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LUÍS CLÁUDIO NASCIMENTO DA SILVA

AVALIAÇÃO DA EXPRESSÃO DE CITOCINAS, ÓXIDO NÍTRICO E DE RECEPTORES TOLL LIKE EM MACRÓFAGOS PERITONEAIS TRATADOS IN VITRO COM A LECTINA NATIVA E RECOMBINANTE DE SEMENTES DE *Cratylia mollis*.

Recife, 09 de setembro de 2013

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BIOMÉDICO

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Tese apresentada ao Curso de Pós Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como pré-requisito para a obtenção do título de Doutor em Ciências Biológicas.

Orientadora: **Profª Drª Maria Tereza dos Santos Correia**
Co-Orientadora: **Profª Drª Regina C. B. Q. de Figueiredo**

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“Ó profundidade das riquezas, tanto da sabedoria, como da ciência de Deus! Quão insondáveis são os seus juízos, e quão inescrutáveis os seus caminhos! Pois o coração do homem pode fazer planos, mas sempre a resposta certa vem do Senhor. Dele é o tempo, e há tempo para todo o propósito debaixo do céu. O Senhor não demora em cumprir a sua promessa!”

(Adaptado de Romanos 11:33; Provérbios 16:1; 2 Pedro 3:9; Eclesiastes 3:1)

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RESUMO

As lectinas são proteínas conhecidas por sua capacidade de ligar específica e reversivelmente a carboidratos resultando em uma variedade de propriedades biológicas. Este trabalho tem por objetivo avaliar os efeitos imunomodulador e citoprotetor das lectinas Cramoll 1,4 (pCramoll) e rCramoll 1 (rCramoll). Na determinação da ação imunomoduladora macrófagos peritoneais de camundongos Balb/c foram tratados com diferentes concentrações das lectinas (0,625-10 μM) e foram analisados os efeitos na produção óxido nítrico (NO), viabilidade celular, produção de ânion superóxido, alterações no potencial de membrana mitocondrial (ΔΨm**)** e na fagocitose de *Staphylococcus aureus*. A produção de citocinas pró-inflamatorias (IL-1β, IL-6, INF-γ e TNF-α) foi avaliada em macrófagos infectados e não infectados com *S. aureus.* Ambas lectinas induziram significantemente a produção de NO e de citocinas. As proteínas foram consideradas não-citotóxicas pelo ensaio com MTT, entretanto a análise por Citometria de Fluxo revelaram um aumento de células mortas. A produção de superóxido foi estimulada pelas lectinas o que foi confirmado pelas mudanças induzidas no ΔΨm. A ação fagocítica dos macrófagos foi aumentada em 27,1% e 22,47% após o tratamento destes com pCramoll e rCramoll . Por fim, as lectinas inibiram a expressão de TNF-α e IL-6 e estimularam a produção de IL-1β e INF-γ por macrófagos infectados por *S. aureus.* Paralelamente, o potencial protetor das lectinas contra a morte celular induzida pelo estresse oxidativo foi avaliada. Para tanto, células Vero (fibroblastos renais de macaco) foram pré-tratadas com crescentes concentrações das lectinas (0,625-10 μM) por 30 minutos e em seguida foram expostas ao H_2O_2 (1 mM). Após 24 horas, o efeito citoprotetor foi avaliado. Os mecanismos celulares envolvidos foram determinados por citometria de fluxo envolvendo: proteção contra morte celular, danos nos lisossomos e DNA, produção de ânion superóxido (MitoSOX), alterações no potencial de

membrana mitocondrial (ΔΨm**)** e proliferação celular. pCramoll e rCramoll atenuaram a citotoxicidade induzida por H_2O_2 de forma dose-dependente, os efeitos máximos foram 96.85 ± 15.59% (rCramoll) e 59.48 ± 23.44% (pCramoll). A análise com Live/Dead mostrou redução da morte celular de 65.04 ± 3.29% (H_2O_2) para 39.77 ± 2.93% (pCramoll) e 13.90 ± 9.01% (rCramoll). Os efeitos deletérios de H_2O_2 na proliferação celular foram reduzidos em 10.83% (pCramoll) e 24.17% (rCramoll). As lectinas atenuaram a produção excessiva de superóxido, o collapso do ΔΨm e os danos lisossomais e ao DNA das células Vero expostas à H_2O_2. Em conclusão nossos estudos demonstram que pCramoll e rCramoll possuem elevado potencial imunomodulador e citoprotetor.

