B16-F10 de melanoma murino, aumentando a produção de espécies reativas de oxigênio e promovendo a ativação das caspases 3, 8 e 9, indicando assim a ativação de vias relacionadas à apoptose (LUZ et al., 2017).

A lectina de *Polygonatum cyrtonema* pode ser internalizada em células de melanoma e, em seguida, induzir a apoptose pela regulação positiva da proteína pró-apoptótica Bax e regulação negativa das proteínas anti-apoptóticas Bcl-2 e Bcl-x_L, causando colapso do potencial de membrana mitocondrial e extravasamento do citocromo c, bem como ativação de caspase-3 e caspase-9 (LIU et al., 2009). A lectina de *P. cyrtonema* também induz morte em células tumorais de forma autofágica através da ativação da via mitocondrial ROS-p38-p53, bem como bloqueando as vias Ras-Raf e P13K-Akt, sugerindo uma relação entre autofagia e

apoptose (WANG et al., 2011). A lectina de *Canavalia ensiformis* (ConA) também foi capaz de induzir autofagia em células de hepatomas através de uma via mediada por mitocôndria. Essa lectina liga-se a manose presente nas glicoproteínas da membrana celular e é internalizada para a mitocôndria via endocitose mediada por clatrinas, inicinado a autofagia (CHANG et al., 2007).

A lectina de *Mytilus galloprovincialis* (MytiLec), quando em contato com células tumorais de linfoma de Burkitt, ativa várias vias celulares, dentre essas a expressão do fator de necrose tumoral (TNF)-α e ativação de mitocôndria controlada por caspase-9 e carpase-3 (HASAN et al., 2015). A lectina purificada do veneno de *Bothrops leucurus* induziu morte em células de melanoma (B16-F10) via necrose. Acredita-se que o efeito dessa lectina seja decorrente do aumento da concentração de íons de cálcio no citosol e formação de superóxido mitocondrial, promovendo assim a abertura do poro de transição de permeabilidade mitocondrial (ARANDA-SOUZA et al., 2014).

2.6. ATIVIDADE ANTIMICROBIANA DE LECTINAS

2.6.1. Ação antibacteriana

Bactérias são procariotos (Domínio Bacteria, Reino Eubacteria) e possuem no citoplasma uma região chamada nucleóide e diversos grânulos. O citoplasma é envolvido por uma membrana plasmática a qual está envolta por uma camada espessa e rígida denominada parede celular. Essa parede bacteriana é formada por uma rede de peptideoglicano, o qual é constituído por uma porção dissacarídica formada por repetições de dissacarídeos (*N*-acetilglicosamina e ácido *N*-acetilmurâmico) e por polipeptídeos que unem esses dissacarídeos para formar a rede. As bactérias Gram-positivas possuem muitas camadas de peptideoglicano, formando estruturas mais rígidas e espessas, enquanto que as Gram-negativas contêm somente uma camada de peptideoglicano (Figura 5). A parede celular compreende diversas estruturas externas como glicocálix, flagelos, filamentos axiais, fímbrias e pili. A membrana plasmática bacteriana possui moléculas receptoras, proteínas transportadoras e complexos da cadeia respiratória (JUNQUEIRA & CARNEIRO, 2012).

A maioria das bactérias é inofensiva e muitas são benéficas por desempenharem diversas relações ecológicas, simbiontes e mutualísticas; entretanto, algumas podem causar graves infecções em humanos e outros animais, como meningite, endocardites, gastroenterites

e infecções no trato urinário (MOURA et al., 2012). Em bovinos e caprinos, infecções bacterianas nas glândulas mamárias conhecidas como mastites resultam em prejuízos econômicos, pois levam à redução da produção de leite bem como contaminação do leite (GUIMARÃES et al., 2017). Tal redução ocorre devido à destruição dos tecidos produtores de leite (SCHROEDER, 2012; MUSHTAQ et al., 2018). A contaminação do leite pode estar associada com a presença de enterotoxinas que podem prejudicar a saúde humana bem como a disseminação de microrganismos resistentes a antibióticos no ambiente (SCALI et al., 2015). Dentre as bactérias patogênicas, *Staphylococcus aureus* é uma das maiores responsáveis por mastites clínica e subclínica (ZADOKS & FITZPATRICK, 2009; PEIXOTO et al., 2010; KLEIN et al., 2015), no entanto outras bactérias Gram-positivas e Gram-negativas como estreptococos, *Escherichia coli* e *Klebsiella pneumoniae* também podem causar essa infecção (CONTRERAS e RODRÍGUEZ, 2011).

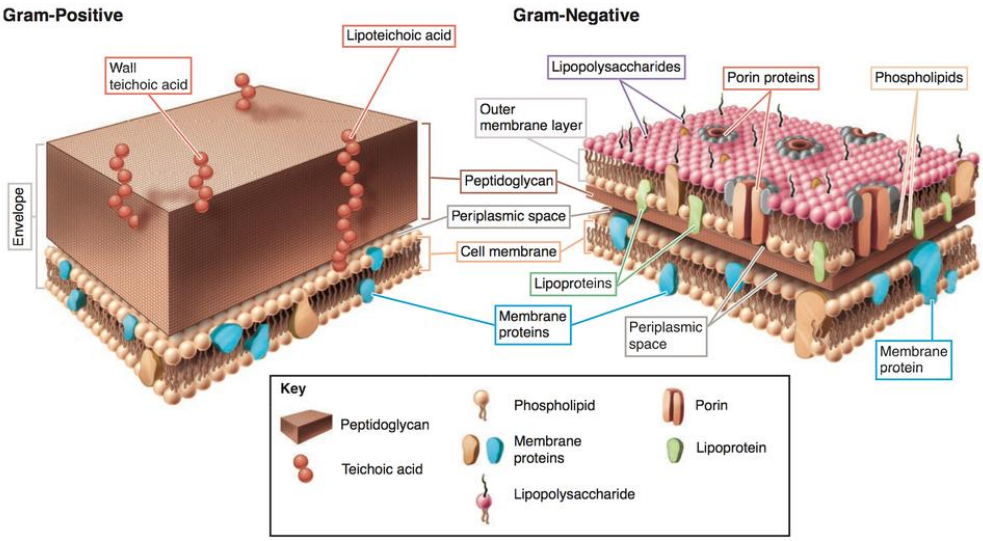

Figura 5: Componentes da parede celular de bactérias Gram-positivas e Gram-negativas.

Fonte: http://microbioenergetica.squarespace.com

O uso indiscriminado de substâncias antimicrobianas vem selecionando bactérias resistentes, devido à pressão seletiva que elas exercem sobre esses organismos favorecendo as cepas resistentes. Isso ocorre principalmente em ambientes hospitalares, no entanto, os antibióticos também estão presentes nos mais diversos ambientes como no alimento e na água, aumentando ainda mais a disseminação da resistência (LERBECH et al., 2014). São diversos os mecanismos de resistência tais como: destruição e inativação enzimática do antibiótico (como por exemplo, as bactérias produtoras de β-lactamases); prevenção da entrada da droga no sítio-alvo dentro do microrganismo; alteração no sítio-alvo da droga e

acelereção do efluxo da droga. Essa resistência pode ser também trasmitida entre células bacterianas através da transferência horizontal de genes de bactérias doadoras por conjugação, transformação (absorção de DNA livre) ou transdução (através de bacteriófagos). Krewer et al. (2013) encontraram resistência simultanêa a três ou mais drogas em 65,6% dos isolados *Staphylococcus* causadores de mastite. Sendo assim, é necessária cautela no uso de antibióticos para não promover o estabelecimento de bactérias resistentes (COSTA et al., 2013; MORITZ & MORITZ, 2016), bem como o estudo de novos agentes antibacterianos que possam ser usados de forma isolada ou associados a antibióticos já existentes (LEWIS e AUSUBEL, 2006; MUSHTAQ et al., 2018).

A atividade antibacteriana de lectinas *in vivo* pode ser evidenciada por proteínas endógenas, tais como a lectina humana do tipo C REGIIIα, que reconhece seus alvos bacterianos através da ligação ao peptideoglicano (LEHOTZKY et al., 2010). Lectinas tipo C[2] ligam-se também aos fosfolípideos da membrana e matam bactérias Gram-positivas através da formação de um poro de permeabilização (MUKHERJEE et al., 2014). Lectinas do tipo-C de invertebrados participam não apenas na etapa inicial de reconhecimento de patógenos, através da identificação de carboidratos, mas também em funções antimicrobianas como imobilização, fagocitose, encapsulamento, formação de nódulos, ativação do sistema de melanização da profeniloxidase e outras incluindo atividade antimcrobiana direta (RABINOVICH et al., 2012; WANG et al., 2014).

Hasan et al. (2014) isoloram uma lectina de uma variedade de batata (*Solanum tuberosum* L.) conhecida por "Deshi", a qual possui atividades bacteriostática e bactericida contra patógenos Gram-positivo (*Listeria monocytogenes)* e Gram-negativos (*Escherichia coli, Salmonella enteretidis* e *Shiguella boydii*). A produção de biofilme pela bactéria *Pseudomonas aeruginosa* foi reduzida a uma concentração dose-dependente em 5-20% em 24h após exposição a essa lectina.

A atividade antibacteriana de lectinas decorre da interação entre essas proteínas com diversos componentes da parede bacteriana que, como mencionado anteriormente, incluem diferentes carboidratos, tais como *N*-acetil-glucosamina, ácido *N*-acetilmurâmico (MurNAc), tetrapeptídeo ligados ao MurNAc e lipopolissacarídeos (GOMES et al., 2013). Os efeitos diretos da ação de lectinas sobre bactérias incluem: aglutinação, inibição do crescimento, alteração de permeabilidade celular, redução da captação de nutrientes, desencadeamento de respostas intracelulares (incluindo inibição da respiração celular) por interação com

[2]Lectinas que possuem domínio ligador de carboidratos que requer cálcio para estabelecer a ligação.

receptores de membrana, indução de danos à membrana levando a extravasamento do conteúdo intracelular, entre outros (PROCÓPIO et al., 2017).

2.6.2. Ação antifúngica

Fungos são organismos eucariotos não fotossintetizantes e possuem parede celular constituída por celulose ou quitina (TORTORA et al., 2012). Esses microrganismos são ubíquos, encontrados no solo, água, vegetais, homem e detritos em geral (TRABULSI, 2000). Interagem com a natureza das mais variadas maneiras, podendo agir tanto de forma benéfica quanto provocando doenças. Os fungos podem crescer na forma filamentosa ou leveduriforme, no entanto, alguns fungos apresentam dimorfismo térmico podendo crescer tanto na forma de hifa quanto na forma de levedura, dependendo da temperatura ambiente (KUMAR et al., 2013).

Os fungos leveduriformes são unicelulares, não filamentosos, tipicamente esféricos ou ovais. Diferentemente dos fungos filamentosos que podem ser multinucleados, as leveduras possuem apenas um núcleo. A reprodução normalmente ocorre por brotamento, no entanto, existem espécies capazes de formar pseudo-hifas e isso ocorre quando as células de leveduras permanecem conectadas umas as outras após a reprodução por brotamento. O conjunto de pseudo-hifas forma pseudomicélio. Algumas espécies de fungos leveduriformes patogênicos são dimórficas. Esse dimorfismo pode ser importante na capacidade infectiva do fungo; por exemplo, *Candida albicans* se fixa em células epiteliais humanas na forma de levedura, porém necessita estar na forma de pseudo-hifa para invadir os tecidos mais profundos (TORTORA et al., 2012, NARASINGHAN et al., 2014).

As taxas de infecções fúngicas aumentaram durante a última década, representando 10% das infecções adquiridas em hospitais e em pacientes imunocomprometidos (TORTORA et al., 2012). Houve também um aumento considerável nas infecções fúngicas profundas não apenas em pacientes imunocomprometidos, mas também na população saudável (EGGIMANN et al., 2003; SILVA et al., 2011; WACHTLER et al., 2012).

O gênero *Candida* pertence ao Reino Fungi, Filo Ascomycota, Classe Hemiascomycetes, Ordem Saccharomycetales e Família Candidaceae (CALDERONE, 2002). Esse gênero contém cerca de 200 espécies das quais apenas 20 estão associadas a infecções humanas e são potencialmente causadoras de candidíase. As infecções por leveduras, ou candidíases, frequentemente são causadas por *Candida albicans* e podem ocorrer como

candidíase vulvovaginal ou como “sapinho”, uma candidíase mucocutânea. A candidíase com frequência ocorre em recém-nascidos, pacientes com AIDS e indivíduos em tratamento com antibióticos de amplo espectro (TORTORA et al., 2012). Juntamente com *C. albicans*, outras espécies como *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* e *Candida krusei* são responsáveis por mais de 90% das candidíases (PFALLER et al., 2007).

Normalmente as drogas usadas no tratamento de infecções fúngicas também afetam as células de mamíferos, tornando difícil o tratamento. Além disso, o uso extensivo de antifúngicos pode resultar no estabelecimento de cepas resistentes. Esses problemas tornam necessário o desenvolvimento de novos tratamentos visando reduzir os efeitos negativos sobre o paciente e atenuar o desenvolvimento de resistência contra essas novas substâncias (PETRIKKOS & SKIADA, 2007; CANUTO & RODERO, 2002; REZANKA et al., 2012; PAIVA et al., 2013).

A atividade antifúngica de lectinas pode estar relacionada à ligação dessas proteínas a constituintes da parede celular como quitina ou oligômeros de quitina ou outros sacarídios. Lectinas que se ligam a quitina podem prejudicar a síntese e/ou deposição de quitina na parede celular; outras lectinas podem atravessar a parede celular e agir diretamente na membrana plasmática (KLAFKE et al., 2013; PAIVA et al., 2013). Lectinas podem se ligar às hifas, inibindo o crescimento fúngico, devido a perda na absorção de nutrientes bem como interferindo nos processos de germinação de esporos (HAMID et al., 2013; PAIVA et al., 2013; REGENTE et al., 2014).

A lectina isolada da esponja marinha *Aplysina fulva* foi capaz de inibir o crescimento de cepas de *C. albicans*, *C. tropicalis* em concentrações variando de 128 a 512 μg/mL (GOMES-FILHO, 2014). A lectina de *Opuntia ficus indica* apresentou alta atividade contra *C. albicans* reduzindo o crescimento fúngico em 59% (SANTANA et al., 2009). A lectina de folhas de *Schinus terebinthifolius*, uma lectina ligadora de quitina, inibiu e prejudicou o crescimento de *C. albicans* em baixas concentrações (CMI: 6,5 μg/mL e CMF: 26 μg/mL) (GOMES et al., 2013). A lectina ligadora de quitina da sarcotesta de *Punica granatum* (PgTeL) apresentou atividade antifúngica para as espécies *C. albicans* (CMI: 25 μg/mL; CMF: 50 μg/mL) e *C. krusei* (CMI and CMF of 12.5 μg/mL) e seu mecanismo de ação envolve estresse oxidativo, colapso energético, dano na parede celular e ruptura das células dos fungos (SILVA et al., 2018).

2.7. BIOFILMES

Biofilmes são comunidades complexas e estruturadas de microrganismos inseridos em uma matriz polimérica auto-produzida e composta por exopolissacarídeos, proteínas, ácidos teicoicos, enzimas e DNA extracelular (KLEIN et al., 2015). Esse estilo de vida fornece a esses microrganismos uma proteção contra adversidades ambientais e um aumento na resistência e tolerância (10-1000 vezes) a antibióticos quando comparado à forma planctônica (CERCA et al., 2005; KUMAR, 2017). Acredita-se que o desenvolvimento do biofilme pode estar relacionado com a baixa eficácia de alguns tratamentos usados em mastite bovina bem como na dificuldade do tratamento de infecções recorrentes (MELCHIOR et al., 2006; BATONI et al., 2016). Lectinas têm sido relatadas por possuírem tanto a capacidade de prevenir a formação de biofilme quanto em erradicar biofilmes previamente estabelecidos (MOURA et al., 2015, 2017).

A terapia antiadesão usando lectinas tem se mostrado uma estratégia promissora contra o desenvolvimento de biofilmes em superfícies bióticas e abióticas, prevenindo a adesão inicial de bactérias e consequentemente o crescimento de biofilmes (TEIXEIRA et al., 2007; CAVALCANTE et al., 2013). Pesquisas sobre o potencial biotecnológico de lectinas no combate de biofilmes tem sido estimuladas pela necessidade por compostos não-tóxicos, biocompatíveis e efetivos na prevenção de infeccções associadas a dispositivos, principalmente em ambientes hospitalares (TEIXEIRA et al., 2007; CAVALCANTE et al., 2011; HASAN et al., 2014). A atividade antibiofilme de lectinas, além de estar relacionada aos mecanismos envolvidos na atividade antibacteriana em geral, também se dá pela capacidade dessas proteínas de interagir com os sinais *quorum sensing*, surfactantes, enzimas e polissacarídeos envolvidos na formação de biofilmes (CAVALCANTE et al., 2011; 2013; HASAN et al., 2014). Adicionalmente, tem sido reportada a capacidade de lectinas, em sinergismo ou não com antibióticos, de erradicar biofilmes pré-existentes de bactérias patogênicas (ISLAM et al., 2009; KLEIN et al., 2015).

A lectina ligadora de quitina de *S. tuberosum* inibiu a formação de biofilme de *P. aeruginosa* e essa atividade foi atribuída à ligação a resíduos de *N*-acetilglicosamina (HASAN et al., 2014). A lectina ligadora de glicose/manose de sementes de *Trigonella foenumgraecum* preveniu a adesão e subsequente formação de biofilme de *Streptococcus mutans* em concentrações sub-inibitórias; adicionalmente, o tratamento de biofilmes pré-formados com essa lectina alterou a morfologia das células, causando invaginações e destruição da matriz

(ISLAM et al., 2009). A propriedade erradicadora dessa lectina pode ser devido à interferência no arranjo e na integridade estrutural do biofilme. O efeito antibiofilme da lectina do veneno de *Bothrops jararacussu* foi demonstrado de forma dose-dependente contra células de *S. aureus* e *Staphylococcus epidermidis*, sem interferir no crescimento bacteriano; Essa lectina também apresentou atividade em biofilmes pré-formados quando usada a 100 µg/mL durante 2 h de incubação, rompendo mais de 50% do biofilme (KLEIN et al., 2015).

Teixeira et al. (2007) estudaram duas lectinas de algas vermelhas, *Bryothamnion seaforthii* e *Bryothamnion triquetrum*, e reportaram que elas foram capazes de formar um revestimento forte e uniforme em esferas de hidroxiapatita revestidas com saliva, impedindo a fixação inicial de espécies cariogênicas de *Streptococcus* (*S. oralis*, *S. sanguis*, *S. mitis*, *S. mutans* e *S. sobrinus*).

2.8. SISTEMA IMUNOLÓGICO E IMUNOMODULAÇÃO

O sistema imunológico tem a finalidade de evitar ou limitar muitas infecções e prevenir a proliferação de células transformadas do próprio organismo, como células tumorais. É formado por um conjunto diverso de células e moléculas que atuam de forma coletiva e coordenada para produzir a resposta imune. O sistema imune se divide em imunidades inata e adaptativa (ABBAS et al., 2015; KUMAR, 2016).

A imunidade inata é a primeira linha de defesa do organismo contra patógenos e consiste em uma defesa geral e não específica, sendo incapaz de se lembrar de uma invasão anterior pelo mesmo patógeno. Contudo, é responsável em ativar a resposta imune adaptativa, na qual será gerada a memória imunológica (BOTTAZZI et al., 2010; MANTOVANI et al., 2011). A imunidade adaptativa constitui uma defesa mais específica, na qual antígenos são reconhecidos por receptores específicos presentes na membrana de linfócitos T e B, os quais são capazes de distinguir um determinado antígeno dentre diferentes substâncias. Através da memória gerada, esses linfócitos são capazes de responder a uma segunda exposição de forma mais rápida e vigorosa (ABBAS et al., 2015).