Palavras-chave: ativação de macrófagos, morte celular, estresse oxidativo, lectinas, efeito protetor.

ABSTRACT

This study aims to evaluate the immunomodulatory effects on macrophages and cytoprotector action against oxidative stress of Cramoll 1.4 (pCramoll) and rCramoll 1 (rCramoll). In the determination of the immunomodulory action, peritoneal macrophages from Balb/c mice were treated with different concentrations of lectins (0.625 to 10 mM) and we analyzed the effect on NO production (Griess Reagent), cell viability (MTT Reagent), induction of apoptosis (Kit Live/Dead and Acridine orange), superoxide anion production (MitoSOX), changes in mitochondrial membrane potential (ΔΨm) and phagocytosis of *Staphylococcus aureus*. The production of proinflammatory cytokines (IL-1β, IL-6, INF-γ e TNF-α) was analyzed in *S. aureus* infected and non-infected macrophages. Both lectins significantly enhanced Macrophages NO production and cytokines. The lectins were not cytotoxic as observed by MTT assay. However Live/Dead analysis revealed an increase of apoptosis in treated cells, the reduction of lysosomal activity was also reduced. The superoxide production was stimulated by both lectins, which was confirmed with the reduction on ΔΨm. *S. aureus* phagocytic activity of macrophages were enhanced in 27.1% and 22.47% by pCramoll and rCramoll, respectively. Finally, pCramoll and rCramoll downregulated the production of IL-6 and TNF-α and upregulated the expression of IL-1β, INF-γ during *S. aureus* infection of macrophages. In addtion, the protective effects of lectins against cell death induced by oxidative stress were evaluated. For this purpose, Vero cells (monkey kidney fibroblasts) were pretreated with increasing concentrations of lectins (0.625 to 10 µM) for 30 minutes and then were exposed to H_2O_2 (1mM). After 24 hours, the cytoprotective effect was evaluated and the cellular mechanisms involved were determined by flow cytometry involving: protection against cell death (Live/Dead kit), damage to lysosomes (Acridine Orange) and DNA (TUNEL), superoxide anion production (MitoSOX), changes in mitochondrial membrane potential (ΔΨm) (Rhodamine 123) and cell proliferation. pCramoll and rCramoll significantly attenuated the H_2O_2-induced cytotoxicity in a dose-dependent way, the maximum protective effects were 96.85 ± 15.59% (rCramoll) and 59.48 ± 23.44% (pCramoll). The Live/Dead analysis showed a reduction in apoptotic cells from 65.04 ± 3.29% to 39.77 ± 2.93% (pCramoll) and 13.90 ± 9.01% (rCramoll). The deleterious effects of

H_2O_2 on cell proliferation were reduced 10.83% (pCramoll) and 24.17% (rCramoll). The lectins attenuated the excessive superoxide production, the collapse of $\Delta\Psi m$, lysosomal and DNA damage that occurred in Vero cells exposed to H_2O_2. In conclusion, our results show that pCramoll and rCramoll have high immunomodulatory potential on macrophages and cytoprotective effects against H_2O_2.
Keywords: macrophages activation, cell death, oxidative stress, lectins, protective effects.

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SUMÁRIO

1. INTRODUÇÃO

O uso de plantas como fonte de nutrientes e no tratamento de patologias é comum desde o início da humanidade. As civilizações primitivas foram selecionando, ainda que empiricamente, plantas comestíveis, assim como espécies com capacidade medicinais dotadas de pouca toxicidade. Atualmente, as plantas medicinais são extensamente utilizadas, principalmente em regiões onde o acesso ao cuidado de saúde formal é limitado, e a sua seleção e uso dependem dos sintomas, da disponibilidade de espécies na região e de aspectos culturais e educacionais (AMOROZO, 2002). Tem sido notável, o ressurgimento dos estudos e uso de plantas medicinais. O crescente interesse em drogas derivadas de plantas é devido principalmente à convicção, nem sempre verdadeira, que "medicamentos naturais" são mais econômicos e seguros do que drogas sintéticas, muitas das quais são tóxicas e apresentam efeitos colaterais adversos (DA SILVA et al., 2011; DA SILVA et al, 2012). Dentre os produtos vegetais as lectinas têm se destacado pelo seu elevado potencial farmacológico (ANDRADE et al, 2004; MELO et al., 2011; OLIVEIRA et al., 2013).

1.1. LECTINAS: DEFINAÇÃO, HISTÓRICO E CLASSIFICAÇÃO

A ocorrência de proteínas com atividade hemoaglutinante é conhecida desde o 1888 quando Stillmark observou a capacidade de extrato protéico parcialmente purificado obtido de sementes de mamona (*Ricinus communis*) de aglutinar eritrócitos humanos. A proteína que exerceu tal ação ficou conhecida como ricina. As proteínas purificadas de plantas com atividade hemaglutinante foram inicialmente denominadas de aglutininas de plantas, fitohemaglutininas, hemaglutininas ou fitoaglutininas (SHARON; LIS 2004).

Em 1954, Boyd & Shapleigh propuseram o termo "lectina" (originado do latim "lectus", que significa selecionado, escolhido) para fazer a designação a esse grupo de proteínas que apresentam essa seletividade na interação com carboidratos como característica comum. Este mesmo termo foi generalizado em 1972, por Sharon e Lis, englobando todas as proteínas presentes em fontes de natureza variada, de origem não imunológica, capazes de ligar-se a carboidratos, com especificidade ou não para determinado grupo sanguíneo. Inicialmente, pouca atenção foi dispensada a essas proteínas, que se acreditava estar presente apenas no reino vegetal (SHARON; LIS, 1988; SHARON; LIS 2004).

Lectinas são proteínas que têm a habilidade de se ligar especificamente a carboidratos e aglutinar células (PEUMANS; VAN DAMME, 1995; SHARON, 2008). Elas possuem um ou mais domínios de reconhecimento a carboidratos. As lectinas podem possuir outros domínios com diferentes atividades levando sua classificação em outras categorias (BOYD, 1954; KOCOUREK; HOREJSI, 1993; GABIUS, 1997). Apesar de originalmente comparadas com anticorpos devido a similaridades na especificidade de ligação, as lectinas não são imunoglobulinas (BOYD, 1954; BOYD ; SHAPLEIGH, 1954). A síntese de lectinas pode ser aumentada por estímulos externos, mas não pela presença de antígenos, como as imunoglobulinas (PEUMANS; VAN DAMME, 1995, 1998a; PEUMANS et al., 2001).

A ligação lectina-carboidrato é realizada por ligações não-covalentes como pontes de hidrogênio, interações de van der Walls e hidrofóbicas, estando o(s) domínio(s) de ligação a carboidratos dispostos preferencialmente na superfície protéica (PEUMANS; VAN DAMME, 1995; SHARON, 2008). Outra característica das lectinas é a não modificação bioquímica e estrutural dos carboidratos aos quais se ligam. Essa propriedade as diferencia das glicosiltransferases, glicosidases e enzimas que introduzem um substituinte (grupo sulfatos, por exemplo) em um carboidrato. Essa distinção é necessária porque algumas glicosidases podem se ligar aos carboidratos e, sob algumas condições, como a baixa temperatura, podem aglutinar células rapidamente ao se ligar a carboidratos na superfície celular (BRECHTEL et al., 2001). Em contrapartida, algumas lectinas de plantas e animais podem apresentar atividade enzimática, porém essa atividade está relacionada a um domínio estrutural distinto do sítio de ligação a carboidrato (TRAINOTTI et al., 2001; RAO et al., 2012).

A partir da década de 1960, com a exploração de habilidade de reconhecer e ligar especificamente a carboidratos simples ou complexos, as lectinas de plantas começaram a ganhar evidência, tornando potentes ferramentas para a biologia e medicina (SHARON, 2008). Nowel observou em 1960 que a lectina isolada de sementes de *Phaseolus vulgaris* (PHA) estimulava a divisão de linfócitos. Mais tarde, a descoberta que lectinas WGA (germe de trigo) e Concanavalina A (*Canavalia ensiformis*) aglutinavam preferencialmente células malignas permitiu correlacionar mudanças nos carboidratos presentes na superfície celular com o desenvolvimento do câncer (SHARON; LIS, 2004). As lectinas também foram importantes para a descoberta ou confirmação de várias características fundamentais das membranas celulares. Estas incluem a demonstração que oligassacarídeos da membrana plasmática estão distribuídos assimetricamente e confinados na superfície externa da célula

(NICOLSON; SINGER 1971). Experimentos com lectinas mostraram também que os oligassacarídeos podem se mover no plano da membrana, confirmando assim o modelo estrutural do mosaico fluído (NICOLSON, 1974).