As células que constituem as imunidades inata e adaptativa estão em constante comunicação e são dependentes umas das outras para seu processo de ativação e regulação. A comunicação celular acontece por meio de citocinas, moléculas que orquestram uma variedade de processos, tais como regulação daS inflamações local e sistêmica, proliferação celular, quimiotaxia e reparo tecidual (DUQUE & DESCOTEAUX, 2014).

As citocinas são produzidas principalmente por macrófagos e linfócitos embora possam também ser produzidas por células polimorfonucleares, células endoteliais e epiteliais, adipócitos e células do tecido conjuntivo (DUQUE & DESCOTEAUX, 2014). Essas moléculas podem ser classificadas em pró e anti-inflamatórias (NEURATH et al., 2014, ROSSI et al., 2017), no entanto, devido aos efeitos pleiotrópicos das citocinas essa classificação muitas vezes pode não ser aplicável a determinadas condições. São exemplos de citocinas os fatores de necrose tumoral (TNFs), as interleucinas (ILs), os interferons (IFNs) e os fatores estimulantes de colônias (CSFs) (ESQUIVEL-VELÁZQUEZ et al., 2015).

Os TNFs são considerados mediadores pró-inflamatórios essenciais para homeostase imune, pois podem tanto iniciar uma forte resposta inflamatória quanto atuar como modulador da extensão e duração dos processos inflamatórios, sendo relevante para a inibição de doenças autoimune e formação de tumor (AKDIS et al., 2016). A citocina TNF-α promove a inflamação sistêmica através da indução de febre, da resposta de fase aguda e da ativação de macrófagos e neutrófilos. É também conhecida por suas propriedades antitumorais (para linfoma e mieloma, por exemplo) e vem sendo usada no tratamento de pacientes com sarcomas avançados e melanomas metastáticos (BERTAZZA & MOCELLIN, 2010).

Os IFN são moléculas conhecidas por atuarem na inibição da replicação viral e regulação das respostas imunes. IFN-γ possui um papel importante em respostas imunes celulares e é responsável pela ativação de células T citotóxicas CD8+, células assassinas naturais (NK, do inglês *natural-killer*) e macrófagos. Ainda, polariza as células T CD4+ nativas (NELSON & COX, 2014).

As IL participam da regulação da resposta imune e têm como a principal característica a comunicação entre os leucócitos. A IL-2 exerce papeis centrais na geração, ativação e homeostase dos linfócitos T. Estudos relatam que a intensidade e a duração do sinal de IL-2 controlam tanto a expansão primária como a secundária da população de células T $CD8^+$ (BOYMAN & SPRENT, 2012). Além disso, a IL-2 regula respostas Th1, Th2 e a diferenciação de células Th17 através de seu efeito modulador na expressão de outras citocinas (LIAO et al., 2011). IL-4 é conhecida pela sua capacidade de induzir a comutação do isotipo de IgE em células B e de iniciar e manter a diferenciação de células que comandam a produção de anticorpos (ZHU et al, 2010).

A IL-6 é proliferativa e estimuladora de células imunitárias (MIHARA et al., 2012). Apesar de ser considerada como pró-inflamatória, essa citocina também apresenta atividades regenerativa ou anti-inflamatória (SCHELLER et al., 2011). A citocina anti-inflamatória IL-

10 é conhecida por suas propriedades imunossupressoras, que têm efeitos sobre a regulação imune e inflamação (OUYANG et al., 2011) e seu aumento pode atuar suprimindo a produção de citocinas pró-inflamatórias (IZCUE et al, 2009). IL-17 estimula as células epiteliais das mucosas a secretar citocinas pró-inflamatórias, quimiocinas e peptídeos antimicrobianos (DOMINGUEZ-VILLAR & HAFLER, 2011). A IL-17 pode influenciar significativamente a patogênese de várias doenças auto-imunes (lúpus eritematoso sistêmico, psoríase, esclerose múltipla) (SHAH et al., 2010; DHAMA et al., 2015).

O óxido nítrico (NO) é um radical livre de meia vida curta, e apresenta uma grande variedade de atividades, tais como no controle de pressão arterial, na sinalização celular, na transdução de sinal neural, na função plaquetária e também apresenta ações antitumoral e antimicrobiana, a morte microbiana ocorre através da geração de peroxinitrito, $ONOO^-$ (FANG et al., 2010; LO FARO et al., 2014).

Os agentes imunomoduladores possuem a capacidade de alterar o funcionamento do sistema imunológico de forma a restaurar a função normal do sistema imune, bem como estimulá-la (imunoestimulação) ou inibí-la (imunossupressão) para fins terapêuticos (MAHIMA et al., 2012; DHAMA et al., 2015). A produção de NO, citocinas e quimocinas, bem como a expansão clonal e ativação dos linfócitos podem ser modulados para combater o câncer e infecções microbianas (JACKSON et al., 2014; BERINSTEIN et al., 2015) ou minimizar resposta imune em doenças autoimune ou em transplantes (GOWDY et al., 2015).

Lectinas podem modular o funcionamento do sistema imune, pois exercem tanto efeito imunosupressivo quanto imunoestimulador (SOUZA et al., 2013; JANTAN et al., 2015). Essa ação imunomoduladora ocorre por meio da interação das lectinas com porções glicídicas da superfície de células imunológicas desencadeando a produção e/ou liberação de citocinas (KATRLÍK et al., 2010; GAO et al., 2013; SUNG et al., 2013). Lectinas podem estimular respostas pró-inflamatórias (PRASANNA & VENKATESH, 2015; MARINKOVIĆ et al., 2016) ou anti-inflamatórias (CAMPOS et al., 2016). Ainda, as lectinas podem ativar células do sistema imunológico, tais como macrófagos (DIAS-NETIPANYJ et al., 2016), mastócitos (BARBOSA-LORENZI et al., 2016) e linfócitos (MELO et al., 2011). E induzir a secreção de NO, contribuindo parcialmente para suas habilidades antitumorais (WONG & NG, 2006; KITAGAKI et al., 2009; NÓBREGA et al., 2012; SINGH et al., 2017).

As citocinas, induzidas por lectinas, podem variar de acordo com o modelo utilizado, o tipo de célula imunológica e a via de administração utilizada (SANSONE et al., 2016). Em estudos com lectina de *Musa paradisiaca* (BanLec) foi observada indução de resposta pró-

inflamatória em esplenócitos murinos com aumento de IFN-γ, IL-2 e TNF-α (CHEUNG et al., 2009), bem como estímulo da secreção de IL-4 e IL-10 em linfócitos (STOJANOVIĆ et al., 2010). No entanto, um outro estudo revelou uma diminuição do nível de IFN-γ após exposição *in vivo* à BanLec, sem diferenças significativas para IL-2 ou IL-4 no sangue periférico de ratos. BanLec recombinante, administrada por via retal, também induziu a produção de IFN-γ, IL-2 e IL-4 (PEUMANS et al., 2002).

As lectinas podem estimular de forma específica um tipo de célula imunológica. Por exemplo, a lectina de *Viscum album coloratum* (VCA-B) estimula a maturação e a ativação de células dendríticas (CDs), iniciando resposta Th1. A ativação das CDs e o aumento das respostas imunitárias específicas sugerem que VCA-B apresenta ação imunocoadjuvante como terapêutico tumoral (KIM et al., 2014). Por outro lado, a lectina do cogumelo *Agrocybe aegerita* (AAL) exerceu função imunomoduladora controlando a produção de citocinas inflamatórias e exacerbando a resposta pró-inflamatória na sepse induzida por ruptura cecal, tendo como prováveis células-alvo os macrófagos (ZHANG et al., 2015).

3. OBJETIVOS

3.1. GERAL

Purificar lectina a partir dos foliólulos das folhas de *C. surinamensis* (CasuL), caracterizá-la estruturalmente e investigá-la quanto às atividades citotóxica, antibacteriana, antibiofilme, anti-*Candida* e imunomoduladora.

3.2. ESPECÍFICOS

- ✓ Estabelecer procedimento de purificação para CasuL.
- ✓ Caracterizar CasuL quanto à especificidade de ligação a carboidratos, ponto isoelétrico, composição em subunidades e estabilidade frente a variações de temperatura e pH.
- ✓ Determinar a estrutura primária de CasuL.
- ✓ Determinar a toxicidade de CasuL sobre células humanas normais e tumorais.
- ✓ Avaliar as atividades antibacteriana e antibiofilme de CasuL contra patógenos humanos e causadores de mastite em bovinos e caprinos.
- ✓ Determinar os efeitos antifúngico e antibiofilme de CasuL sobre espécies de *Candida*, bem como alterações causadas na parede celular.
- ✓ Avaliar os efeitos de CasuL sobre o crescimento, viabilidade, ultraestrutura e capacidade formadora de biofilme de isolados de mastite bovina e caprina.
- ✓ Determinar o potencial sinérgico entre CasuL e antibióticos comerciais contra os isolados de mastite.
- ✓ Avaliar a toxicidade de CasuL para esplenócitos de camundongos.
- ✓ Investigar a capacidade de CasuL em promover a ativação (produção de espécies reativas de oxigênio) dos esplenócitos.
- ✓ Avaliar a atividade imunomoduladora de CasuL sobre os esplenócitos através da determinação da funcionalidade das células, capacidade de expansão clonal e liberação de citocinas e óxido nítrico.

4. RESULTADOS

Os resultados de pesquisa são apresentados em forma de artigo.

4.1. ARTIGO 1 - CASUL: A NEW LECTIN ISOLATED FROM *Calliandra surinamensis* LEAF PINNULAE WITH CYTOTOXICITY TO CANCER CELLS, ANTIMICROBIAL ACTIVITY AND ANTIBIOFILM EFFECT

Artigo publicado no periódico *International Journal of Biological Macromolecules*, volume 98, p. 419-429, 2017

Fator de impacto: 3.909 (JCR-2017)

International Journal of Biological Macromolecules 98 (2017) 419–429

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

CasuL: A new lectin isolated from *Calliandra surinamensis* leaf pinnulae with cytotoxicity to cancer cells, antimicrobial activity and antibiofilm effect

Thamara Figueiredo Procópio[a], Leydianne Leite de Siqueira Patriota[a], Maiara Celine de Moura[a], Pollyanna Michelle da Silva[a], Ana Patrícia Silva de Oliveira[a], Lidiane Vasconcelos do Nascimento Carvalho[b], Thâmarah de Albuquerque Lima[a], Tatiana Soares[c], Túlio Diego da Silva[c], Luana Cassandra Breitenbach Barroso Coelho[a], Maira Galdino da Rocha Pitta[a,b], Moacyr Jesus Barreto de Melo Rêgo[a,b], Regina Celia Bressan Queiroz de Figueiredo[d], Patrícia Maria Guedes Paiva[a], Thiago Henrique Napoleão[a,*]

[a] Departamento de Bioquímica, Centro de Biociências, Universidade Federal de Pernambuco, 50670-420 Recife, Brazil
[b] Laboratório de Imunomodulação e Novas Abordagens Terapêuticas, Núcleo de Pesquisa em Inovação Terapêutica (NUPIT), Universidade Federal de Pernambuco, 50670-420 Recife, Pernambuco, Brazil
[c] Centro de Tecnologias Estratégicas do Nordeste, Recife, Pernambuco, Brazil
[d] Laboratório de Biologia Celular de Patógenos, Departamento de Microbiologia, Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Cidade Universitária, 50670-420 Recife, Brazil

ARTICLE INFO

Article history:
Received 5 January 2017
Received in revised form 2 February 2017
Accepted 3 February 2017
Available online 5 February 2017

Keywords:
Leguminous
Plant lectin
Cancer cells
Antifungal activity
Biofilm

ABSTRACT

This work describes the isolation of a lectin (CasuL) from the leaf pinnulae of *Calliandra surinamensis* and the evaluation of its cytotoxic, antimicrobial and antibiofilm properties. Proteins from pinnulae extract were precipitated with ammonium sulphate (60% saturation) and submitted to Sephadex G-75 chromatography, which yielded isolated CasuL (purification factor: 113). Native CasuL is an acidic protein (pI 5.82) with a relative molecular mass of 48 kDa. This lectin is also an oligomeric protein composed of three subunits and mass spectrometry revealed similarities with a *Sorghum bicolor* protein. CasuL did not undergo unfolding when heated but changes in conformation and hemagglutinating activity were detected at basic pH. CasuL did not reduce the viability of human peripheral blood mononuclear cells but was toxic to leukemic K562 cells (IC_{50} 67.04 ± 5.78 μg/mL) and breast cancer T47D cells (IC_{50}: 58.75 ± 2.5 μg/mL). CasuL (6.25–800 μg/mL) only showed bacteriostatic effect but was able to reduce biofilm formation by *Staphylococcus saprophyticcus* and *Staphylococcus aureus* (non-resistant and oxacillin-resistant isolates). CasuL showed antifungal activity against *Candida krusei* causing alterations in cell morphology and damage to cell wall. In conclusion, the pinnulae of *C. surinamensis* leaves contain a thermo-stable lectin with biotechnological potential as cytotoxic, antibiofilm, and antifungal agent.

1. Introduction

Lectins are proteins that bind specifically to both free and cell surface attached carbohydrates. This property may trigger various cellular responses and confers to these proteins several biological activities [1,2]. In plants, lectins are reported to be involved in defense against pathogens, herbivores and predators. This physiological role has stimulated the investigation of their biotechnological potential in the control of microbial infections and pest insects [3–6]. In addition, vasorelaxant effect [7], immunomodulatory action [8] as well as cytotoxic and antitumor activities [9–11] are some properties among the wide range of biological activities described for plant lectins.

Lectins are found in many plants of the family Fabaceae (Leguminosae), to which belongs the *Calliandra* genus (subfamily Mimosoideae). This genus comprises plants widely used in the tra-

* Corresponding author at: Departamento de Bioquímica, Centro de Biociências, Universidade Federal de Pernambuco, 50670-420 Recife, Brazil.
E-mail address: thiagohn86@yahoo.com.br (T.H. Napoleão).

http://dx.doi.org/10.1016/j.ijbiomac.2017.02.019
0141-8130/

ditional medicine for treatment of infectious diseases as malaria and leishmaniasis [12]. Leaves and branches of *Calliandra calotyrsus* and *Calliandra porturicensis* are described as sources of compounds with activity against intestinal nematodes of ruminants [13] besides analgesic, anticonvulsant, and anti-ulcer properties [14,15]. *Calliandra surinamensis* (popularly known as "pink powder puff", "pompon du marin" or "esponjinha-rosa", among other names) is a perennial, tropical and low-branching shrub widely used as ornamental species in Brazil. However, few studies are available about their biological properties and biotechnological potential. The use of *C. surinamensis* as an ornamental plant, resistant to pathogens and insects, stimulates the evaluation of this plant as source of bioactive molecules such as lectins.

Plant lectins have shown remarkable anticancer properties *in vivo* and *in vitro* and clinical studies have shown that they can function as an alternative in cancer therapy [16–18]. In addition, drug delivery systems strategies have been investigated to increase the bioavailability of antitumor lectins [19–21]. Lectins can be toxic to cancer cells via different mechanisms, which are generally initiated by interaction with specific receptors, glycosylated or not, on the membrane of cancer cells. After this step, the lectins can be internalized through endocytosis and addressed to different compartments leading to activation of signaling pathways related to cell death [18,22].

Lectins with antibacterial activity can interact with peptidoglycans, lipopolysaccharides and other molecules present in the cell wall of Gram-positive and Gram-negative species, interfering with cell growth and viability as well as blocking interaction sites with host cells preventing infection [6,23,24]. Lectins also exert antifungal effect, which may be linked to interaction with chitin, cellulose, glucans and mannoproteins present in the cell wall, interfering with fungal growth, cell homeostasis and spore germination [25,26].

Bacterial biofilms are multicellular communities enclosed in a self-produced polymeric matrix and able to attach to abiotic and biotic surfaces. Biofilms are involved in the most of bacterial infections in humans. An additional difficulty to control the biofilm formation is that several antibacterial molecules are unable to penetrate deeply into the biofilm being often entrapped by the extracellular polymeric matrix. Furthermore, under antibiotic pressure, bacterial cells in the biofilm can express resistance mechanisms making them recalcitrant to a considerable number of antibiotics [27]. In this sense, natural compounds, including lectins, have been evaluated for their ability to inhibit biofilm formation by pathogenic bacteria [28–30].

This work reports the isolation and characterization of a lectin from *C. surinamensis* leaf pinnulae (*C. surinamensis* lectin: CasuL) as well as the evaluation of its cytotoxic activity against human normal and cancer cells, antibacterial and antibiofilm activities against Gram-positive and Gram-negative pathogens, and antifungal activity against *Candida* species.

2. Materials and methods

2.1. Lectin purification

Leaves of *C. surinamensis* were collected in Recife (Pernambuco, Brazil) and put to dry at 28 °C during 15 days. Next, the pinnulae were detached and powdered using a blender. This powder was suspended in 0.15 M NaCl (10%, w/v) and homogenized during 16 h using a magnetic stirrer. Next, the suspension was filtered through gauze and centrifuged (12,000*g*, 15 min, 4 °C) to obtain the crude extract. The extract was treated with ammonium sulphate at 60% saturation [31] during 4 h under magnetic stirring. After this period, the material was centrifuged (3000*g*, 15 min) and the precipitated (PF) and supernatant (SF) fractions were collected and dialyzed against distilled water (4 h) followed by 0.15 M NaCl (4 h). The extract and fractions were evaluated for protein concentration and hemagglutinating activity (HA) as described in the next section.

PF (3.0 mg of protein) was loaded onto a Sephadex G-75 column (30 × 1 cm) equilibrated with 0.15 M NaCl. After sample loading, the column was irrigated with 0.15 M NaCl, 0.5 M glucose, 0.5 M mannose, and 1.0 M NaCl, in this order. The elution of proteins was monitored by measuring the absorbance at 280 nm. The protein peak eluted with 0.15 M NaCl corresponded to CasuL, which was then evaluated for protein concentration and HA.

2.2. Protein concentration and hemagglutination assay

Protein concentration was determined according to Lowry et al. [32] using bovine serum albumin (31.25–500 μg/mL) as standard. The hemagglutinating activity (HA) was determined using a suspension of rabbit erythrocytes (2.5% v/v) previously treated with glutaraldehyde [33]. Ethics Committee on Animal Experimentation of the *Universidade Federal de Pernambuco* approved the erythrocytes collection method (process 23076.033782/2015-70). The sample (50 μL) was serially two-fold diluted in 0.15 M NaCl in a row of a 96-well microplate and then 50 μL of the erythrocyte suspension were added to each well. Erythrocytes incubated only in 0.15 M NaCl were used as control. Each assay was performed in duplicate. The number of HA units (HAU) was determined as the reciprocal of the highest dilution of the lectin that promoted full agglutination of rabbit erythrocytes. The specific HA was calculated as the ratio between the HAU and the protein concentration (mg/mL).

For HA inhibition assay, the lectin samples were incubated for 15 min with monosaccharides (glucose, mannose methyl-α-D-glucopyranoside, *N*-acetylglucosamine or galactose at 0.2 M), the disaccharide maltose (0.2 M) or glycoproteins (fetuin, ovalbumin or bovine serum albumin at 0.5 mg/mL) prior to the addition of erythrocyte suspension.

2.3. Evaluation of protein homogeneity and estimation of native molecular mass

CasuL was submitted to PAGE for native basic proteins (12% acrylamide gel, w/v) according to Reisfeld et al. [34] and 1% (w/v) Amido Black in 10% (v/v) acetic acid was used as staining solution. The lectin was also submitted to PAGE for acidic proteins in 12% acrylamide gel (w/v) prepared according to Davis [35] and the polypeptide bands were detected with 0.02% (v/v) Coomassie Blue in 10% (v/v) acetic acid.