As lectinas são encontradas de forma ubíqua na natureza, estando presente em vírus (GRANELLI-PIPERNO et al., 2006), bactérias (REYNOLDS et al., 2012), fungos (HAMSHOU et al., 2012), plantas (PEREIRA et al., 2012) e animais (NUNES et al., 2012), incluindo humanos (KAMIYA et al., 2008). Ainda que, não claramente definidos diversos papéis têm sido propostos para essa classe de proteínas, por exemplo: transporte de carboidratos (KAMIYA et al., 2008; HU et al., 2009), proteínas de estocagem (KUSOLWA; MYERS, 2011), mediadoras de interações célula-célula e parasito-hospedeiro (AUDFRAY et al., 2012), chaperonas moleculares envolvidas no controle de qualidade do enovelamento protéico (HOSOKAWA et al., 2010; GARG et al., 2012), renovação de glicoproteínas do soro (CARVALHO et al., 2012) e na resposta imune inata (DEGN et al., 2012).

As lectinas apresentam uma área ampla e variada de aplicabilidade podendo ser valiosas em processos biotecnológicos, nas áreas de pesquisa médica, biológica, farmacológica e bioquímica (SHARON; LIS, 2004, SHARON, 2008).

1.1.1. Classificação

Por representar um grupo heterogêneo de proteínas as lectinas diferem fortemente em relação à estrutura molecular, especificidade ao carboidrato e atividades biológicas. As lectinas de plantas exibem ampla variedade de especificidade de ligação a carboidratos através de domínios conservados (VAN DAMME et al., 2008) que são utilizados para sua classificação (tabela 1).

Em outra abordagem, Peumans & Van Damme (1998) classificaram as lectinas de acordo com sua estrutura geral em: merolectinas, hololectinas, quimerolectinas e superlectinas (figura 1).

- **Merolectinas**: consistem em um único domínio de ligação a carboidrato e, seu caráter monovalente as torna incapazes de precipitar glicoconjugados ou aglutinar células.
- **Hololectinas**: composta exclusivamente de domínios de reconhecimento a carboidrato, mas possuem pelo menos dois domínios de ligação e estes podem ser

idênticos ou muito similares e, assim, podem aglutinar células e precipitar glicoconjugados.

Tabela 1: Domínios de ligação a carboidratos das lectinas de plantas (adaptado de Vandenborre et al. 2011).

Domínio	Especificidade	Exemplo
Domínio típico da aglutinina de *Agaricus bisporus*	Antígeno-T	ABA (PDB: 1Y2T)
Domínio tipo Amarantina	Antígeno-T	Amaranthin (PDB: 1JLY)
Domínio homológo a Quitinases Class V	Grupo sanguíneo B; Carboidratos com resíduos de manose	TCLL (PDB: 4B16)
Cyanovirin domain	Carboidratos com resíduos de manose	CV-N (PDB: 2JZJ)
Domínio típico da aglutinina de *Euonymus europaeus*	Grupo sanguíneo B; Carboidratos com resíduos de manose	EEA
Domínio típico da aglutinina de *Galanthus nivalis*	Manose, oligomanosídeos, ácido siálico	PCL (PDB: 3A0C)
Domínio típo Heveina	N-acetil-D-glicosamina	WGA (PDB: 2UVO)
Domínio típo Jacalina	Manose	JAC (PDB: 3P8S)
Domínio das lectinas de legumes	Glicose/Manose	Cramoll (PDB: 1MVQ)
Domínio LysM	Quitina	LysM
Domínio típico da aglutinina de *Nicotiana tabacum*	N-acetil-D-glicosamina	NICTABA
Domínio típo Ricin-B	Galactose/ N-acetil-D-glicosamina	ML-I (PDB ID: 1M2T)

PDB: Protein data bank (http://www.rcsb.org/pdb/home/home.do)

- **Quimerolectinas:** não consistem exclusivamente de domínios de reconhecimento a carboidrato, mas são formadas por domínio de ligação a carboidratos e outro domínio que possui uma atividade catalítica ou com outra função que age independentemente

do domínio de ligação a carboidratos. As quimerolectinas comportam-se como mero ou hololectinas dependendo do número de sítios de ligação a carboidrato.