To determine the lectin native molecular mass, CasuL (2.0 mL, 2.0 mg of protein) was chromatographed onto a Hiprep 16/60 Sephacryl S-100HR column (16 mm × 60 cm) coupled to the AKTAprime plus system (GE Healthcare Life Sciences, Uppsala, Sweden) and equilibrated with 0.15 M NaCl. A flow rate of 0.5 mL/min was maintained and fractions of 3.0 mL were collected. A mixture of bovine serum albumin (66 kDa), ovalbumin (45 kDa) and lysozyme (14 kDa) from Sigma-Aldrich (USA) was chromatographed at the same conditions. The relative molecular mass of CasuL was calculated by comparison with migration of these molecular mass markers.

2.4. Determination of isoelectric point and subunit composition

To determine the isoelectric point of CasuL, the protein (150 μg) was solubilized in a rehydration solution [2% (w/v) CHAPS, 1% (v/v) IPG buffer pH 3–10, 0.002% (w/v) bromophenol blue] for 20 min at 28 °C. The sample was taken up into the strip (linear pH gradient 3–10; 7 cm) passively during rehydration for 16 h at 25 °C. The isoelectric focusing was performed using the IPGphor III system (GE

Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer's instructions. Next, the strip was stained with a solution of 0.02% (w/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid. The isoelectric point was calculated using the software ImageMaster 2D Platinum (GE Healthcare Life Sciences).

Electrophoresis under denaturing conditions, in the presence of sodium dodecyl sulphate (SDS-PAGE), was performed on 12% acrylamide gel according to Laemmli [36]. Polypeptide bands from CasuL and molecular mass markers (12–225 kDa, GE Healthcare Life Sciences) were stained with Coomassie Brilliant Blue in 10% acetic acid (0.02%, v/v).

2.5. Mass spectrometry analysis

The polypeptide spot from isoelectric focusing was cut from the gel and washed three times with 25 mM ammonium bicarbonate/acetonitrile 1:1 (v/v). The material was covered with 100% acetonitrile for 10 s and then replaced by 25 mM ammonium bicarbonate and incubated for 45 min at 56 °C. A digestion buffer, containing 10 mg/mL trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega Corporation, USA) in 25 mM ammonium bicarbonate, was added to the gel, incubated for 45 min, and replaced by 50 mM ammonium bicarbonate without the enzyme. The sample was incubated at 37 °C overnight and then the peptides were extracted by incubation in 5% trifluoracetic acid/acetonitrile 1:1 (v/v) for 30 min followed by removal and storage of the supernatant. The extraction step was repeated twice and all supernatant fractions were pooled and dried in a vacuum concentrator (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The peptides were analysed in a MALDI-TOF/TOF Autoflex III smartbeam spectrometer (Bruker Daltonics, USA). The spectra of peptides were compared with sequences of Viridiplantae proteins in NCBInr database using MASCOT database search (http://www.matrixscience.com). The analysis was performed three times.

2.6. Effects of pH and temperature on CasuL HA and conformation

To determinate the effects of pH variation on CasuL HA, the agglutination assay was performed as described above but replacing the 0.15 M NaCl solution by the following buffer solutions: 10 mM citrate phosphate buffer, pH 5.0 and 6.0, 10 mM sodium phosphate pH 7.0, and 10 mM Tris–HCl, pH 8.0 and 9.0. The effect of temperature on CasuL HA was evaluated using lectin samples previously heated for 30 min at 30–100 °C.

The effects of pH and temperature on CasuL conformation were evaluated by fluorimetric analysis using the extrinsic probe bis-ANS [(bis)(8-anilinonaphthalene-1-sulfonate)], which emits fluorescence when binds to hydrophobic regions surrounded by positively charged residues. Fluorescence measurements were performed using CasuL samples (2 μM) incubated with 50-fold molar excess of bis-ANS in the dark. The following samples were analyzed: CasuL in water, CasuL incubated for 30 min at 25 °C at pH 3.0, 4.0 (citrate phosphate buffer), 5.0, 6.0 (potassium phosphate buffer), and 8.0 (Tris buffer), and CasuL incubated for 30 min at different temperatures (30–100 °C). The bis-ANS fluorescence spectra were recorded on a Jasco spectrofluorometer FP-6300 (Jasco Corporation, Japan) by setting the excitation wavelength at 360 nm and emission at 400 to 600 nm.

2.7. Cytotoxicity assays

Cytotoxicity of CasuL was evaluated on human peripheral blood mononuclear cells (PBMCs) and on the cancer cell lines K562 (chronic myelogenous leukemia) and T47D (breast cancer). The cancer cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in an atmosphere with 5% CO_2. PBMCs were obtained from blood of five healthy volunteer donors (Human Research Ethics Committee of the *Universidade Federal de Pernambuco* – CEP/CCS/UFPE N° 145/09), by density-gradient centrifugation over Ficoll-Hypaque (GE Healthcare Life Sciences, Sweden). The viability of the cells was evaluated by the trypan blue exclusion method. Only cells samples in which the viability was higher than 98% were used in the experiments.

PBMCs (100 μL, 10^6 cells/mL), K562 or T47D cells (100 μL, 10^5 cells/mL), in RPMI 1640 medium supplemented with 20% (v/v) fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, were plated in 96-well microtiter plates. After 24 h, CasuL (100 μL from dilutions of a 1.0 mg/mL stock solution in water) was added to each well to achieve concentrations ranging 1–100 μg/mL. After incubation for 72 h, 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5.0 mg/mL) was added to each well [37,38]. After 3 h, the formazan product was dissolved in dimethyl sulphoxide and the absorbance at 540 nm was measured using a microplate reader. Non-treated cells and cells treated with etoposide phosphate (0.625–10 μg/mL) were used as negative and positive controls, respectively. The assays were performed in triplicate in three independent experiments. The concentration that inhibited cell growth by 50% (IC_{50}) was determined by probit analysis using the program SPSS 8.0 (IBM, New York, NY, USA) for Windows.

2.8. Antimicrobial assays

CasuL was evaluated for antimicrobial activity against human pathogenic bacteria and fungi. The bacteria tested were *Escherichia coli* ATCC-25922, *Staphylococcus aureus* (standard strain ATCC-6538 and oxacillin-resistant strain UFPEDA-670), and *Staphylococcus saprophyticcus* UFPEDA-833, obtained from the culture collection (WDCM114) of the *Departamento de Antibióticos* from the *Universidade Federal de Pernambuco*. The fungi tested were *Candida albicans* (URM 5901), *Candida glabrata* (URM 4246), *Candida krusei* (URM 6391) and *Candida parapsilosis* (URM 6345) obtained from the culture collection University Recife Mycologia from the *Departamento de Micologia* of the *Universidade Federal de Pernambuco*, Brazil.

For evaluation of antimicrobial activity, the microorganisms were cultured in Mueller Hinton Broth (MHB) at 37 °C (bacteria) or Sabouraud Dextrose Broth (SDB) at 28 °C (fungi) overnight under gentle shaking. For the experiments, the density of microorganism cultures was adjusted turbidimetrically at a wavelength of 600 nm to 3×10^6 CFU/mL. In each row of a 96-well microplate, 100 μL of CasuL (1.6 mg/mL in Milli-Q water) were two-fold serially diluted in culture medium until a final ratio of 1:1024. Next 20 μL of microbial culture were added to each well. Wells containing only the culture medium or only the microorganisms in culture medium were used as sterility and 100% culture growth controls, respectively. The optical density (OD_{600}) was measured at time zero at 600 nm and then the samples were incubated at 37 °C (bacteria) or 28 °C (fungi) for 24 h. After this period, the OD_{600} was recorded again. The increase in OD_{600} in comparison with time zero was considered as microbial growth. The minimal inhibitory concentration (MIC) was determined as the lowest CasuL concentration able to promote a reduction of OD_{600} higher or equal to 50% in comparison with the 100% growth control. Tetracycline (8 μg/mL) was used as positive control. Each assay was achieved in triplicate and three independent experiments were performed.

To determine the minimal bactericidal (MBC) or fungicidal (MFC) concentration, the supernatant (10 μL) from each well where the growth inhibition was higher or equal to 50% was transferred to Mueller Hinton Agar or Sabouraud-Dextrose Agar plates, and incu-

bated for 24 h. The MBC or MFC, corresponding to the lowest CasuL concentration able to reduce the number of CFU in 99.9% in comparison with the initial inoculum, was determined. Each assay was carried out in triplicate in three independent experiments.

2.9. Antibiofilm assay

The bacteria cultures were grown in MHB medium overnight at 37 °C and then suspended in sterile saline solution (0.9%, w/v, NaCl) to obtain a suspension equivalent to 10^8 CFU/mL (OD_{600}). To each well of a 96-well polystyrene microplate, it was added 40 μL of MHB medium, 80 μL of the bacterial suspension and 80 μL of ultrapure Milli-Q water (control) or 80 μL of CasuL (6.25–800 μg/mL, in Milli-Q water).The OD_{600} was recorded at this time and the microplate was further incubated at 28 °C for 24 h. After this period, the OD_{600} was read again, the culture medium was removed and the plate wells were washed three times with saline solution. The remaining attached cells were heat-fixed at 60 °C for 60 min and the adherent biofilm layer was stained with 0.4% (w/v) crystal violet for 25 min at 25 °C. After washing with water, the stain bound to the biofilm was solubilized with ethanol (15-min incubation) and the absorbance was measured at 570 nm [39]. Tetracycline (8 μg/mL) was used as positive control. Three independent experiments were performed in triplicate.

2.10. Fungal cell wall analysis

In order to investigate possible damages caused by CasuL on fungal cell wall, it was used the Calcofluor White M2R (LIVE/DEAD® Yeast Viability Kit, Molecular Probes, USA), a fluorochrome that binds cellulose and chitin in the cell walls of fungi. Fifty μL of the yeast culture were added to 1 mL of 10 mM Na-HEPES pH 7.2 prepared in sterile 0.2 μm-filtered water. After centrifugation (10,000*g*, 5 min), the pellet was resuspended in 1 mL of this same buffer. The cells were then stained with 25 mM Calcofluor White M2R according to manufacturer's instructions. The samples were observed using a confocal microscope (Leica SPII AOBS, Leica Microsystems, Wetzaler, Germany) at excitation and emission wavelengths of 355 and 440 nm, respectively. The images were collected and analyzed using the LITE 2.0 software (Chem-Table Software, Moscow, Russia).

2.11. Statistical analysis

The data were expressed as the mean or the percent mean ± standard deviation (SD) and statistical differences were determined using Tukey's test; a p value <0.05 was considered statistically significant.

3. Results and discussion

The procedure for CasuL purification started with the extraction of proteins from *C. surinamensis* leaf pinnulae in saline solution. The extract presented HA (Table 1), which was inhibited by the monosaccharides mannose and glucose as well as by glycoproteins (ovalbumin, fetuin and bovine serum albumin), confirming the presence of lectins.

The fractions PF and SF obtained from the extract were evaluated for protein concentration and HA. The PF presented a purification factor of 7.5 while the SF showed specific HA lower than that found for the extract (Table 1), evidencing that most of lectin molecules present in the extract were precipitated by treatment with ammonium sulphate. The PF was then chosen to continue the purification process.

PF was chromatographed onto a column of Sephadex, a matrix that is useful for protein separation according to molecular size [40], but it may also serve as a support for affinity chromatography of some lectins [41], as the Sephadex gel is composed by cross-linked dextran (glucose polymer). Therefore, the procedure included a gel filtration step using 0.15 M NaCl and then three elution steps using 0.5 M glucose, 0.5 M mannose and 1.0 M NaCl, in this order. The chromatogram (Fig. 1A) showed a single protein peak obtained during the gel filtration step. No other peaks were obtained in the elution steps with glucose (a competitor for lectins possibly linked to the matrix glucose components), mannose (a monosaccharide able to interact with lectins present in the *C. surinamensis* extract and that could have adsorbed to the matrix) and 1.0 M NaCl (high ionic strength to elute proteins adsorbed to the matrix in a biospecifically manner or not). This result shows that the lectins present in PF were not able to bind to Sephadex G-75. The single peak obtained in this chromatography step presented HA and showed a purification factor of 113 (Table 1) being then investigated for protein homogeneity.

Gel filtration chromatography of the sample from Sephadex G-75 showed a single protein peak with relative molecular mass of 48 kDa (Fig. 1B). Electrophoresis under native conditions for acidic proteins revealed a single polypeptide band (Fig. 1C), whereas no band was visualized in electrophoresis for basic proteins. Since only one protein was detected, the peak from Sephadex G-75 was named CasuL. Isoelectric focusing revealed CasuL as a single polypeptide band with isoelectric point 5.82 (Fig. 1D). All these results confirm the homogeneity of CasuL.

The SDS-PAGE showed the presence of three polypeptide bands with approximate molecular masses of 27, 13 and 10 kDa (Fig. 1E). Together with the results from gel filtration chromatography, this result allows us to state that CasuL is an oligomeric protein composed by three distinct subunits. The lectins from leguminous rarely are monomeric proteins, being often found in oligomeric forms [42].

CasuL was digested with trypsin and the derived peptides were analyzed by mass spectrometry. The peptides revealed similarity of CasuL with a protein from *Sorghum bicolor* (XP_002466464.1) with undefined function (Table 2). The peptides with molecular mass (Da) 993.5751, 1065.6127, 1066.1182, 1093.6200, 1118.6182, 1140.6635, 1165.6758, 1179.7050, 1194.7023, 1213.6638, 1239.7451, 1252.6940, 1320.7170, 1323.7743, 1340.7875,1342.7338, 1360.7256, 1367.7404, 1383.7893, 1385.7487, 1417.7971, 1434.8608, 1460.8517, 1476.8599, 1495.8274, 1519.8201, 1699.9318, 1816.1196, 1867.0204, 1869.0134, 2384.0374 showed no matches with sequences of plant proteins in the database.

The hemagglutinating activity of CasuL was not inhibited by the monosaccharides glucose, mannose, methyl-α-D-glucopyranoside, *N*-acetylglucosamine and galactose or by the disaccharide maltose but was neutralized in the presence of the glycoproteins ovalbumin, fetuin and bovine serum albumin. This explain the fact that CasuL did not adsorb to the Sephadex G-75 matrix and suggest that other lectins able to bind mannose are present in the extract but were not concentrated in PF.

The stability of the CasuL carbohydrate-binding property towards temperature and pH variations was evaluated. The HA of lectin (256 HAU) was maintained after heating at all tested temperatures (30–100 °C). It is common that plant lectins display resistance to heating at high temperatures [43–46] but some of them are not heat-stable [11]. The thermostability of a protein can vary according to the dependence degree of protein structure on hydrogen bonds [47], presence of glycosylation [48] as well as complexity of its tertiary structure [49].

Concerning its stability at different pH values, CasuL showed highest HA values at pH 5.0 (64 HAU) and 6.0 (128 HAU), being also active at pH 7.0 (16 HAU). However, this lectin was not able to agglutinate erythrocytes when incubated at pH 8.0 and 9.0. Simi-

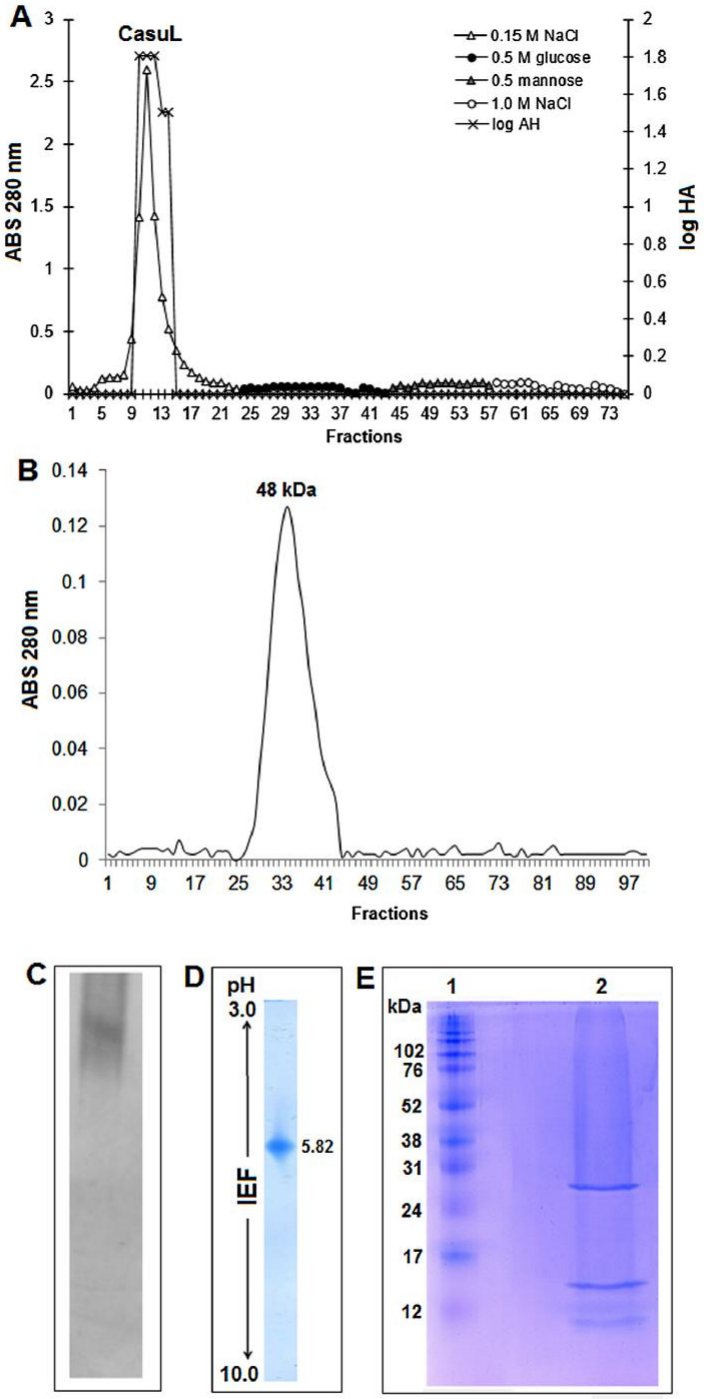

Fig. 1. Purification of the lectin from *C. surinamensis* leaf pinnulae (CasuL). (A) Chromatography of the precipitated fraction (PF), obtained after treatment of leaf extract with ammonium sulphate at 60% saturation, on Sephadex G-75 column. (B) Gel filtration chromatography of CasuL (2.0 mg of protein) on a Hiprep 16/60 Sephacryl S-100HR column coupled to AKTAprime plus system. (B) Electrophoresis of CasuL under native conditions for acidic proteins. (C) Isoelectric focusing (IEF) of CasuL under native conditions. The isoelectric point of lectin is 5.82. (D) Electrophoresis under denaturing conditions (SDS-PAGE) of molecular mass markers (1) and CasuL (2).

Table 1
Summary of purification of *Calliandra surinamensis* lectin (CasuL).

Sample	Volume (mL)	Protein (mg/mL)	HAU	SHA	Total HAU	Purification factor	Yield (%)
Extract	170	5.59	64	11.45	10,880	1.0	100
PF	11.5	1.49	256	85.91	2944	7.5	27
SF	185	0.52	4	7.69	740	0.67	7
CasuL	90	0.025	32	1280	2880	111.8	26.4

HAU: hemagglutinating activity units. SHA: specific hemagglutinating activity, which corresponds to the ratio between HAU and protein concentration (mg/mL). Purification factor was calculated by the ratio between the SHA in the stage and the SHA of the extract. Yield corresponded to the percentage of total HAU recovered in each step.

Table 2
Sequence similarities between peptides derived from trypsin digestion of CasuL and the sequence of protein from *Sorghum bicolor* (XP_002466464.1).

Peptide	Mass (Da)	Sequence	Position in *S. bicolor* protein.
1	1234.7721	KTVVASLGSIYPKD	45–56
2	1126.6111	KTETTKMLMR + Oxidation (M)	164–172
3	1493.8398	KMLMRYLAFMGGR + 3 Oxidation (M)	169–180
4	2501.3680	RTVEQQVLESNPVLEAFGNAKTVK	187–209
5	1019.5744	KTVKNNNSSR	207–215
6	1037.6028	RTYLLERSR	237–244
7	1104.6338	RDDKSVYHLK	348–356
8	1308.7586	KTVAELLMCDEK	357–367
9	1123.6272	RDALAKTVYSR	399–408
10	1701.8985	KDYVVAEHQALLNSSR	568–582
11	1637.9151	RFGMLAPELVDSSDEK	681–695
12	1105.6225	KMEARETGALK	879–888
13	1365.7476	KEAAKLAIEQAPPK	949–961
14	2239.2220	KIVEVPVVDNAKLEELTTQNK	962–981
15	1707.8698	KQSDELSQETQEQASK	1007–1021
16	1082.6204	KKSAAACIAYK	1161–1170
17	1107.6293	KASSSLSKGTNR	1226–1236
18	999.5503	RVRPSRGSLK	1325–1333
19	1184.6818	KNNYVPPVIIR	1365–1374

Polypeptide band of CasuL from isoelectric focusing was used for analysis. The similarity score was 74 and the sequence coverage was 15%.

larly to CasuL, a lectin from the seeds of the leguminous *Bauhinia forficata* showed highest HA at pH 6.0 and lost this activity at basic pH range starting from 8.5; this lectin also showed a pI of 5.4, next to that of CasuL [46]. The lectins from *Myracrodruon urundeuva* bark and heartwood were also unable to agglutinate erythrocytes at pH 9.0 [50]. Variations of pH may affect the activity of proteins due to minor or major changes in the protein structure resulting from breaking or formation of ionic interactions as well as loss of solubility. In this regard, we investigated possible conformational alterations induced by the heat or pH variation on CasuL.

Bis-ANS is useful to detect stages of protein unfolding since its fluorescence is reduced when the protein hydrophobic patches to which this probe binds are exposed to the aqueous environment [51]. The spectrofluorimetry analysis showed no significant reduction of fluorescence in all temperature tested, suggesting that CasuL did not undergo unfolding with consequent exposure of hydrophobic sites to water (Fig. 2A and B). Similar spectra were obtained for the samples heated at temperatures up to 70 °C. In other hand, a progressive increase of the bis-ANS spectral area was observed when the lectin sample was heated at 80, 90 and 100 °C. This may be associated with conformational change that led to higher closure of hydrophobic sites. Nonetheless, these changes did not compromise the ability of the carbohydrate-binding site of CasuL in agglutinate rabbit erythrocytes.

When the lectin was incubated at pH 8.0 (Fig. 2C), a remarkable shift in maximum emission wavelength of the bis-ANS as well as reduction in fluorescence emission since 450 nm can be observed, indicating that at this condition unfolding may have occurred reflecting in the loss of HA. The fluorescence spectra of bis-ANS were similar when the lectin was incubated at pH 3.0–6.0, with some differences in the intensity of fluorescence. These results show that the conformation state of CasuL at these pH values did not interfere in the ability of this protein to bind carbohydrates at erythrocytes surface and promote agglutination.

CasuL did not affect PBMCs viability when used in concentrations lower than 100 μg/mL. This result prompted the evaluation of cytotoxic potential of this lectin against cancer cells. In this case, CasuL was able to reduce the viability of K562 and T47D cells (Fig. 3) with IC_{50} of 67.04 ± 5.78 and 58.75 ± 2.5 μg/mL, respectively. The cytotoxicity degree of lectins is usually variable and its potential for effective use as antitumoral agent is dependent on its specificity towards cancer cells comparing to the normal cells. For example, the mistletoe lectin I was highly toxic to K562 cells (IC_{50} = 75 pg/mL) and was also able to reduce T47D viability in 36.2% at 100 ng/mL. However, it is well known that this protein is also very toxic to mammals [52,53]. On the other hand, similarly to CasuL, the lectin from *Cliona varians* sponge showed an IC_{50} value of 70 μg/mL for K562 cells without toxicity to blood lymphocytes at this concentration [54].

Several bacteria are of great medical relevance because they cause serious infections in humans, such as meningitis, endocarditis, gastroenteritis and urinary tract infections [55]. The misuse of antibiotics has led to an increase of antimicrobial resistance reports because of the selective pressure that favors resistant strains, mainly in hospital environments; however, the presence of antibiotics in food and water, for example, also increases the emergence and dissemination of antimicrobial-resistant bacteria that infect humans and animals [56]. This scenario stimulates the search for new antibacterial agents that may be used alone or in association with antibiotics in order to increase their efficacy.

The Fig. 4 shows the effects of CasuL on bacterial growth (black bars). It is possible to note that all the lectin concentrations tested were unable to inhibit the growth of the bacteria in more than 50%, then the MIC and MBC values could not be determined. *E. coli* did not have its growth affected by lectin treatment at any concentration tested. On the other hand, significant ($p < 0.05$) growth inhibition was detected for *S. saprophyticcus* and the oxacillin-resistant isolate of *S. aureus*, although it was not higher than 30%. The best results

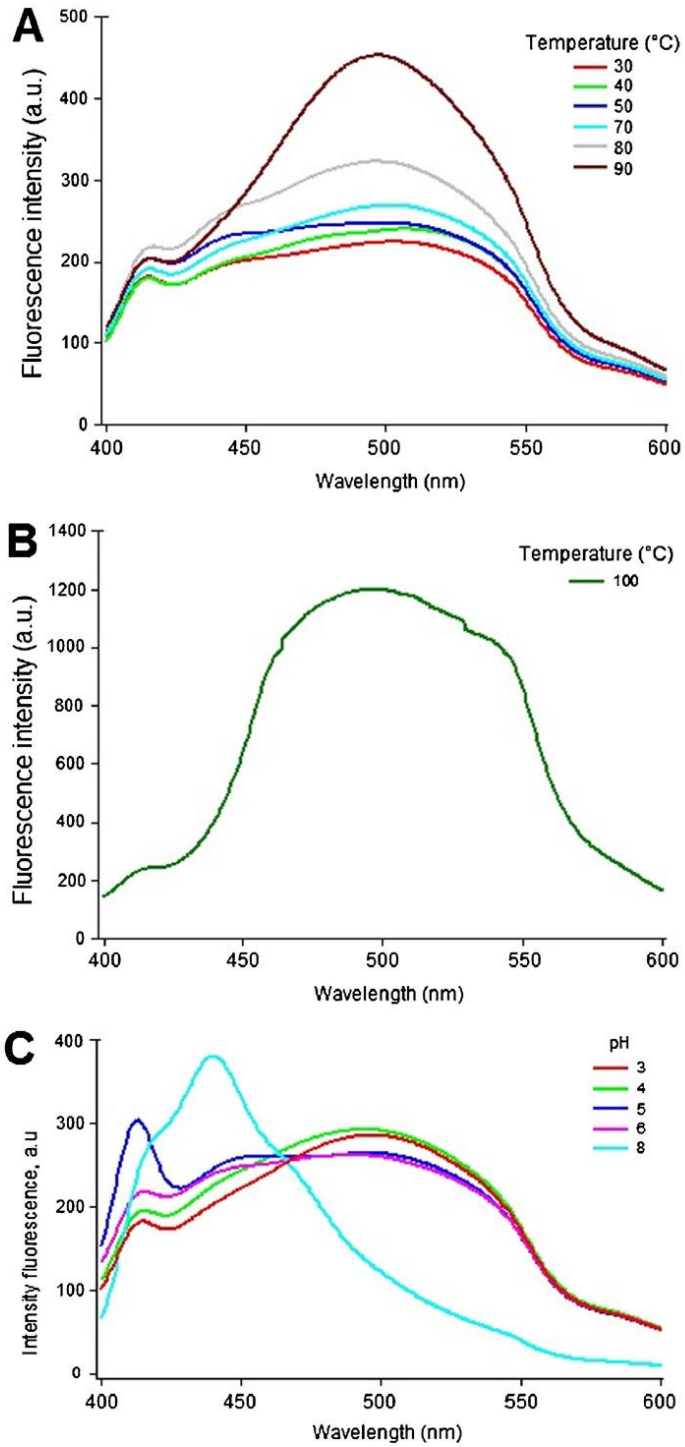

Fig. 2. Fluorescence spectra of bis-ANS in presence of CasuL pre-heated at different temperatures (A and B) or incubated at different pH values (C).

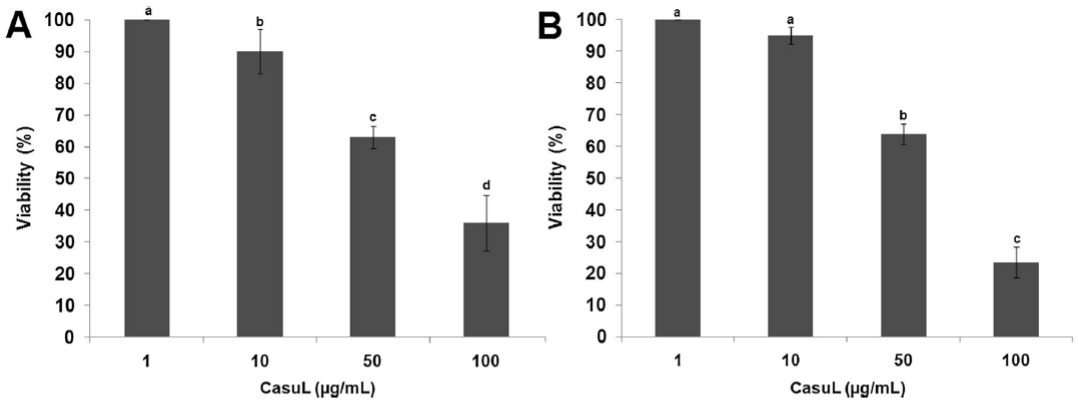

Fig. 3. Effects of different concentrations of CasuL on the viability of K562 chronic myelogenous leukemia (A) and T47D breast cancer (B) cells. Results are expressed in comparison with the 100% growth control (non-treated cells). Different letters indicate significant differences ($p < 0.05$) between the treatments.

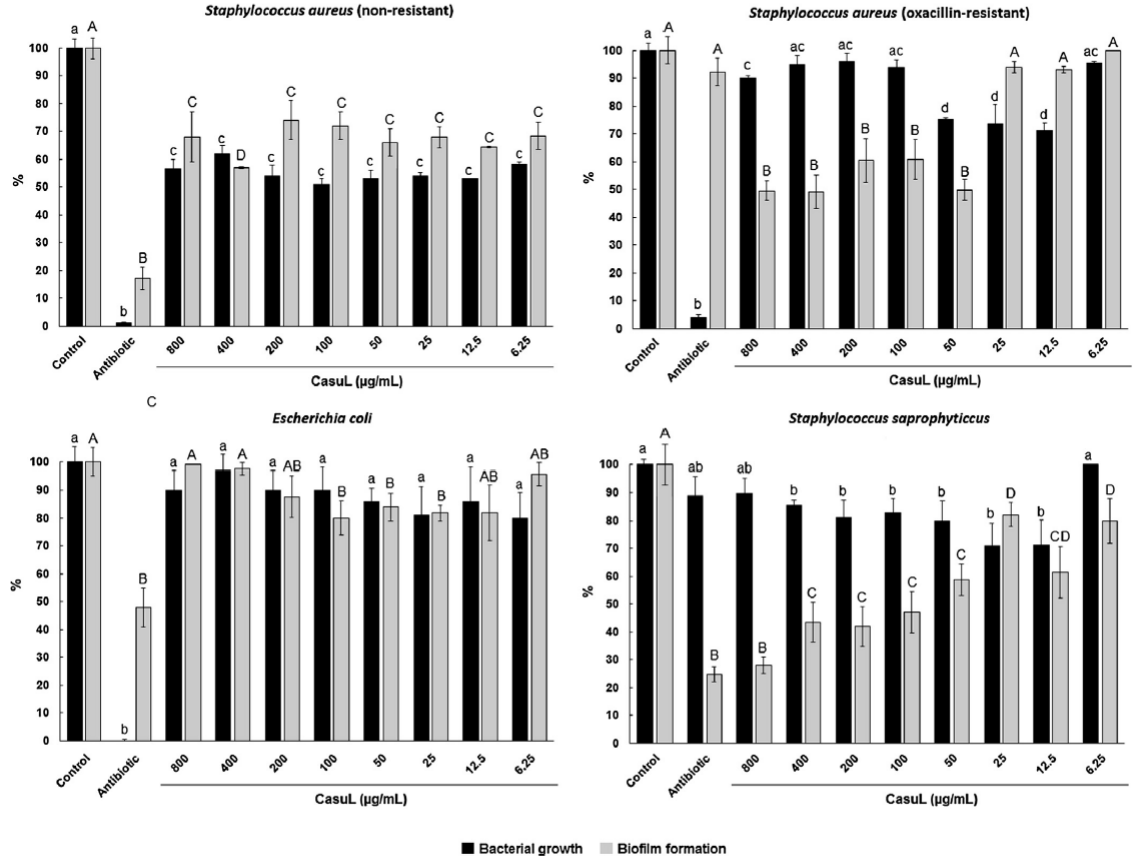

Fig. 4. Effects of CasuL on the bacterial growth and biofilm formation by pathogenic bacteria, evaluated by OD_{600} and crystal violet assay, respectively. The values obtained for untreated control corresponded to 100% of bacterial growth or biofilm formation. The antibiotic tetracycline at 8 µg/mL was used as reference drug. Different lowercase letters indicate significant differences ($p < 0.05$) between the bacterial growth in the treatments. Different uppercase letters indicate significant differences ($p < 0.05$) between the biofilm formation in the treatments.

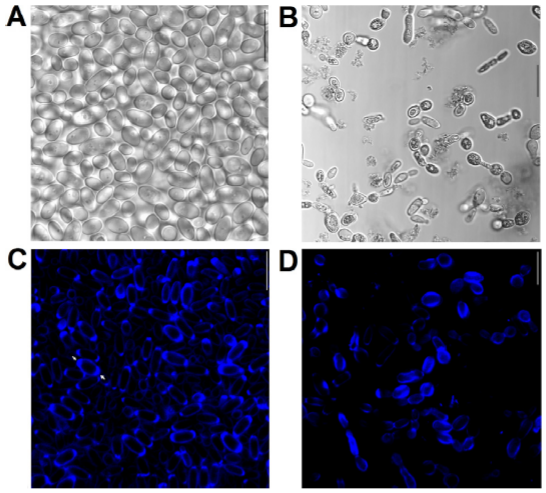

Fig. 5. Differential interference contrast (A-B) and laser confocal (C-D) microscopies of *Candida krusei* cells untreated (A and C) and treated with CasuL at MIC for 24 h (B and D). Cells with regular shape and size are observed in negative control (A) while drastic morphological alterations can be observed in cells treated with CasuL (B). Staining with Calcofluor White (blue fluorescence): (C) control cells showing continuous blue labeling with brighter fluorescence at the region of bud scars (arrows); (D) CasuL-treated cells showing a diffuse blue fluorescence indicative of loss of cell wall integrity. Scale bars correspond to 16 μm.

were obtained for non-resistant *S. aureus* isolate, whose growth was significantly reduced ($p < 0.05$) at all concentrations with inhibition values ranging from 38% to 49%. The *S. saprophyticcus* isolate was not sensitive to tetracycline treatment, used as reference drug.

The antibiofilm assay (Fig. 4, gray bars) showed that CasuL was able to reduce significantly ($p < 0.05$) the biofilm formation by all tested bacteria, although the results for *E. coli* were not very expressive. The best result was found for *S. saprophyticcus*, with a reduction of 72% in biofilm formation at 800 μg/mL. This inhibition value was similar to those obtained with the reference drug tetracycline. Inhibitory effect higher than 50% was found from 100 μg/mL for this bacterium and since 400 μg/mL for the oxacillin-resistant isolate of *S. aureus*. The increase of CasuL concentration raised the magnitude of the antibiofilm effect only for *S. saprophyticcus*, revealing a dose-dependent effect.

Antibiofilm agents that do not affect bacterial survival have been considered more relevant since the emergence of microbial resistance to these molecules will be minimized [57]. For this reason, compounds that specifically target the biofilm growth have been searched for the establishment of future anti-biofilm therapies [27]. In this sense, CasuL demonstrates an interesting potential to be evaluated in the future to cover surfaces thus preventing biofilm formation. Similarly to CasuL, the synthetic peptide VRLIVAVRIWRR was not a good agent against planktonic bacteria; on the other hand, it was an effective inhibitor of oral multispecies biofilms formed by bacteria from supragingival plaque [58].

Leveduriform fungi of the *Candida* genus are also of great medical importance since they cause several types of infections, especially in the mouth, eyes and genital mucosa, as well as systemic infections in immunocompromised individuals as HIV or cancer patients [59]. The development of resistance by these fungi to chemotherapeutic agents is also reported, which encourages the search for new antifungal agents [60,61]. Antifungal activity of CasuL was assessed against four *Candida* species but only *C. krusei* was sensitive to this lectin (MIC and MFC of 125 and 250 μg/mL, respectively). Differential interference contrast (DIC) microscopy revealed that control cells presented an uniform size and regular oval shape (Fig. 5A). Drastic morphological alterations, with retraction of cytoplasmic content and the presence of ruptured cells and cellular debris could be observed in *C. krusei* cells treated with CasuL at the MIC; cells undergone incomplete budding/division were also observed (Fig. 5B). Because Calcofluor is a well-known fluorochrome that binds to chitin present in the wall of yeast, it is useful to identify and quantify fungal parasites as well as to reveal changes in yeast and hyphae wall integrity and chitin content [62,63]. Staining of control-*C. krusei* yeasts with Calcofluor revealed a continuous blue fluorescence signal at cell wall indicative of preserved cell integrity (Fig. 5C). Intense and bright fluorescence could be observed in the budding region, indicating that this region is rich in chitin [63]. Conversely, CasuL-treated cells presented a decrease of fluorescence signal, with discontinuous wall labeling and diffusion of staining towards the cytoplasm (Fig. 5D). This result indicates that CasuL affected the integrity of *C. krusei* cell wall since diffuse fluorescence of Calcofluor White in yeast cells is linked to damage or even complete disintegration of the cell wall [64]. Another possibility is that the lectin binds to chitin impairing the synthesis *de novo* of cellular wall during yeast development and division [25,26].

In conclusion, the pinnulae of *C. surinamensis* leaves contain a thermo-stable and acidic lectin with cytotoxic activity against cancer cells, bacteriostatic and antibiofilm properties on human pathogenic bacteria, as well as fungistatic and fungicidal effects on *C. krusei*.

Acknowledgements

The authors express their gratitude to the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq; 446902/2014-4) for research grants and fellowship (MGRP, LCBBC, RCBQF and PMGP), the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES; AUXPE 1454/2013) and the *Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco* (FACEPE; APQ-0108-2.08/14; APQ-0661-2.08/15) for financial support. TFP would like to thank FACEPE for graduate scholarships (IBPG-0088-2.08/14). MCM would like to thank CAPES for post-doctoral scholarship. We are thankful to the Program for Technical Development of Health Inputs (PDTIS)/FIOCRUZ for the use of its facilities.

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4.2. ARTIGO 2 - LOOKING FOR ALTERNATIVE TREATMENTS FOR BOVINE AND CAPRINE MASTITIS: EVALUATION OF THE POTENTIAL OF *Calliandra surinamensis* LEAF PINNULAE LECTIN (CASUL), BOTH ALONE AND IN COMBINATION WITH ANTIBIOTICS

Artigo submetido para publicação

Looking for alternative treatments for bovine and caprine mastitis: Evaluation of the potential of *Calliandra surinamensis* leaf pinnulae lectin (CasuL), both alone and in combination with antibiotics

Thamara Figueiredo Procópio[a], Maiara Celine de Moura[a], Elinaldo Francisco de Lima Bento[b], Tatiana Soares[b], Luana Cassandra Breitenbach Barroso Coelho[a], Raquel Pedrosa Bezerra[c], Rinaldo Aparecido Mota[d], Ana Lúcia Figueiredo Porto[c], Patrícia Maria Guedes Paiva[a], Thiago Henrique Napoleão[a,*]

[a]*Departamento de Bioquímica, Centro de Biociências, Universidade Federal de Pernambuco, 50670-420, Recife, Pernambuco, Brazil.*

[b]*Centro de Tecnologias Estratégicas do Nordeste, 50740-545, Recife, Pernambuco, Brazil.*

[c]*Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, 52171-900, Recife, Pernambuco, Brazil.*

[d]*Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, 52171-900, Recife, Pernambuco, Brazil.*

*Corresponding author. Tel: +558121268540; fax: +558121268576.