- **Superlectinas**: são compostas de no mínimo dois domínios de ligação a carboidrato, porém estes domínios de ligação não são idênticos ou similares, reconhecendo açúcares estruturalmente diferentes (PEUMANS et al., 2001).

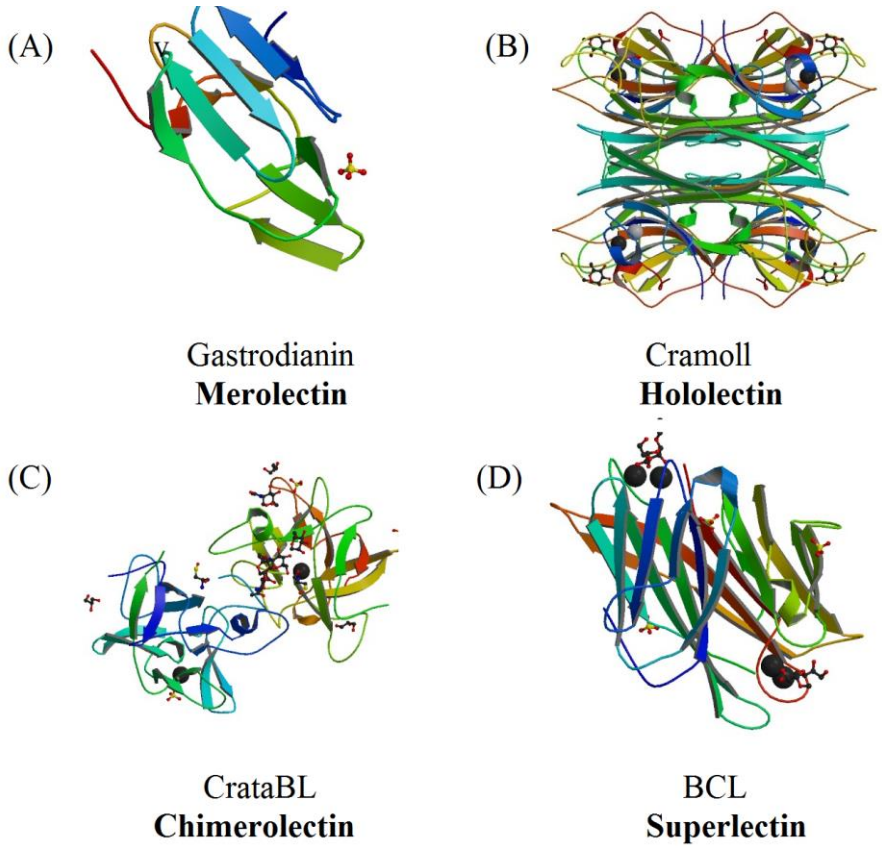

Figura 1: Estruturas de representantes das Merolectinas, Hololectinas, Quimerolectinas e Superlectinas. (A) Gratrodianina de *Gastrodia elata* (PDB: 1XD6); (B) Cramoll de *Cratylia mollis* (PDB: 1MVQ); (C) CrataBL de *Crataeva tapia* (PDB: 4IHZ); (D) *Burkholderia cenocepacia* lectin A (PDB: 2XR4). PDB: Protein data bank (http://www.rcsb.org/pdb/home/home.do)

As diferenças estruturais entre as lectinas são devido à sequência primária, variação no número de subunidades por molécula e natureza dos polipeptídios. Pontes dissulfeto, pontes de hidrogênio e também as interações hidrofóbicas podem estar presentes nas subunidades de

associação (KENNEDY et al., 1995) e parecem desempenhar um papel determinante na estabilidade dessas proteínas (SUSEELAN et al., 2002).

1.1.2. *Cratylia mollis* e suas Isolectinas

Cratylia mollis Mart. é uma espécie endêmica da Região Semi-Árida do sertão do Estado de Pernambuco (Figura 2). Esta planta pertence à pertencente à família Phaseoleae, subfamília Dioclinae, sendo popularmente conhecida como feijão camaratuba ou camaratú. É uma planta de porte arbustivo e que produz grandes quantidades de sementes. Diante da sua elevada resistência a dessecação é amplamente utilizada como forrageira para alimentação de rebanhos bovinos e caprinos, especialmente em estações de estiagem prolongada (SANTOS et al., 2004; TEIXEIRA et al., 2006; LIMA et al., 2009).

Figura 2: *Cratylia mollis* Mart. (feijão camaratu)

A presença de lectinas em sementes de *C. mollis* foi descrita inicialmente nos trabalhos de Paiva & Coelho (1992) e Correia & Coelho (1995). Estas pesquisadoras detectaram quatro isoformas denominadas Cramoll (Cramoll 1, Cramoll 2, Cramoll 3, Cramoll 4). A classificação das isolectinas foi feita de acordo com a migração eletroforética em gel para proteínas básicas nativas, sendo a Cramoll 1 a mais básica (maior migração). Considera-se isolectinas um grupo intimamente relacionado de aglutininas, resultantes da expressão de

famílias gênicas e que possuem diferenças estruturais, na especificidade a carboidrato, diferença de carga, modificações pós-traducionais, atividade hemaglutinante ou outra ação biológica (SHARON; LIZ, 1990; REN et al., 2009; SULTAN et al., 2009; LAM; NG, 2011). Em resumo, o termo isoforma é utilizado para designar múltiplas formas de lectinas presentes na mesma espécie ou variedade de origem genética não definida (PAIVA; COELHO, 1992). A presença de duas ou mais proteínas com atividade hemaglutinante tem sido detectada em várias espécies, tais como as isolectinas de germe de trigo (WGA-1, WGA-2 e WGA-3) (OHBA et al., 2003).

Em sementes de *C. mollis*, a isoforma Cramoll 1 é a isolectina mais abundante, sendo co-purificada, a partir da fração de precipitado salino F40-60%; junto com a isoformar 4 (dando origem a preparação denominada Cramoll 1,4). A separação destas duas isolectinas se dá por cromatográfica de troca iônica (Correia e Coelho, 1995). As demais isolectinas (Cramoll 2 e Cramoll 3) foram isoladas por Paiva & Coelho (1992). Em relação ao carboidrato de ligação, Cramoll 1,4, Cramoll 1 e Cramoll 2 são proteínas específicas para glicose/manose, enquanto a Cramoll 3 é uma glicoproteína galactose específica.

A proteína Cramoll 1 é a melhor caracterizada com sequência de aminoácidos determinada e estrutura tridimensional resolvida por cristalografia de raios-X (Figura 3) (TAVARES et al., 1996; SOUZA et al., 2003). Esses estudos demonstraram a relação de proximidade taxonômica entre Cramoll 1 e a bem conhecida lectina Concanavalina A (ConA), isolada de *Canavalia ensiformis* (também pertencente à subfamília Dioclinae). Essas lectinas apresenta a mesma especificidade monossacarídica (GOLDSTEIN et al., 1965; CORREIA e COELHO, 1995) mas diferem no reconhecimento de carboidratos complexos (LIMA et al., 1997), certamente devido a diferenças nos aminoácidos próximos aos domínio de ligação a carboidratos (SOUZA et al., 2003). De fato, compartilham 82% de homologia em suas estruturas primárias, o que fortemente reflete na grande similaridade nas estruturas terciárias e quaternárias (SOUZA et al., 2003).

O potencial biotecnológico de isolectinas de *C. mollis* tem sido demonstrado por diversos estudos. Cramoll 1,4 foi utilizada na caracterização de tecidos cancerígenos (BELTRÃO et al., 1998; LIMA et al., 2010), como uma matriz para isolar glicoproteínas de importância biológica (LIMA et al., 1997; SILVA et al., 2011) e no desenvolvimento de sensores para detecção de lipopolissacarídeo bacteriano e de sorotipos do vírus da dengue (OLIVEIRA et al., 2011a,b).