E-mail address: thiagohn86@yahoo.com.br

Abstract

Mastitis is an infection that leads to a reduction in milk production and can be associated with the presence of enterotoxins in milk. CasuL is a lectin (carbohydrate-binding protein) that was isolated from *Calliandra surinamensis* leaf pinnulae. It has previously been shown to display both bacteriostatic and antibiofilm properties when applied to human pathogenic bacteria. In the present study, we investigated the antibacterial and antibiofilm activities of CasuL against fifteen different bacterial isolates that are known to cause mastitis in caprine and bovines. The lectin inhibited the growth of *Staphylococcus aureus* (Sa) and *Staphylococcus* sp. (Ssp) isolated from either bovine (isolates Ssp6PD and Sa) or caprine mastitis (isolates Ssp5D and Ssp01). The minimal inhibitory concentrations (MIC) of CasuL ranged from 3.75 to 15 µg/mL. A synergistic effect was observed for CasuL-tetracycline against Sa and Ssp6PD and CasuL-ampicillin against Ssp01. A reduction in both cell number and cells that were under incomplete division in CasuL treatments (MIC), but no structural damage, was observed under the scanning electron microscope. Flow cytometry analysis using thiazol orange and propidium iodide probes demonstrated that CasuL was unable to reduce the cell viability of any of the four isolates tested. At inhibitory and sub-inhibitory concentrations, CasuL reduced biofilm formation by Sa and Ssp5D, but did not display any antibiofilm effect against Ssp6PD or Ssp01. However, the combinations CasuL-tetracycline and CasuL-ampicillin inhibited biofilm formation by Ssp6PD and Ssp01, respectively. In conclusion, CasuL is a bacteriostatic and antibiofilm agent that is effective against mastitis isolates and displayed a synergistic potential when used in combination with either ampicillin or tetracycline both at sub-inhibitory concentrations.

Keywords: *Staphylococcus*; antibacterial activity; bovine mastitis; caprine mastitis; synergism; bacteriostatic agent.

1. Introduction

Mastitis is an infection that is caused by certain microorganisms that are present in the mammary glands. It leads to functional impairment resulting from the destruction of milk-producing tissues [1, 2]. Reduced milk production caused by cases of mastitis in bovines and caprines has an enormous economic impact on the dairy industry [3]. The presence of enterotoxins in milk and the spread of antibiotic-resistant microorganisms are further problems associated with cases of mastitis [4].

Although fungi, viruses, and algae can cause mastitis, bacteria are responsible for the highest infection rates in the mammary glands of cows and goats [5, 6]. *Staphylococcus aureus* is one of the main causes of clinical and subclinical mastitis [7–9]. However, other bacteria such as streptococci, *Escherichia coli*, and *Klebsiella pneumoniae* are also responsible for this infection [10]. Mastitis treatment and prevention consists mainly of the use of antibiotics and proper animal handling to prevent the spread of the disease to healthy animals. However, the use of antibiotics requires caution in order to avoid the emergence of resistant bacteria [11, 12]. Krewer et al. [13] found simultaneous resistance to three or more antibiotics in 65.6% of *Staphylococcus* isolates that cause mastitis.

Biofilms are complex and structured communities of microorganisms enclosed in a self-produced polymeric matrix that contains exopolysaccharides, proteins, teichoic acids, enzymes, and extracellular DNA [9]. Biofilms give these microorganisms protection against environmental adversities and a higher tolerance (10–1000 times) to antibiotics as compared to planktonic forms [14, 15]. It is believed that biofilm development may contribute to the low efficacy of certain therapies used in bovine mastitis treatment, as well as to the difficulties of treating recurrent infections [16, 17].

Lectins are proteins of non-immunological origin that bind specifically and reversibly to free or conjugated carbohydrates. These proteins have significant antibacterial potential which is attributed to their ability to bind molecules present in the surface of gram-positive and gram-negative cells, leading to damage to the cell wall, loss of metabolic stability, inhibition of cell growth, and reduction in cell viability [18]. Lectins can also interfere with adhesion and invasion of host cells by bacteria [19]. Finally, lectins have been reported to be able to both prevent biofilm formation and eradicate already established biofilms [20, 21].

CasuL is a thermo-stable and acidic lectin that was previously isolated from the leaf pinnulae of *Calliandra surinamensis* (pink powder puff", "pompon du marin" or "esponjinha-

rosa"). This lectin displayed cytotoxic activity against human cancer cells, fungistatic and fungicidal effects on *Candida krusei,* and bacteriostatic and antibiofilm properties against human pathogenic bacteria [22].

In view of a previous report on the antibacterial effects of CasuL and the problems associated with bovine and caprine mastitis, this work aimed to evaluate the bacteriostatic and bactericide effects of CasuL on fifteen mastitis isolates, to determine the synergistic potential between CasuL and commercially available antibiotics, and to investigate the effects of CasuL alone or combined with antibiotics on bacterial ultrastructure and antibiofilm activity.

2. Materials and methods

2.1. Lectin purification

Calliandra surinamensis leaves were collected at Recife (Pernambuco, Brazil) and dried for two weeks at 28ºC. The pinnulae were then detached and ground using a blender. CasuL was then purified from the pinnulae powder according to the protocol described by Procópio et al. [22]. Briefly, an extract was prepared by suspending 10 g of the powder in 100 mL of 0.15 M NaCl with magnetic stirring for 16h, followed by filtration and centrifugation (12,000 *g*, 15 min, 4 ºC). The extract was then treated with ammonium sulfate at 60% saturation [23] and the precipitated fraction obtained was dialyzed against distilled water (4 h) and 0.15 M NaCl (4 h), and then loaded onto a Sephadex G-75 column (30.0 × 1.0 cm) equilibrated with 0.15 M NaCl. Elution was monitored by absorbance at 280 nm and CasuL was recovered in fractions 9 to 15.

2.2. Protein concentration

Protein concentration was determined according to Lowry et al. [24] using bovine serum albumin (31.25–500 µg/mL) as the standard.

2.3. Hemagglutinating activity

The hemagglutinating activity (HA) assay was used to determine the carbohydrate-binding ability of CasuL. A 2.5% (v/v) suspension of glutaraldehyde-treated rabbit

erythrocytes in 0.15 M NaCl was used. The Ethics Committee on Animal Use of the *Universidade Federal de Pernambuco* approved the method that was used to collect erythrocytes (process 23076.033782/2015-70). The HA was determined as described by Procópio et al. [22] and the number of HA units (HAU) was determined as the reciprocal of the highest dilution of the lectin that was able to agglutinate erythrocytes. Specific HA was calculated by determining the ratio of HA to protein concentration (mg/mL). An HA inhibitory assay was performed by incubating CasuL for 15 min with fetuin prior to the addition of erythrocyte suspension.

2.4. Bacterial isolates

Fifteen mastitis bacterial strains isolated from goats and cows (Table 1) were obtained from the collection maintained by the *Laboratório de Tecnologia de Bioativos* of the *Departamento de Morfologia e Fisiologia Animal* from the *Universidade Federal Rural de Pernambuco* (UFRPE). The majority of the isolates tested belonged to the *Staphylococcus* genus (*S. aureus*, Sa; *Staphylococcus* sp., Ssp). One of the isolates belonged to the *Corynebacterium* genus and one of the isolates was an *Escherichia coli* strain. The stock cultures were maintained at -20ºC in sterile Mueller Hinton Broth (MHB) with 10% (v/v) glycerol. For use in the assays, the bacteria were cultured in Mueller Hinton Agar (MHA) overnight at 37°C and the culture density was adjusted turbidimetrically at 600 nm (OD_{600}) to 1×10^8 colony forming units (CFU) per mL in sterile 0.15 M NaCl. This suspension was subsequently diluted to 1×10^6 CFU/mL to yield approximately 1×10^5 CFU/mL as the final concentration used in the antibacterial assay described below.

2.5. Determination of minimal inhibitory (MIC) and bactericidal (MBC) concentrations

The broth microdilution assay was used to determine MIC and MBC values. First, a two-fold serial dilution of either CasuL (37.5 µg/mL) or antibiotic (ampicillin and tetraciclin) (8 µg/mL) in 80 µL of distilled water was made from the third to the twelfth wells in a row of a 96-well microplate. In the second well, 80 µL of distilled water was added (100% growth control). Next, 40 µL of MHB was added to all of the wells, including the first well, which contained only medium (sterility control). Next, 80 µL of bacterial culture (1×10^6 UFC/mL) were added to each of the wells, except for the first well. The OD_{600} was measured at time

zero and following incubation at 37ºC for 24 h. The MIC was determined as the lowest sample concentration that was able to promote a reduction of OD_{600} by 50% or higher in comparison with the 100% growth control. Each assay was performed in duplicate and three independent experiments were performed.

To determine the MBC, the supernatants from each well containing CasuL at concentration ≥MIC were smeared onto MHA medium and the plates were then incubated for 24 h at 37ºC The MBC corresponded to the lowest sample concentration that was able to reduce the number of CFU in 99.9% in comparison with the initial inoculum.

2.6. Synergism assay

Possible synergistic effects between CasuL and antibiotics (ampicillin or tetracycline) were evaluated using the method described by Pillai et al. [25]. Lectin-susceptible isolates (Sa, Ssp6PD, Ssp5D, and Ssp01) were tested in the assays. Each experiment corresponded to two rows of a 96-well microplate. CasuL was added (80 µL at 8×MIC) to the fourth well of the first row and a serial two-fold dilution in sterile Milli-Q water was performed until the penultimate well of the second row. Next, the antibiotic was added (80 µL at 4×MIC) to the penultimate well of the second row and a two-fold serial dilution was carried out in the opposite direction until the fourth well of the first row. The third well of the first row contained only CasuL (4×MIC) and the last well of the second row contained only the drug (4×MIC). Forty µL of MHB was added to all wells, except the first, which contained 200 µL of culture medium and served as a sterility control. The second well corresponded to the 100% growth control. Each well, except the first, was inoculated with microbial suspension (80 µL at 1 x 10^6 CFU/mL) and incubated at 37ºC. The experiment was monitored by measuring the OD_{600} at time zero and after 24h. An evaluation of the interaction between the different treatments was performed by determining the fractional inhibitory concentration index (FICI), as follows: FICI = (MIC of CasuL in combination/MIC of CasuL alone) + (MIC of antibiotic in combination/MIC of antibiotic alone). The combinations were classified as synergistic ($FICI \leq 0.5$), additive ($0.5 < FICI \leq 1$), indifferent ($1 < FICI \leq 2$), or antagonistic ($FICI > 2$).

2.7. Growth curves

Six-hour growth curves were determined for CasuL-sensitive isolates using either the lectin alone, or synergic combinations of CasuL with antibiotics. This assay was performed in 96-well microtiter plates according to Gaidamashvili and Van Staden [26]. Eighty µL of the inoculum (1 x 10^6 CFU/mL) in the exponential growth phase were incubated with 40 µL of MHB and 80 µL of the lectin, ampicillin, or tetracycline (at MIC), or with a synergic combination (CasuL-ampicillin/tetracyclin). In the 100% growth control, sterile distilled water (negative control) was used instead of CasuL. The plates were incubated at 37ºC and the OD_{600} was measured every hour.

2.8. Scanning electron microscopy (SEM)

Three-dimensional images of bacterial cells were obtained by SEM. The cells (1.2 mL; 10^8 CFU/mL) were incubated with MHB (0.6 mL) and 400 µL of CasuL (at MIC), CasuL-antibiotic synergic combination, or distilled water (negative control). After incubation (24 h at 37°C), the samples were centrifuged (300 × *g*; 10 min, 25°C) and the cell pellet was washed three times with 0.1 M phosphate buffered saline (PBS) pH 7.0, followed by three washings in 0.1 M cacodylate buffer, and then fixation in 2.5% glutaraldehyde/4% paraformaldehyde/5 mM $CaCl_2$ in 0.1 M cacodylate buffer pH 7.2 for 30 min at 28ºC. The cells were then allowed to adhere to stubs (Ø 12.7 mm, 9 mm length; Ted Pella Inc., Redding, CA, USA) and post-fixed for 1 h with 1% osmium tetroxide/0.8% potassium ferricyanide/5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2. The cells were dehydrated in graded acetone, critical-point-dried with CO_2, coated with a 20 nm-thick gold layer, and observed with a Quanta 200F (FEI Company, Hilsboro, OR, USA) scanning electron microscope.

2.9. Flow cytometry

The viability of bacterial cells treated with CasuL was evaluated using the Cell Viability Kit of BD Biosciences (San Jose, CA, USA). The isolates were incubated with the lectin at the MIC as described in section 2.5. The negative control was prepared by adding distilled water instead of CasuL. For the positive control, cells were treated with 70% (v/v) isopropyl alcohol for 1 h. Following incubation (24 h, 37°C), the samples were centrifuged (300 × *g*, 10 min, 25°C) and the cell pellets were washed three times with 0.1 M PBS pH 7.0. Next, 42 µM thiazole orange (5 µL) and 4.3 mM propidium iodide (5 µL) were added to the

assays, which were vortexed and incubated for 5 min at 25ºC. Data acquisition was performed in a BD Accuri C6 cytometer (BD Biosciences) with an SSC threshold of 200 and stopped after gating *20,000* events for each sample. Analysis was performed in the BD Accuri C6 Software.

2.10. Antibiofilm assay

Forty µL of MHB medium, 80 µL of the bacterial suspension (1 x 10^8 CFU/mL) and 80 µL of ultrapure Milli-Q water (control) or 80 µL of CasuL (1/8, 1/4, 1/2, and 1 × MIC, in Milli- Q water) were added to each well of a 96-well polystyrene microplate. The OD_{600} was recorded at this time and the microplate was further incubated at 37°C for 24 h. After this period, the OD_{600} was read again, the content of the wells was removed, and the plate wells were washed three times with saline solution. They were then fixed with absolute methanol for 30 min. The remaining attached cells were heat-fixed at 50°C for 60 min and stained with 0.4% (w/v) crystal violet for 25 min at 25°C. After washing with water, the stain bound to the biofilm was solubilized with absolute ethanol (25-min incubation) and the absorbance was measured at 570 nm [27]. Tetracycline and/or ampicillin (1/8, 1/4, 1/2, and 1 × MIC, in Milli-Q water) was used as positive control. In addition, the synergic combinations (regarding bacteriostatic effects) were evaluated for their antibiofilm effect. Three independent experiments were performed in triplicate.

2.11. Statistical analysis

The data were expressed as the mean or the percent mean ± standard deviation (SD) and statistical differences were determined using Tukey's test. A p value of <0.05 was considered to be statistically significant.

3. Results and discussion

Mastitis is the most frequent type of inflammation that occurs in milk-producing animals and the disease that has the greatest impact on dairy farming [28]. *S. aureus* is the main species of bacteria that causes mastitis and its pathogenesis is attributed to a combination of extracellular, virulence factors and properties, such the ability to form

biofilms and to develop resistance to antibiotics [29–31]. A previous report on the bacteriostatic and antibiofilm activities of CasuL on human pathogenic *Staphylococcus saprophyticcus* and *S. aureus* isolates [22] encouraged us to evaluate whether this lectin could exert antibacterial effects on *Staphylococcus* and other bacterial isolates from bovine and caprine mastitis.

CasuL was isolated according to the protocol previously established by Procópio et al [22]. The isolated lectin showed a specific HA of 1,420 and was inhibited by fetuin, as in the previous report, confirming that the carbohydrate-binding activity of the sample was effective.

CasuL was able to inhibit the growth of four isolates, all of which were of the *Staphylococcus* genus. Two of the strains were isolated from bovine mastitis (Ssp6PD and Sa) and the others two of the strains were isolated from caprine mastitis (Ssp5D and Ssp1). The MIC values are presented in Table 1 and ranged from 3.75 to 15 µg/mL. It was not possible to determine the MBC as none of the concentrations tested prevented bacterial growth in agar. The MIC values for the reference drugs (ampicillin and tetracycline) are also shown in Table 1. Eight isolates (three bovine and five caprine) were found to lack sensitivity to at least one of the antibiotics.

Combining antimicrobial phytochemicals with commercial drugs expands the field for the application of these natural compounds and can minimize the impact of pathogen resistance [2, 32]. Therefore, the potential synergy between CasuL and antibiotics was evaluated, and the results are shown in Table 2. A synergistic effect was observed for the combinations CasuL-tetracycline against the isolates Sa and Ssp6PD, and for the combination CasuL-ampicillin against the isolate Ssp01. An additive effect was observed against the isolate Ssp5D for the combination CasuL-Tetracycline, while antagonism was observed for the combinations CasuL-ampicillin and CasuL-tetracycline, against the strains Sa and Ssp01, respectively. Since it was not possible to determinate the MIC in the combinations.

Six-hour growth curves were determined for the lectin-sensitive isolates in the absence and presence of either CasuL or antibiotics (Figure 1). When incubated with the lectin, all of the isolates grew similarly to the negative control (100% growth), indicating that the bacteriostatic effect only appears later on (after the six hour period evaluated). The presence of tetracycline and ampicillin led to a reduction in the growth of the Sa isolate at 3 h of incubation (Figure 1A), while the isolate Ssp6PD had its growth reduced after 5 h of incubation with both antibiotics (Figure 1B). For the isolate Ssp01, neither antibiotic showed any inhibitory effect in the first 6 h of incubation (Figure 1C), similarly to CasuL. Finally, for

the isolate Ssp5D, tetracycline was shown to be able to inhibit growth from the third hour of incubation onwards (Figure 1D). This isolate was not sensitive to ampicillin.

Growth curves for synergistic combinations of CasuL and antibiotics were also determined in order to analyze whether the time-effect relationship of these antibacterial agents could be improved when combined. Figure 2A shows that the growth of isolate Sa was reduced by treatment with the CasuL-tetracycline combination after four hours. This result demonstrates that the combination was able to affect the bacterial cells within a short incubation period, similar to that observed for the antibiotic alone, though at a concentration four times greater. For the isolates Ssp01 (Figure 2B) and Ssp6PD (Figure 2C), treatment with the combinations CasuL-ampicillin and CasuL-tetracycline did not result in an inhibition of growth within short incubation periods. These results demonstrate that treatment with CasuL combined with antibiotics at synergic concentrations does not enhance the bacteriostatic effect.

The antibacterial activity of lectins has previously been associated with their ability to bind to peptidoglycans, lipopolysaccharides, and other molecules present in the cell wall, and by interfering with cell growth and viability and promoting structural damage [18, 33]. To determine whether CasuL acts by disrupting the integrity of bacterial cell surface, the isolates were incubated with the lectin at either the respective MIC, or with synergic combinations of CasuL with either tetracycline or ampicillin. The cells were then visualized by SEM. Figure 3 shows a remarkable reduction in cell number and cells under incomplete division following treatment with CasuL. These observations corroborate with the bacteriostatic effect of lectin. However, no bacterial surface alteration was observed following treatment with either CasuL or with the CasuL-antibiotic combinations in comparison with the negative control. These results indicate that the bacteriostatic effects of CasuL and of the synergistic combinations do not involve causing structural alterations.

The results of SEM prompted us to evaluate whether CasuL, in spite of the absence of structural damage, could be affecting bacterial viability. Flow cytometry analysis was performed using the dye thiazol orange to stain all bacterial cells and propidium iodide to stain non-viable cells. The results demonstrate that CasuL did not reduce the cell viability of all the four susceptibility isolates. The mean fluorescence of propidium iodide (FL3 channel) of lectin-treated cells was similar to that of the negative control while cells incubated with positive control (isopropyl alcohol) were intensely stained by this probe (Figure 4). These results suggest that CasuL probably acts by inhibiting the replication of bacterial cells without

killing them. Bacteriostatic agents and bactericidal drugs are both relevant in many clinical situations and can have both disadvantages and advantages depending on the particular case. For example, the lytic action of bactericidal agents may result in an endotoxin surge and the production of a large number of cell wall fragments leading to exacerbated inflammatory reaction. On the other hand, the use of bacteriostatic drugs can minimize the risk of exotoxin-related shock-syndrome. An example of this is the effective use of clindamycin to treat *S. aureus* infections [34].

Biofilm-forming bacteria are usually highly tolerant to conventional antibiotics and are often resistant to the host immune response [35]. Recurrent mastitis infections are often attributed to biofilm growth [16]. Therefore, we evaluated the antibiofilm activity of CasuL alone at inhibitory and sub-inhibitory concentrations as well as the antibiofilm activity of CasuL-antibiotic combinations. Biofilm formation by the Sa isolate was reduced by approximately 30% by treatment with CasuL at 1/2×MIC and MIC (Figure 5A) and by up to 60% following treatment with ampicillin (Figure 5C) but was not affected by treatment with tetracycline (Figure 5B). Treatment with the synergic combination CasuL-tetracycline led to a 26% decrease in biofilm development (Figure 5D). CasuL was found to be less effective than a C-type lectin from *Bothrops jararacussu* (3.12–100 µg/mL), which inhibited biofilm formation by a *S. aureus* isolate from bovine mastitis by over 50% [9].

Treatment with CasuL led to an increase in biofilm development by isolate Ssp6PD at all of the concentrations tested (Figure 5E). Neither of the commercial antibiotics was found to act as antibiofilm compounds against Ssp6PD, also, stimulating biofilm formation (Figures 5F and 5G). Biofilm formation can be used as a defensive strategy by bacteria to escape the effects of antimicrobial agents [36]. Interestingly, the combination, CasuL-tetracycline, inhibited biofilm formation by almost 60% (Figure 5H). This result is impressive as treatment with this combination not only reduced the amount of CasuL and antibiotic required to inhibit bacterial growth, but also neutralized the biofilm stimulatory effect of both of the antimicrobial agents. A synergistic effect in antibiofilm activity was also detected for the antimicrobial peptide coprisin when it was applied in combination with the antibiotics ampicillin, vancomycin, or chloramphenicol [37].

Antibiofilm activity was not observed following treatment of the isolate Ssp01 with CasuL (Figure 5I). While tetracycline was found to stimulate biofilm development (Figure 5J), ampicillin had no effect (Figure 5K). Similar to the result observed for the isolate Ssp6PD, the CasuL-ampicillin combination showed an antibiofilm effect, inhibiting formation

by approximately 35% (Figure 5L). CasuL also reduced biofilm formation by isolate Ssp5D at 1/4×MIC (Figure 5M) and tetracycline reduced biofilm formation at all of the concentrations tested (Figure 5N).

4. Conclusions

CasuL displayed bacteriostatic activity against four mastitis isolates from the *Staphylococcus* genus as well as synergistic potential when given in combination with either ampicillin or tetracycline. The antibacterial activity of CasuL does not cause structural damage or impairment of cell viability. This correlates with the absence of bactericidal action and defines it as a bacteriostatic drug. At sub-inhibitory concentrations, CasuL acted as an antibiofilm agent against *S. aureus* and one *Staphylococcus* sp. isolate. It is important to highlight that two lectin-sensitive isolates displayed an ability to respond to the presence of an antibacterial compound by forming biofilm. However, the CasuL-antibiotic combinations were able to prevent this response. The results of our studies suggest that it would be worthwhile to carry to further studies to evaluate the *in vivo* effects of CasuL for the treatment of mastitis, particularly when used in conjunction with antibiotics.

Acknowledgements

The authors express their gratitude to the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq; 446902/2014-4) for research grants and fellowships (LCBBC, RAM, ALFP, PMGP and THN). We would also like to thank the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) and *Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco* (FACEPE; APQ-0108-2.08/14; APQ-0661-2.08/15) for their financial support. TFP would like to thank FACEPE for the award of graduate scholarship, IBPG-0088-2.08/14. MCM would like to thank CAPES and FACEPE (BCT-0059-2.08/18) for the post-doctoral scholarship.

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Figure captions

Figure 1. Growth curves of the mastitis isolates Sa (A), Ssp6PD (B), Ssp01 (C), and Ssp5D (D) in absence or presence of CasuL, ampicillin, or tetracycline at their respective minimal inhibitory concentrations (MIC). The optical density (OD) at 600 nm was determined every hour for a period of 6h. For the negative control, cells were treated with distilled water instead of antibacterial agent. Data were expressed as the mean ± standard deviation (SD). All the MIC values can be seen in Table 1.

Figure 2. Growth curves of the mastitis isolates Sa (A), Ssp01 (B), and Ssp6PD (C) in absence or presence of CasuL-tetracyline (A, C) or CasuL-ampicillin (B) synergic combinations. The optical density (OD) at 600 nm was determined every hour for a period of 6 h. For the negative control, cells were treated with distilled water instead of antibacterial agent. Data were expressed as the mean ± standard deviation (SD). The concentration of CasuL and antibiotics in the synergic combinations can be seen in Table 2.

Figure 3. Scanning electron microscopy of bacterial cells of the isolates Sa, Ssp6PD, Ssp01, and Ssp5D following exposure to either CasuL at the minimal inhibitory concentration (MIC), or to CasuL-antibiotic synergic combination (except for Ssp5D isolate). For the negative control, cells were treated with distilled water instead of antibacterial agent. The synergistic combinations used were as follows: CasuL-tetracycline for isolates Sa and Ssp6PD, and CasuL-ampicillin for the isolate, Ssp1. Reduction in cell number and cells under incomplete

division can be seen in CasuL treatments, but no bacterial surface alteration was observed following treatments with either lectin or with synergic combinations. The MIC values of CasuL can be seen in Table 1. The concentrations of CasuL and antibiotics in the synergic combinations can be seen in Table 2.

Figure 4. Analysis of cell viability of the isolates Ssp01 (A), Ssp5D (B), Ssp6PD (C), and Sa (D) in absence or presence of CasuL at the minimal inhibitory concentration (MIC) by flow cytometry. Cells incubated in absence of lectin corresponded to the negative control (NC). Isopropyl alcohol (70%, v/v) was used as positive control (PC). Overlay histograms (at left) shows the distribution of unviable cells stained with propidium iodide (FL3 channel) in NC, PC and CasuL groups. Bar charts (at right) display the mean fluorescence in FL3 channel. Data are expressed as the mean ± standard deviation (SD). All the MIC values are given in Table 1.

Figure 5. Evaluation of the antibiofilm effect against the isolates Sa (A–D), Ssp6PD (E–H), Ssp01 (I–L) and Ssp5D (M–N) of CasuL (A, E, I, M), tetracycline (B, F, J, N) or ampicillin (C, G, K), all at sub-inhibitory concentrations, as well as of CasuL-tetracycline (D, H), and CasuL-ampicillin (L) combinations. Different letters indicate significant differences ($p<0.05$) between the treatments and the negative control. The minimal inhibitory concentrations (MIC) of CasuL and antibiotics are given in Table 1. The concentrations of CasuL and antibiotics in the combinations are given in Table 2.

Figure 1

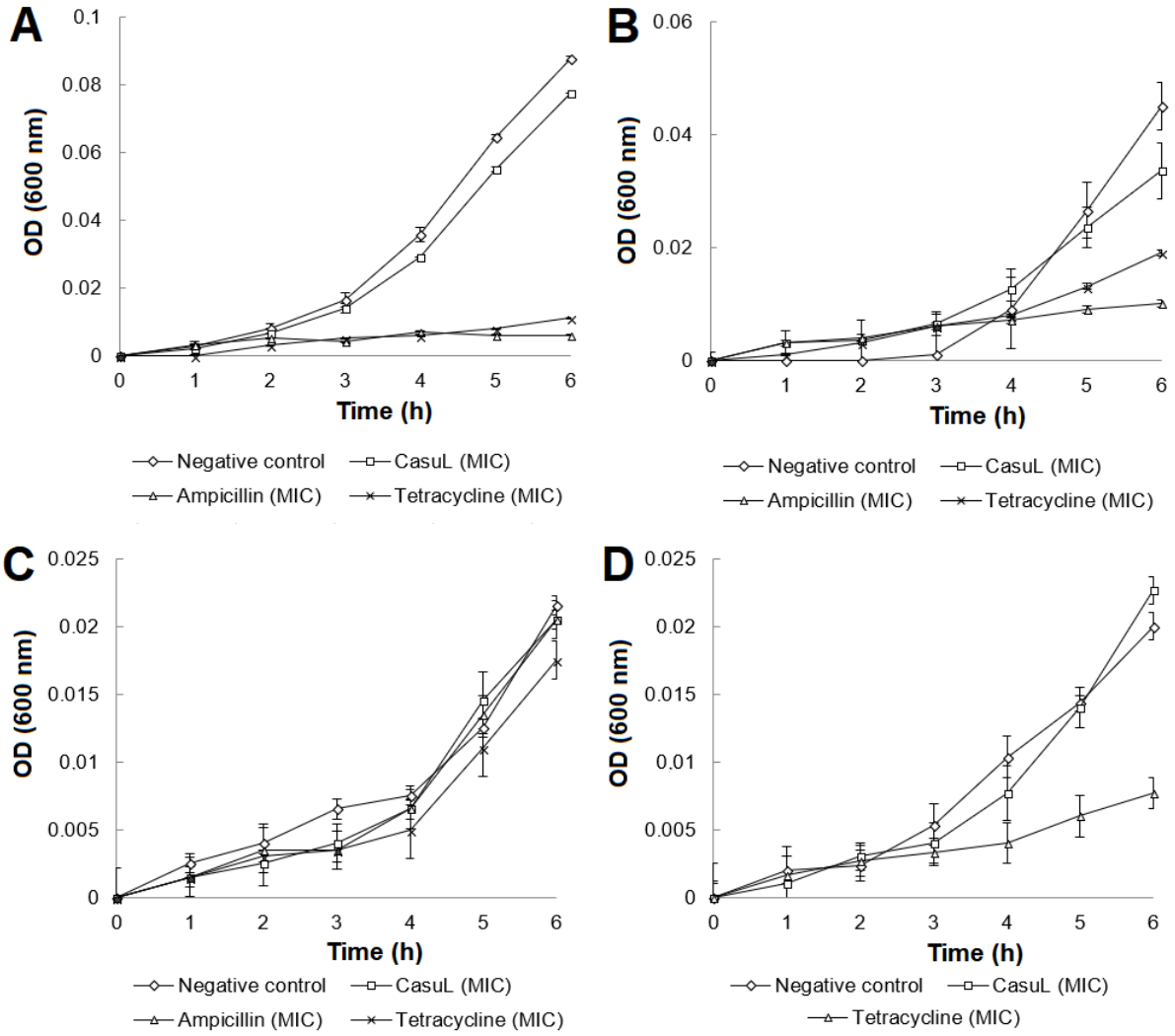


Figure 2

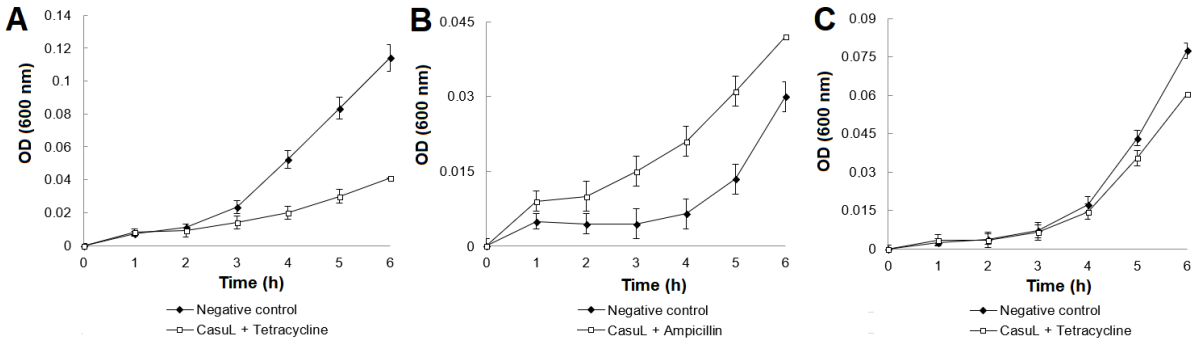


Figure 3

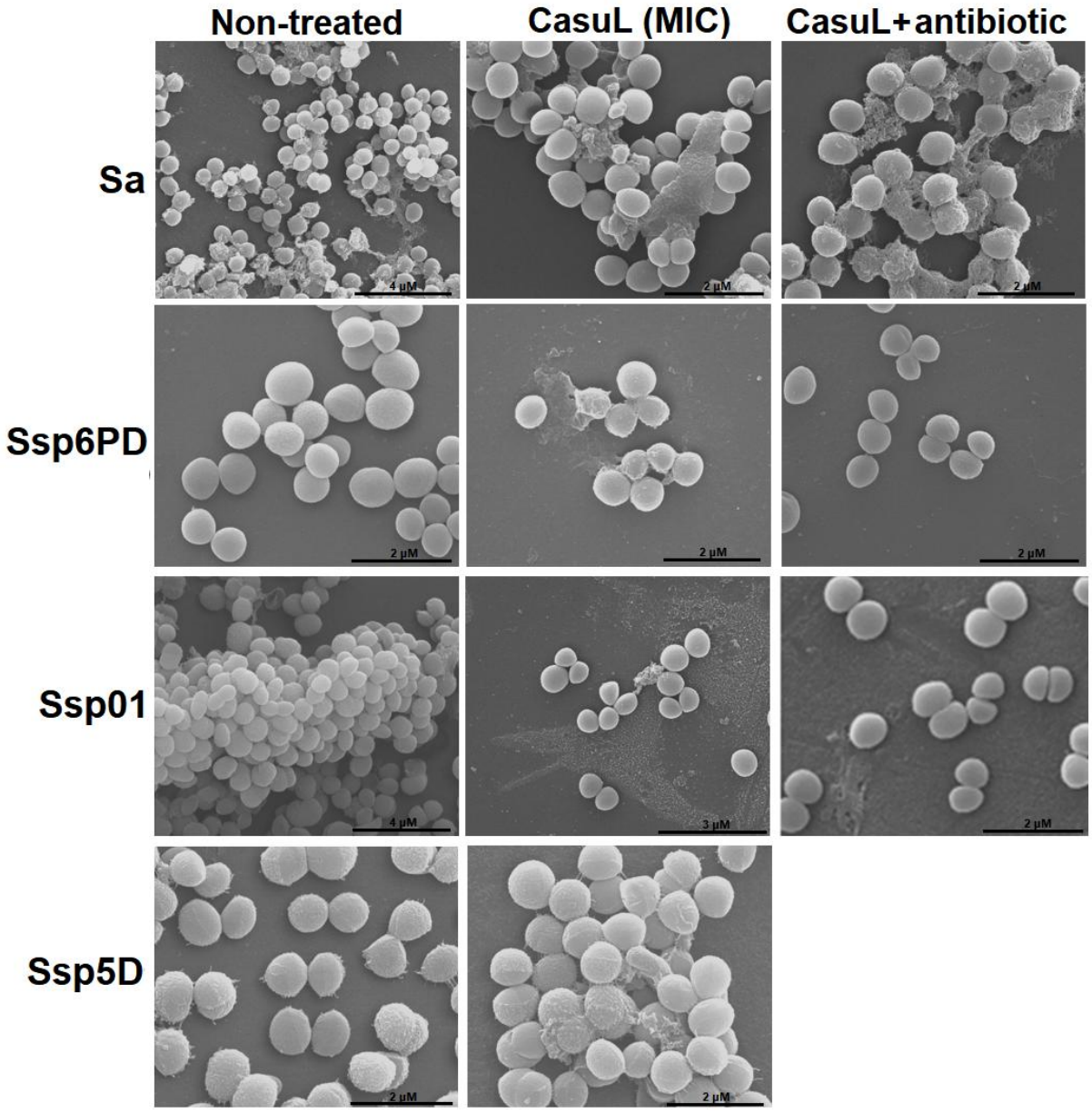

Non-treated
CasuL (MIC)
CasuL+antibiotic
Sa
Ssp6PD
Ssp01
Ssp5D
4 µM
2 µM
2 µM
2 µM
2 µM
2 µM
4 µM
3 µM
2 µM
2 µM
2 µM