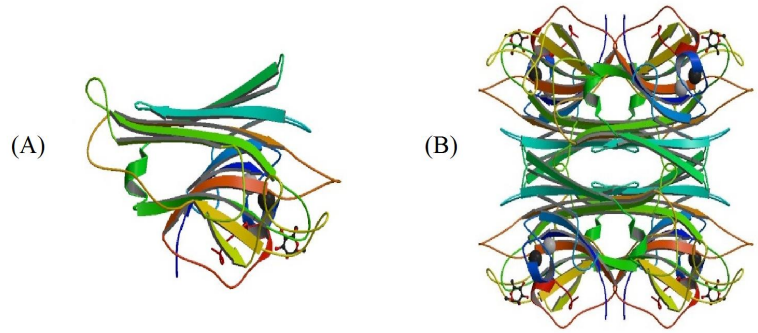

Figura 3: Estrutura da Cramoll 1 (PDB ID: 1MVQ). (A) Monômero and (B) Tetrâmero

Em relação às aplicações farmacológicas, esta proteína tem apresentado elevada ação antitumoral (ANDRADE et al., 2004), imunomoduladora em linfócitos humanos (MACIEL et al., 2004), murinos (MELO et al., 2010a,b; MELO et al., 2011a), antiparasitária *in vivo* e *in vitro* (FERNANDES et al., 2010; MELO et al., 2011b). Esta lectina possui ainda uma marcante ação cicatrizante, promovendo o efetivo fechamento e reparo de feridas, acelerando a granulação, o processo de reepitelização e a retração destas feridas, inclusive em modelos com imunodepressão induzida (MELO et al., 2011; PEREIRA et al., 2012).

Recentemente, uma lectina recombinante e funcional da isoforma 1 (rCramoll) foi obtida e purificada por nosso grupo. Nesta abordagem a sequência do gene codificante da Cramoll 1 foi sintetizada *in vitro* no vetor de expressão pET-28α. Para a expressão foram utilizadas linhagens isogênicas da bactéria *Escherichia coli* Rosetta (DE3). rCramoll apresenta a mesma especificidade (glicose/manose) observada para a pCramoll, aglutina eritrócitos de coelho e formas epimastigotas de *Trypanosoma cruzi* (PAZ et al., 2010).

1.2. MACRÓFAGOS

O sistema imune inato constitui a primeira barreira de defesa, inespecífica que protege o organismo contra agentes estranhos como microorganismos, células alteradas ou transformadas. Este sistema tem influência no desenvolvimento da imunidade específica formada posteriormente. A resposta imune inata consiste, portanto, de uma complexa rede de interações celulares envolvendo diversas vias de sinalização responsáveis pelo reconhecimento e erradicação de patógenos (PLUDDEMANN et al., 2011).

Macrófagos constituem o principal grupo de leucócitos fagocitários e são cruciais para a imunovigilância contra patôgenos invasores e neoplasias. Foram inicialmente descritos por Élie Metchnikoff (Prêmio Nobel de Fisiologia, 1908) como células fagocitárias responsáveis pela eliminação de patôgenos em organismos vertebrados e invertebrado, evidenciando que células fagocitárias mononucleares obtidas de animais resistentes à infecção de uma bactéria eram mais capazes de matar essa e outras bactérias, estabelecendo a base para o conceito de "ativação de macrófagos" como chave para imunização (METCHNIKOFF, 1905; MARTINEZ; HELMING; GORDON, 2009).

Desde esta descoberta, os imunologistas se ocuparam com o conceito dos macrófagos como células imunes efetoras e com a compreensão de como estas participam na defesa do hospedeiro. Entretanto, por focar somente nas funções imunes dos macrófagos, ignoraram seu papel vital na homeostasia, que é independente de sua participação em respostas imunes. Quando ativos os macrófagos expressam um ou mais moléculas efetoras citotóxicas como peroxidade, proteases citolíticas, óxido nítrico (NO) e citocinas pro-inflamatórias (MANTOVANI; SICA, 2010).

Macrófagos também estão envolvidos na remoção de restos celulares gerados durante remodelação tecidual, além de remover rapidamente e eficientemente células que sofreram apoptose (MARTINEZ; HELMING; GORDON, 2009; GEISSMANN et al., 2010).