Figure 4

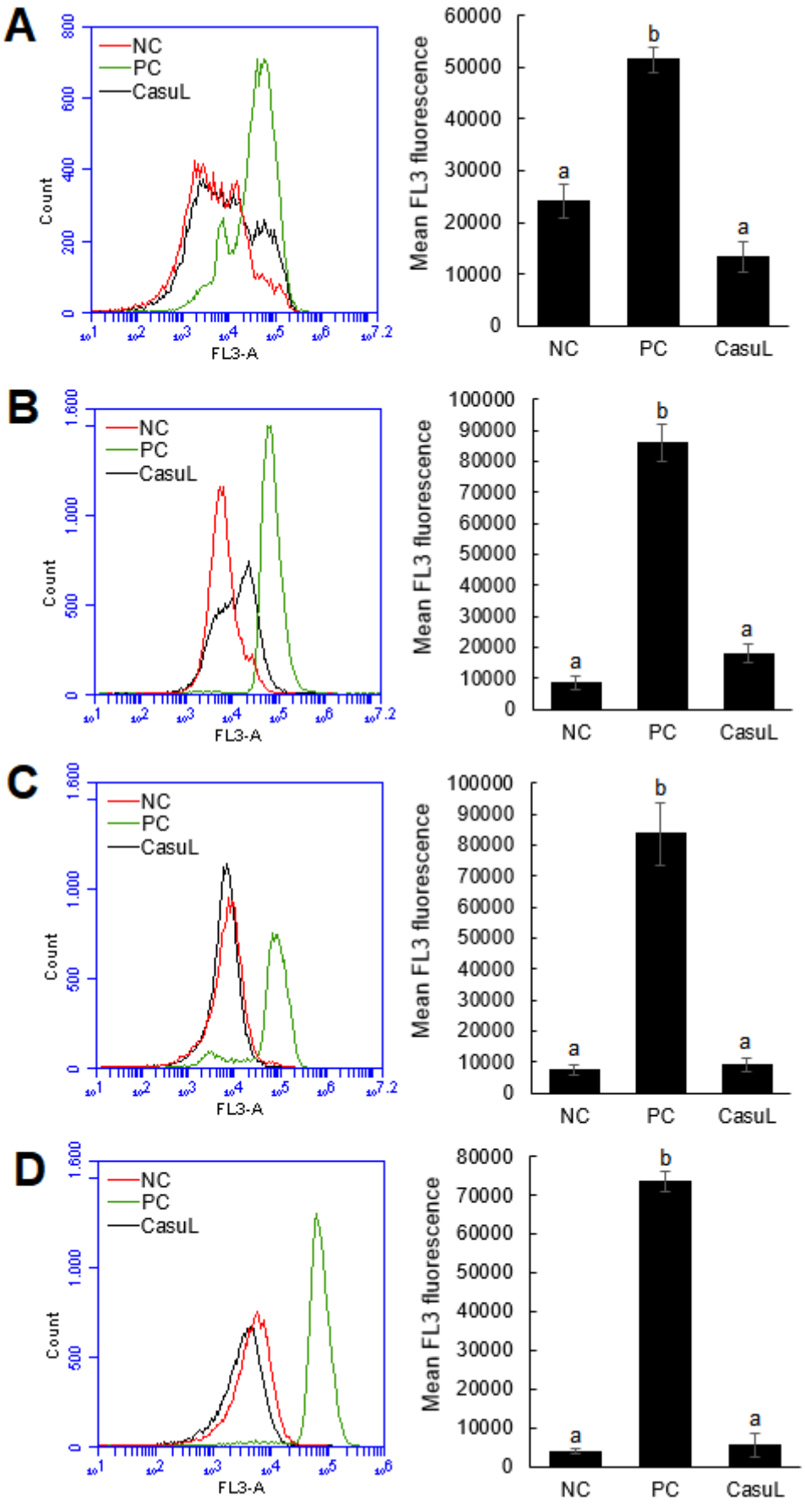

A
B
C
D
NC
PC
CasuL
Count
FL3-A
Mean FL3 fluorescence
a
b
a
NC
PC
CasuL

Figure 5

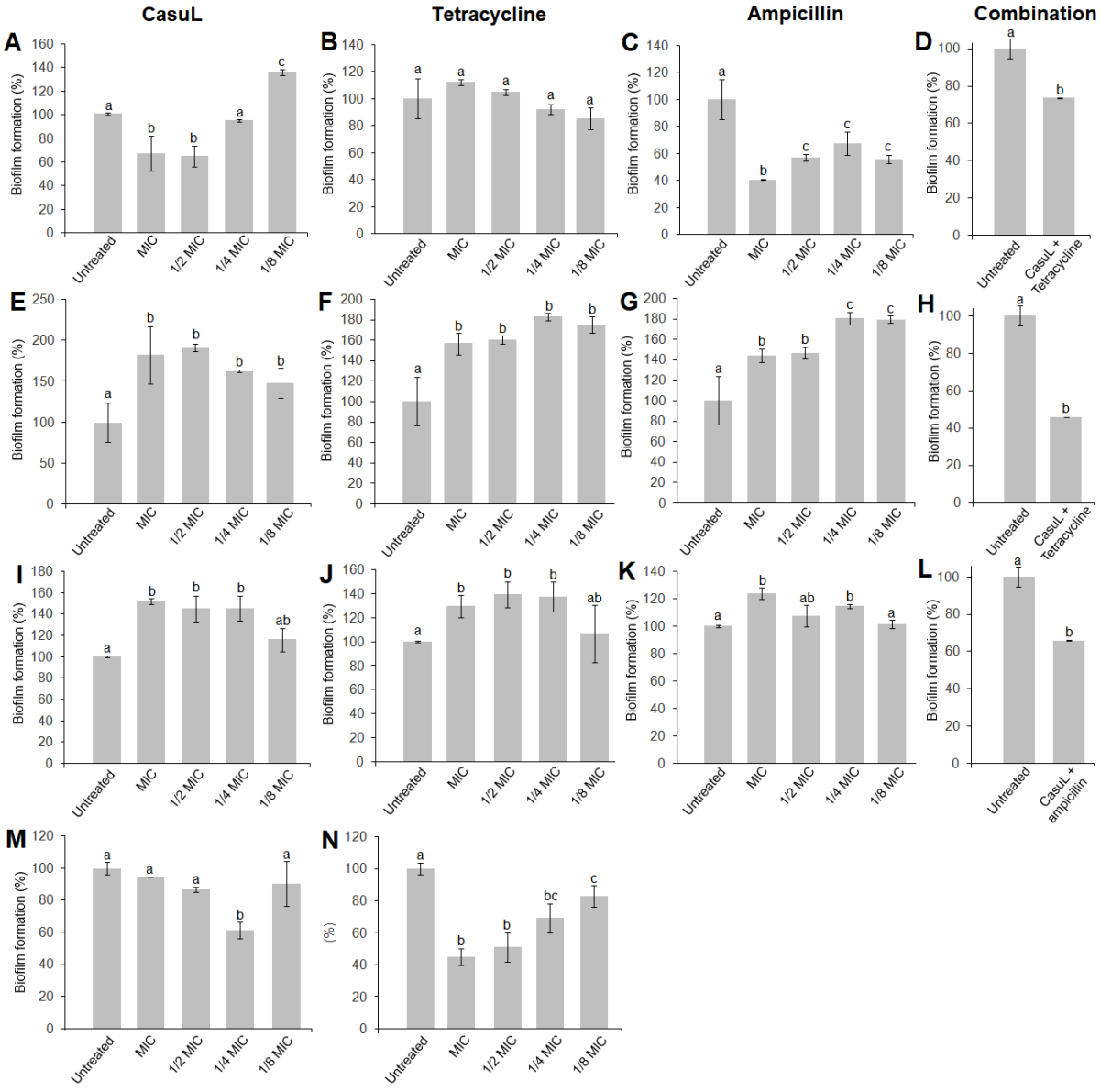

CasuL
Tetracycline
Ampicillin
Combination
A
B
C
D
E
F
G
H
I
J
K
L
M
N
Biofilm formation (%)
(%)
Untreated
MIC
1/2 MIC
1/4 MIC
1/8 MIC
CasuL + Tetracycline
CasuL + ampicillin

Table 1. Mastitis isolates and minimum inhibitory concentration (MIC) values of CasuL, ampicillin and tetracycline.

Isolates	MIC (µg/mL)		
	CasuL	Ampicillin	Tetracycline
Bovine mastitis			
CORY (*Corynebacterium* sp.)	ND	ND	0.25
Ec (*Escherichia coli*)	ND	4.0	0.125
Sa (*Staphylococcus aureus*)	3.75	8.0	0.5
Ssp13PD (*Staphylococcus* sp.)	ND	0.125	0.125
Ssp18PE (*Staphylococcus* sp.)	ND	0.5	8.0
Ssp5PE (*Staphylococcus* sp.)	ND	0,125	ND
Ssp6PD (*Staphylococcus* sp.)	3.75	4.0	2.0
Caprine mastitis			
Sa24 (*Staphylococcus aureus*)	ND	8.0	0.5
Ssp1 (*Staphylococcus* sp.)	15.0	0.5	1.0
Ssp2 (*Staphylococcus* sp.)	ND	ND	2.0
Ssp310 (*Staphylococcus* sp.)	ND	8.0	0.125
Ssp48 (*Staphylococcus* sp.)	ND	0.125	ND
Ssp5D (*Staphylococcus* sp.)	3.75	ND	4.0
Ssp601 (*Staphylococcus* sp.)	ND	0.5	ND
Ssp9 (*Staphylococcus* sp.)	ND	0.5	4.0

ND: not detected.

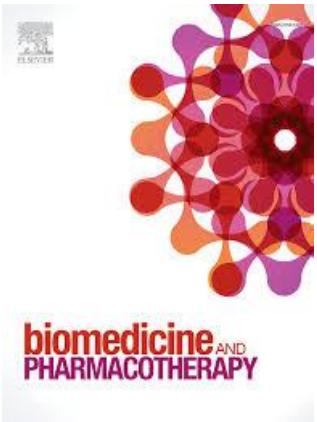
Table 2. Evaluation of antimicrobial activity of CasuL in combination with ampicillin or tetracycline against *Staphylococcus* isolates from bovine and caprine mastitis.

Isolate	MIC (µg/mL)						FICI	Effect
	Alone			In combination				
	CasuL	Ampicillin	Tetracycline	CasuL	Ampicillin	Tetracycline		
Sa (*Staphylococcus aureus*)	3.75	8.0	0.5	ND	ND	---	NC	Antagonism
				0.00023	---	0.125	0.250	Synergism
Ssp6PD (*Staphylococcus* sp.)	3.75	4.0	2.0	0.00003	8.0	---	2.000	Indifferent
				0.00366	---	0.03125	0.016	Synergism
Ssp01 (*Staphylococcus* sp.)	15	0.5	1.0	0.00183	0.01563	---	0.031	Synergism
				ND	---	ND	NC	Antagonism
Ssp5D (*Staphylococcus* sp.)	3.75	ND	4.0	0.00006	---	4.0	1.000	Additive

MIC: minimum inhibitory concentration. ND: not detected. NC: not calculated. FICI: fractional inhibitory concentration index. Classification: synergistic (FICI $\leq$ 0.5), additive (0.5 < FICI $\leq$ 1), indifferent (1 < FICI $\leq$ 2) or antagonistic (FICI > 2).

4.3. ARTIGO 3 - *Calliandra surinamensis* LECTIN (CASUL) DOES NOT IMPAIR THE FUNCTIONALITY OF MICE SPLENOCYTES, PROMOTING CELL SIGNALING AND CYTOKINE PRODUCTION

Artigo publicado no periódico Biomedicine & Pharmacotherapy, volume 107, p. 650-655, 2018



Fator de impacto: 3,457 (JRC-2017)

Biomedicine & Pharmacotherapy 107 (2018) 650–655

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Calliandra surinamensis lectin (CasuL) does not impair the functionality of mice splenocytes, promoting cell signaling and cytokine production

Thamara Figueiredo Procópio[a], Leydianne Leite de Siqueira Patriota[a], Bárbara Rafaela da Silva Barros[b], Lethícia Maria de Souza Aguiar[b], Virgínia Maria Barros de Lorena[c], Patrícia Maria Guedes Paiva[a], Cristiane Moutinho Lagos de Melo[b], Thiago Henrique Napoleão[a,*]

[a] Departamento de Bioquímica, Centro de Biociências, Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife, Brazil
[b] Departamento de Antibióticos, Centro de Biociências, Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife, Brazil
[c] Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, Pernambuco, Brazil

ARTICLE INFO

Keywords:
Leguminous
Plant lectin
Oxidative stress
Tumor necrosis factor
Immunomodulation

ABSTRACT

CasuL is a lectin (carbohydrate-binding protein) isolated from the leaf pinnulae of *Calliandra surinamensis* that is toxic against cancer cells. In this study, the effects of CasuL on the activation of immune cells were evaluated in BALB/c mice splenocytes. Assays measuring the changes in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$), mitochondrial membrane potential ($\Delta\Psi m$), and reactive oxygen species (ROS) levels associated with cell viability, proliferation, and cytokine and nitric oxide production were performed. The lectin (3.12–100 μg/mL) did not induce apoptosis or necrosis of splenocytes, and treatment for 48 h at 12.5 μg/mL stimulated cell proliferation. High cytosolic ROS levels were found in cells incubated with CasuL (12.5 μg/mL), but it did not affect $[Ca^{2+}]_{cyt}$, mitochondrial ROS, and $\Delta\Psi m$ levels. Furthermore, CasuL promoted high IL-2 and TNF-α production but did not affect nitric oxide release. In conclusion, CasuL was able to promote oxidative stress in mouse immune cells without inducing cell damage, and stimulated proliferation and cytokine production. These findings suggest the potential use of CasuL in future antitumoral and immunological targets.

1. Introduction

Calliandra surinamensis (Fabaceae) is a low branching tropical shrub commonly known as “pink powder puff,” “pompon du marin,” or “esponjinha-rosa.” This plant is mainly recognized by its ornamental relevance, but also used in folk medicine to treat coughs, wounds, and inflammation [1]. A methanolic extract from stem bark has been demonstrated to have antimicrobial activity [2], and methanolic extract from leaves and their fractions showed membrane stabilizing activity, antimicrobial effects, and moderate thrombolytic action [3]. Myricitrin, lupeol, ferulic acid, and flavonol glycosides have been detected in *C. surinamensis* bark, leaves, and flowers, but no biological activities have been attributed to them [1,4]. One of the few biomolecules with demonstrated bioactivity isolated from *C. surinamensis* is the lectin, CasuL [5].

Lectins constitute a heterogeneous group of proteins that bind carbohydrates (mono-, oligo-, or polysaccharides) with high affinity and specificity through reversible bonds [6]. CasuL was isolated from the leaf pinnulae of *C. surinamensis* by protein extraction in saline solution, precipitation with ammonium sulfate, and gel filtration chromatography. It is a 48 kDa acidic, oligomeric, and thermostable protein with cytotoxic activity on leukemic (K562) and breast cancer (T47D) cells. In addition, CasuL showed bacteriostatic and anti-biofilm activities on non-resistant and antibiotic-resistant *Staphylococcus* isolates, and was active against *Candida krusei* by disrupting the integrity of the cell wall [5].

Plant-derived compounds with pharmaceutical potential need to be assessed for their toxicity since the natural origin is not a guarantee for their safe use [7]. With regards to the effects of plant compounds on the immune system, both immunotoxic and immunomodulatory effects have been previously reported. For example, the administration of *Senna occidentalis* to rats led to alterations in lymphoid organs and neutrophils with direct toxicity to the thymus, probably due to the action of anthraquinones and lipophilic substances [8]. An ethanolic extract from *Nigella sativa* reduced the viability and proliferation of rat splenocytes inhibiting the production of Th1/Th2 cytokines [9]. On the

* Corresponding author.
E-mail addresses: thiagohn86@yahoo.com.br, thiago.napoleao@ufpe.br (T.H. Napoleão).

https://doi.org/10.1016/j.biopha.2018.08.043
Received 9 June 2018; Received in revised form 6 August 2018; Accepted 10 August 2018

contrary, many plant compounds, including several lectins, are not toxic to immune cells but can modulate their function by exerting both immunosuppressive and immunostimulatory effects [10,11].

Immunomodulators may be used for stimulatory or suppressive effects depending on the therapeutic purpose [12,13]. The production of nitric oxide, cytokines, and chemokines as well clonal expansion and activation of lymphocytes can be modulated to combat cancer and microbial infections [14,15] or to minimize immune responses in autoimmune diseases or transplants [16].

The aim of this work was to evaluate the effects of CasuL on activation of mouse immune cells. Assays measuring the changes in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$), mitochondrial membrane potential ($\Delta\Psi m$), and reactive oxygen species (ROS) levels associated with cell viability, proliferation, and cytokines and nitric oxide production were performed.

2. Materials and methods

2.1. Plant material and lectin isolation

Leaves of *C. surinamensis* were collected in Recife (Pernambuco, Brazil) and placed at 28 °C to dry for 15 days. Plant collection was performed under authorization (36301) of the *Instituto Chico Mendes de Conservaçãoda Biodiversidade* (ICMBio) and the access was recorded (A2E872B) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen). The pinnulae from dried leaves were then detached, powdered using a blender, and homogenized (10 *g*) in 0.15 M NaCl (100 mL) for 16 h at 28 °C under magnetic stirring. The extract was obtained after filtration through gauze and centrifugation at 12,000 ×*g* for 15 min at 4 °C, and then treated with ammonium sulphate at 60% saturation for 4 h [17]. After centrifugation at 3000 ×*g* for 15 min, the precipitated fraction was dialyzed against distilled water and 0.15 M NaCl for 4 h each, and then loaded onto a Sephadex G-75 (GE Healthcare Life Sciences, Sweden) column (30 × 1.0 cm) equilibrated with 0.15 M NaCl. The absorbance at 280 nm was monitored, and CasuL was collected as the first protein peak eluted with 0.15 M NaCl [5] and evaluated for protein concentration [18]. Other three bioactive proteins (two lectins and one protease inhibitor) were evaluated for immunomodulatory activity in the same set of experiments of CasuL.

2.2. Hemagglutinating activity (HA)

HA was evaluated as described by Procópio et al. [5] using a suspension (2.5%, v/v) of glutaraldehyde-treated rabbit erythrocytes in 0.15 M NaCl [19]. The erythrocytes were collected as approved by the Ethics Committee on Animal Use of the *Universidade Federal de Pernambuco*, UFPE (process 23076.033782/2015-70). The HA corresponded to the reciprocal of the highest dilution of CasuL that promoted full agglutination of erythrocytes. The specific HA was calculated as the ratio between HA and the protein concentration (mg/mL).

2.3. Preparation of mice splenocytes

Female BALB/c mice (6–8-week-old) were raised and maintained at the animal facilities of the *Laboratório de Imunopatologia Keizo Asami* at UFPE. Mice were kept under standard laboratory conditions (20–22 °C and 12:12 light/dark cycle) with free access to standard diet (Labina/Purina, Campinas, Brazil) and water. The Ethics Committee on Animal Use of UFPE approved all the experiments performed (process number 0048/2016). The mice were anesthetized with 2% xylazine (10 mg/kg) and 10% ketamine hydrochloride (115 mg/kg), followed by cervical dislocation, and the spleens were removed aseptically and placed in a conical centrifuge tube containing RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) with fetal calf serum. The spleen cells were obtained through dounce equipment and the homogenate was transferred to conical tubes containing 10 mL of incomplete RPMI medium and covered with Ficoll-Paque™ Plus (GE Healthcare Life Sciences, Sweden) with density adjusted to 1.077 g/mL. After centrifugation at 2500 ×*g* at 25 °C for 25 min, the cell layer containing immune cells was recovered, washed twice with PBS, and collected after centrifugation at 500 ×*g* and 25 °C for 10 min. Cells were counted in a Neubauer chamber and viability was determined by the trypan blue exclusion method. Cells were only used when viability was higher than 98%.

2.4. Analysis of CasuL effect on cell viability

Mice splenocytes (10^6 cells) were treated with CasuL (3.12–100.0 μg/mL) in supplemented RPMI 1640 medium and were maintained in 24-well plates for 24 h at 5% CO_2 to analyze the cytotoxicity of lectin. Untreated cells were grown under the same conditions for use as a negative control. Following this, splenocytes were obtained by centrifugation at 450 ×*g* for 10 min at 25 °C. After discarding the supernatant, 1 mL of PBS was added to the precipitate, which was then centrifuged at 450 ×*g* for 10 min at 25 °C. Cell death was analyzed using the FITC Annexin V Apoptosis Detection Kit I from BD Biosciences (San Jose, CA, USA). After discarding the supernatant, the pellet was resuspended in binding buffer, and annexin V conjugated with fluorescein isothiocyanate (FITC) (1:500) and propidium iodide (PI, 20 μg/mL) were added. Flow cytometry was performed in a FACSCalibur platform using Cell Quest Pro software (BD Biosciences). Results were analyzed in graphs by a dot plot. Annexin-FITC negative/PI positive cells were considered necrotic cells, and Annexin-FITC positive/PI negative cells represented splenocytes in the early stage of apoptosis. Double negatives were considered viable cells. Six experiments were performed for statistical analysis.

2.5. Evaluation of cell proliferation

After obtaining the splenocytes, the cell suspension was centrifuged at 300 ×*g* and 25 °C for 5 min and washed with sterile PBS containing 5% (v/v) fetal bovine serum at pH 7.2. After this, the cell solution was adjusted to 10^6 cells/mL and 5 mM of 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) was added. Cells were incubated for 10 min at room temperature in the dark and at 5% CO_2. Cells were centrifuged twice at 300 × g and 25 °C for 5 min with sterile PBS. Stained cells were cultured for 24–48 h at 5% CO_2 in the presence of 3.12–12.5 μg/mL of CasuL or only complete RPMI 1640 medium (negative control). After the culture, cells were centrifuged at 300 × *g* and 25 °C for 5 min, data were acquired for 10,000 events on the FACSCalibur platform, and results were analyzed using Cell Quest Pro software. Six experiments were performed for statistical analysis.

2.6. Measurement of cytosolic Ca^{2+} concentration

Changes in $[Ca^{2+}]_{cyt}$ were measured using Fluo-3AM probe (Thermo Fisher Scientific, Waltham, MA, USA). Splenocytes (10^6 cells/mL) were incubated in plates (24 wells) with RPMI 1640 medium and CasuL at 12.5 μg/mL in a humidified CO_2 incubator (5% CO_2) for 24 h. Cells incubated only with medium were used as the control. After the incubation, splenocytes were washed with PBS and centrifuged at 300 × *g* and 26 °C for 5 min. The cell pellets were plated onto new 24-well plates and incubated with 5 μM of Fluo-3AM containing 1 μM of pluronic acid F-127 (Sigma-Aldrich, St. Louis, MO, USA) and 30 μg/mL of bovine serum albumin (Sigma-Aldrich) at 5% CO_2 and 37 °C for 40 min. After the incubation, cells were washed with PBS centrifuged at 300 × *g* and 26 °C for 5 min and then transferred to cytometer tubes. Maximum fluorescence was measured using the tubes with cells incubated with 1 μM ionomycin for 2 min, and minimal fluorescence was measured using tubes with cells incubated with 8 mM EDTA for 2 min. Fluorescence intensity was analyzed using the FACSCalibur flow

cytometer with excitation at 395 nm and emission at 525 nm A minimum of 10,000 events were collected. Next, $[Ca^{2+}]$cyt was calculated with the following equation: $[Ca^{2+}]cyt = Kd(F_{sample} - F_{min})/F_{max} - F_{sample}$, where F represents the fluorescence value and Kd was 390 nM [20–22]. Six experiments were performed for statistical analysis.

2.7. Measurements of cytosolic and mitochondrial ROS levels

Cytosolic and mitochondrial levels of ROS were measured by flow cytometry using dihydroethidium (DHE) (Sigma-Aldrich) and MitoSox Red (Thermo Fisher Scientific) probes, respectively, as described previously [22]. Splenocytes (10^6 cells/mL) were incubated in plates (24 wells) with RPMI 1640 medium and 12.5 µg/mL CasuL at 5% CO_2 for 24 h. Cells incubated only with medium were used as control. After incubation, splenocytes were washed with PBS and centrifuged at 300 $\times g$ and 26 °C for 5 min. The cell pellets were plated onto new 24-well plates and incubated at 5% CO_2 with 5 µM DHE for 40 min or with 5 µM MitoSox Red for 10 min. After the incubation, cells were washed with PBS and centrifuged at 300 $\times g$ and 26 °C for 5 min and then transferred to cytometer tubes. Fluorescence intensity was analyzed in the FACSCalibur flow cytometer with excitation at 488 nm and emission at 620 nm A minimum of 10,000 events were collected. Six experiments were performed for statistical analysis.

2.8. Determination of the mitochondrial transmembrane potential (ΔΨm) in intact mouse splenocytes

Changes in ΔΨm were measured using MitoStatus probe (BD Biosciences). Splenocytes (10^6 cells/mL) were incubated in 24-well plates with RPMI 1640 medium and 12.5 µg/mL CasuL at 5% CO_2 for 24 h. Cells in only medium were used as the control. After incubation, splenocytes were washed with PBS and centrifuged at 300 $\times g$ and 26 °C for 5 min. The pellet was plated onto new 24-well plates and incubated with 100 nM MitoStatus at 37 °C with 5% CO_2 for 30 min. After incubation, cells were washed with PBS and centrifuged at 300 $\times g$ and 26 °C for 5 min, and then transferred to cytometer tubes. Fluorescence intensity was analyzed in the FACSCalibur flow cytometer with an excitation of 488 nm and emission of 620 nm A minimum of 10,000 events were collected. Six experiments were performed for statistical analysis.

2.9. Cytokine production measurement

Supernatants of splenocyte cultures untreated or treated with CasuL (12.5 µg/mL) for 24 h were collected for quantification of cytokines using the Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (Becton Dickinson Biosciences, USA) for simultaneous detection of interleukins (IL-2, IL-4, IL-6, IL-10, and IL-17 A), tumor necrosis factor (TNF), and interferon-gamma (IFN-γ). The assays were performed according to the manufacturer's instructions and data were acquired on the FACSCalibur platform. Seven individual cytokine standard curves (0–5000 pg/mL) were generated in each assay. The range of detection was between 2 and 5000 pg/mL. The results were analyzed using the FCAP 3.1 software (BD Biosciences). Six experiments were performed for statistical analysis.

2.10. Nitrite analysis

The supernatants from cultures of splenocytes untreated or treated with CasuL (12.5 µg/mL) for 24 h were used for nitrite analysis by the colorimetric Griess method [23]. NO concentration was estimated using a standard curve (3.12–100.0 µmol/mL) and a microplate spectrophotometer (Thermo Fisher Scientific) at 595 nm. Six experiments were performed for statistical analysis.

2.11. Statistical analysis

The Shapiro-Wilk test was applied to test the normal hypothesis on the variables involved in this study. Means of the data obtained with the samples were analyzed using non-parametric tests. The statistical differences between two groups of data were analyzed by the Wilcoxon test or one-way analysis of variance (ANOVA) if there were more than three groups of data. All of the results were considered with a significance level of 5%. Statistical analysis was performed on the GraphPad Prism 5.01 software.

3. Results and discussion

Previous studies demonstrated the antimicrobial and antineoplastic effects of CasuL, which stimulated more studies on its pharmacological potential. Immunotoxicity is one of the endpoints considered in safety reiterations of foods and pharmaceuticals [24,25]. The need for safety assessments, together with the hypothesis that CasuL could act both as a cytotoxic and immunomodulating agent, led us to evaluate its effects on splenocytes. Mice splenocytes were used as a test model for future *in vivo* antitumor assays. Our assays aimed first to investigate whether CasuL promoted cell activation; second, if this activated status could lead to irreversible damage; and third, in the case of CasuL promoting cell activation, if these cells were functional or not such as with the ability to perform effector activities including developing clonal expansions and release chemical mediators.

CasuL did not induce apoptosis or necrosis of splenocytes at the tested concentrations in comparison with negative control (Fig. 1). It was previously reported that CasuL also did not affect the viability of human peripheral blood mononuclear cells (PBMCs) at 100 µg/mL [5], demonstrating that CasuL is non-toxic to mammalian immune cells. Since toxicity to splenocytes was not detected at any concentration, a proliferation assay was performed using concentrations up to 12.5 µg/mL, similar to the concentration range for the immunomodulatory effects of other lectins [26–28].

ROS are free radicals generated by the plasma membrane NADPH oxidase, lipoxygenase, or mitochondrial respiratory chain [22]. Under normal conditions, our cells constantly produce these oxidant molecules by several different mechanisms in both cytosol and mitochondria [29]. These free radicals are produced in large quantities during infections and diseases through enzymatic interactions between phagocytes and microorganisms [30]. However, higher levels of ROS induce oxidative stress and can lead to cell death [31]. The treatment of mice splenocytes with CasuL (12.5 µg/mL) induced a slight increase in cytosolic ROS (Fig. 2A) but did not alter mitochondrial ROS levels (Fig. 2B). From this result, it can be suggested that CasuL has specifically affected ROS production pathways that occur in the cytosol, such

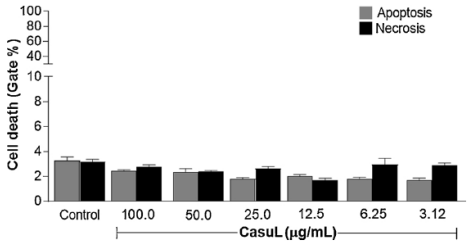


Fig. 1. Investigation of the cytotoxic effect of *Calliandra surinamensis* lectin (CasuL) (3.12–100 µg/mL) on BALB/c mouse splenocytes after incubation for 24 h. Cytotoxic effect of CasuL was assessed by flow cytometry using annexin V (AnnV) and propidium iodide (PI). AnnV−/PI + cells were considered necrotic and AnnV + /PI− cells were considered apoptotic. CasuL did not induce cell death when compared with control cells. Bars represent the mean ± standard deviation of six experiments.

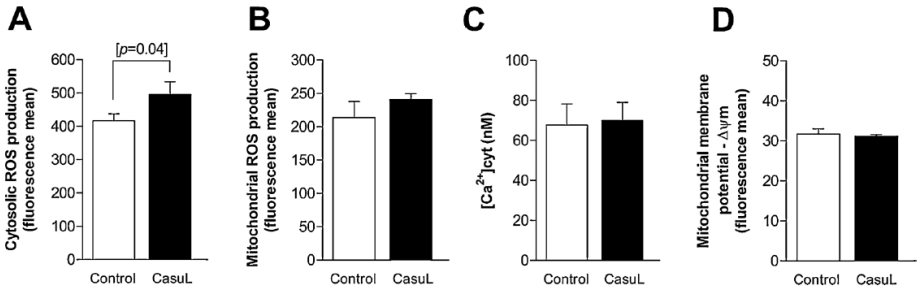


Fig. 2. Evaluation of the effects of *Calliandra surinamensis* lectin (CasuL) (12.5 μg/mL) treatment on cytosolic (A) and mitochondrial (B) reactive oxygen species (ROS) production, cytosolic calcium concentration (C), and membrane mitochondrial potential (ΔΨm) (D) on BALB/c mouse splenocytes after incubation for 24 h. CasuL increased the cytosolic ROS levels. Bars represent the mean ± standard deviation of six experiments.

as that involving the NADPH oxidase. It has been reported that lectins can induce activation of this enzyme in neutrophils through interactions with glycans of the gp91phox subunit, which is the catalytic core of the phagocyte NADPH oxidase [32].

An increase in $[Ca^{2+}]_{cyt}$ is crucial to the activation, differentiation, and effector functions of lymphocytes [33]. Thus, we determined $[Ca^{2+}]_{cyt}$ in untreated and CasuL-treated cells. The data showed that incubation with CasuL did not affect $[Ca^{2+}]_{cyt}$ in comparison with control (Fig. 2C). Melo et al. [22] highlighted that $[Ca^{2+}]_{cyt}$ can signal for both cell proliferation or apoptosis depending on the concentration, with higher levels activating apoptosis or necrosis. In this sense, the maintenance of calcium levels in cells treated with CasuL agrees with the absence of a cytotoxic effect. However, it was previously reported that treatment with mitogenic agents with natural origins can increase $[Ca^{2+}]_{cyt}$ without inducing cell death [22,34].

No change in ΔΨm was observed in CasuL-treated cells in comparison with control cells (Fig. 2D). Together with the ROS, $[Ca^{2+}]_{cyt}$, and cytotoxicity results, CasuL promoted moderate oxidative stress without initiating deeper changes in cell biochemistry that could lead to irreversible damage in splenocytes.

Glycans are present on cell membranes and may be recognized by lectins, and this interaction can trigger several intracellular responses in immune cells, such as proliferation and activation [10]. The effect of CasuL on cell proliferation was evaluated using the CFSE probe, and the results revealed a stimulatory effect after 48 h of treatment at 12.5 μg/mL (Fig. 3). The proliferation assay has been used as an indicator of immunostimulation because most of the cells evaluated in this assay were lymphocytes. After activation, lymphocytes begin to expand clonally in response to stimulus. Lectin from *Cratylia mollis* seeds (Cramoll 1,4), concanavalin A, and brown kidney bean lectin (BKBL) also induced a proliferative response in murine splenocytes, and it was suggested that these lectins can be used as mitogenic agents in immunostimulatory assays [35,36].

Associated with cell proliferation, the production of cytokines and nitric oxide (NO) were determined to evaluate the functionality of the cells treated with CasuL (12.5 μg/mL). Both untreated and CasuL-treated cells were able to release all cytokines and NO (Fig. 4A–H). However, only TNF-α (Fig. 4D) and IL-2 (Fig. 4E) were produced at higher levels compared to control cells. This behavior was expected, because the antineoplastic potential of CasuL [5] can be associated with TNF-α production in immune cells, and the induction of proliferation observed in the present study can be due to IL-2 production, a cytokine mitogenic to T lymphocytes [37].

TNF is considered a proinflammatory mediator essential for immune homeostasis because it can both trigger strong inflammatory responses as well as act as a modulator of the extent and duration of inflammatory processes; thus, it is relevant for the inhibition of autoimmune diseases and tumorigenesis [38]. The cytokine TNF-α is also known for its antitumor properties and has been used for the treatment of patients with advanced sarcomas and metastatic melanomas [39]. The stimulation of TNF by CasuL provides evidence for further studies on the anticancer potential of this lectin. In addition, the increased levels of cytosolic ROS may be related to activation of NADPH oxidase by TNF signaling [40].

The data set obtained of maintained levels values of IL-4 (Fig. 4A), IL-17 A (Fig. 4C), IL-6 (Fig. 4F), IFN-γ (Fig. 4G), and NO (Fig. 4H) production suggest that CasuL would not create an exacerbated inflammatory status. Other lectins promoted the immunomodulatory status: *Musa paradisiaca* lectin induced an increase in TNF-α production [41], and PHA (*Phaseolus vulgaris* lectin) and abrin (*Abrus precatorius* lectin) promoted a proinflammatory response in murine splenocytes [42,43].

In this study, the expression levels of IL-10 cytokine were not increased but maintained in splenocytes after treatment with CasuL (Fig. 4B). Considering the previous report on the antineoplastic activity of CasuL [5], the maintenance or the reduction of IL-10 levels was interesting since enhanced IL-10 regulatory responses were related to improper clearance of tumor cells [44]. The maintenance in IL-10 released by cells treated with CasuL was consistent with the proliferation

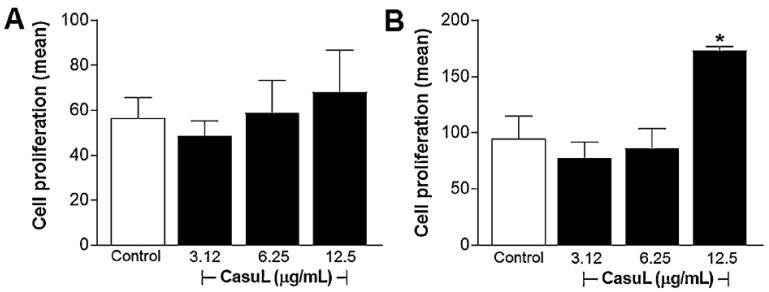


Fig. 3. Evaluation of the proliferative effect of *Calliandra surinamensis* lectin (CasuL) (3.12–12.5 μg/mL) on BALB/c mouse splenocytes after incubation for 24 h (A) and 48 h (B). Proliferation was evaluated using a 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) probe. CasuL showed mitogenic effects at 12.5 μg/mL after a 48-h treatment (*$p < 0.05$). Bars represent the mean ± standard deviation of six experiments.

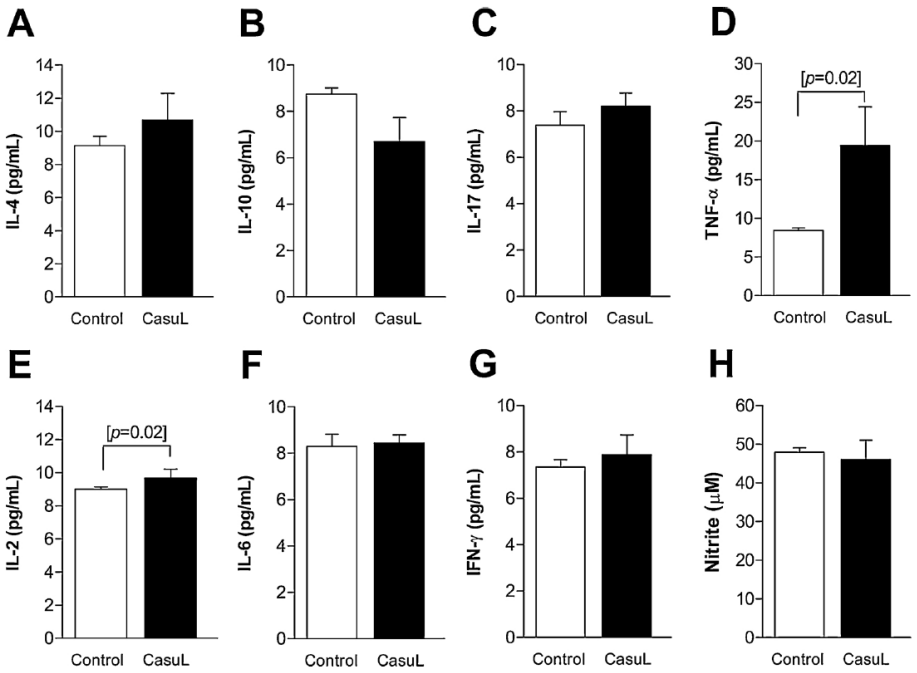


Fig. 4. Evaluation of the effects of *Calliandra surinamensis* lectin (CasuL) (12.5 μg/mL) treatment on the release of cytokines (A–G) and nitric oxide (H) on BALB/c mouse splenocytes after incubation for 24 h. CasuL increased the release of TNF (D) and IL-2 (E). Bars represent the mean ± standard deviation of six experiments.

stimulus and increase in TNF levels. A decrease in IL-10 levels can also aid to combat persistent infections and opportunistic pathogens; however, low levels were associated with autoimmune disorders and impairment of the innate immune system [44].

Lectins can also induce NO production, which can be relevant against microorganisms, parasites, and tumor cells [45,46]. However, high levels of NO are associated with oxidative stress [47]. Lectins from *Microgramma vacciniifolia* fronds and inflorescences of *Alpinia purpurata* stimulated the production of NO by human immune cells [26,27]. CasuL did not induce increase in NO production (Fig. 4H) in treated splenocytes, and this finding was probably associated to a lack of stimuli to produce IFN-γ, the major cytokine inducer of NO release.

As mentioned before, other three bioactive proteins (two lectins and one protease inhibitor) were evaluated in the same set of experiments (unpublished data). One lectin was able to induce ($p < 0.05$) the production of IL-17 (8.45 ± 0.03 *vs.* 7.39 ± 0.57 pg/mL in control), TNF (11.027 ± 0.01 *vs.* 8.43 ± 0.29 pg/mL in control), IFN (8.15 ± 0.03 *vs.* 7.34 ± 0.32 pg/mL in control), IL-4 (10.893 ± 0.04 *vs.* 9.34 ± 0.60 pg/mL in control) and IL-2 (9.46 ± 0.01 *vs.* 9.00 ± 0.14 pg/mL in control). In addition, the treatment with this same lectin reduced ($p < 0.05$) mitochondrial ROS (186.28 ± 26.9 *vs.* 235.77 ± 14.4 in control) and NO (25.28 ± 1.22 *vs.* 32.23 ± 5.55 in control) levels. The protease inhibitor promoted a decrease ($p < 0.05$) of $[Ca^{2+}]_{cyt}$ (27.11 ± 2.30 *vs.* 67.63 ± 10.61 nM in control). These data confirmed that the methodologies used in the study were working.

4. Conclusion

CasuL promoted oxidative stress in mouse immune cells without affecting their viability and functionality, and stimulated the proliferation and production of cytokines IL-2 and TNF. The data obtained here and in previous studies on this lectin stimulates evaluation of *in vivo* antitumor activity and effects on other immunological targets.

Acknowledgements

The authors express their gratitude to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; 446902/2014-4) for research grants and fellowships (PMGP and THN) as well as to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE; APQ-0108-2.08/14; APQ-0661-2.08/15) for financial support. TFP would like to thank FACEPE for graduate scholarship (IBPG-0088-2.08/14) and the Núcleo de Plataformas Tecnológicas of the Instituto Aggeu Magalhães, Fundação Oswaldo Cruz (FIOCRUZ Pernambuco).

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5. CONCLUSÕES

- ✓ CasuL foi isolada com elevado fator de purificação e demonstrou especificidade de ligação a glicoconjugados.
- ✓ CasuL é uma proteína acídica (PI: 5,82) e oligomérica, com massa molecular nativa de 48 kDa e similaridades de sequência com uma proteína de *S. bicolor*.
- ✓ A lectina se mostrou termoestável, mas sofreu modificações estruturais e perdeu sua atividade hemaglutinante quando em meio alcalino.
- ✓ CasuL apresentou ação citotóxica seletiva, sendo ativa contra células cancerosas (leucemia e câncer de mama), mas não reduziu a viabilidade de células de sangue periférico humano.
- ✓ Atividade bacteriostática e antibiofilme foi detectada sobre bactérias patogênicas ao homem (*Staphylococcus saprophyticcus* e isolados de *Staphylococcus aureus* resistente e não-resistente à oxacilina) e sobre isolados do gênero *Staphylococcus* causadores de mastire em bovinos e caprinos.
- ✓ Combinações sinérgicas de CasuL com tetraciclina ou ampicilina inibiram a formação de biofilme contra isolados de mastite.
- ✓ CasuL apresentou atividade fungicida e fungistática sobre *Candida krusei*.
- ✓ CasuL promoveu a ativação (produção de espécies reativas de oxigênio) de esplenócitos de camundongo sem induzir dano celular.
- ✓ A lectina induziu a proliferação celular e estimulou a liberação de citocinas (IL-2 e TNF-α) pelos esplenócitos.

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