1.2.1. Classificação dos Macrófagos

Os macrófagos são classificados como macrófagos M1 (classicamente ativados) ou macrófagos M2 (alternativamente ativados). Os macrófagos M1 sofrem ativação induzida por IFN-γ, desencadeando uma vigorosa resposta pró-inflamatória indispensável para eliminar a infecção causada por patógenos intracelulares. Por outro lado, macrófagos M2 são ativados pela ação de IL-4 e IL-13, estando este fenótipo envolvido na resposta imune aos parasitas (MARTINEZ; HELMING; GORDON, 2009; MOSSER; EDWARDS, 2008).

Outra classificação leva em conta as funções na homeostasia e inflamação, sendo os macrófagos distribuídos em: macrófagos de defesa, de cicatrização e reguladores. Este arranjo favorece a delineação de como os macrófagos podem evoluir para exibir as características que são compartilhadas por mais de uma população de macrófagos (MOSSER; EDWARDS, 2008). Macrófagos de defesa são ativados classicamente e participam das respostas imunes

mediadas por células. Esta população é ativada por Interferon-γ (IFN-γ) e o Fator de Necrose Tumoral (TNF) e tem elevada capacidade microbicida ou tumoricida, secretando níveis elevados de citocinas e de mediadores pró-inflamatórios (MACKANESS, 1977; O´SHEA; MURRAY, 2008).

Macrófagos da cicatrização se desenvolvem em resposta aos sinais inatos ou adaptativos. Durante a injúria tecidual a citocina IL-4 é liberada e desencadeia a ativação desta categoria de macrófagos (LOKE et al., 2007). Outras fontes importantes de IL-4 são os dasófilos e mastócitos, e outros granulócitos (BRANDT et al., 2000). IL-4 age pela estimulação da atividade da enzima arginase, que catalisa a conversão de arginina em ornitina. Este último é um precursor das poliaminas e do colágeno, contribuindo desse modo à produção de matriz extracelular (KREIDER et al., 2007).

Por fim, macrófagos reguladores também estão presentes nas respostas imunes celular e humoral, sendo gerados pela ação hormonal em situações de estresse. Embora as respostas de estresse não sejam consideradas tipicamente parte da imunidade inata, o eixo Hipotálamo-Hipófise-Adrenal (HPA) exerce efeitos acentuados em macrófagos. Glucocorticóides são liberados por células adrenais em resposta ao estresse e inibem a defesa do hospedeiro dependente de macrófagos e suas funções inflamatórias, bloqueando a transcrição de genes de citocinas pró-inflamatórias e diminuindo a estabilidade do RNA mensageiro gerando uma população de macrófagos reguladores (STERNBERG, 2006). No entanto o processo molecular envolvido na diferenciação fenotípica dos macrófagos ainda não foi identificado, embora a sinalização via MAP quinases (Extracellular-sinal-Regulated Kinase - ERK) apareça como um potencial candidato (LUCAS et al., 2005).

1.2.2. Macrófagos e seus Receptores

Uma importante propriedade dos macrófagos refere-se a sua notável plasticidade, o que permite que essas células respondam eficientemente aos sinais ambientais e mudem seu fenótipo; e sua fisiologia pode ser marcadamente alterada pelas respostas inata e adaptativa. A detecção da presença de microorganismos ou restos celulares pelos macrófagos ocorre através dos receptores Toll-like (TLRs), família de receptores homólogos à proteína Toll de *Drosophila*, capazes de distinguir diferentes classes de microorganismos (MEDZHITOV; PRESTON-HURLBURT; JANEWAY, 1997; MIKAMIA et al., 2012).

TLRs reconhecem primariamente características moleculares presentes nos microorganismos, conhecidas como padrões moleculares associados a patógenos (PAMPs), altamente conservados entre as diferentes classes de microorganismos (AKIRA ; HEMMI, 2003; KUMAR; KAWAI; AKIRA, 2011). TLRs e homólogos desempenham função na inicialização da reposta imune contra patógenos em diversos organismos, tais como plantas, insetos e mamíferos (TAKEDA; KAISHO; AKIRA, 2003). São expressos em células imunes ou não imunes, com padrões de expressão distintos em cada tipo de célula (APPLEQUIST; WALLIN; LJUNGGREN, 2001; BOTOS; SEGAL; DAVIES, 2011).

Os TLRs são subdivididos em dois grupos de acordo com a localização celular. TLR1, -2, -4, -5, -6, -10 são expressos na superfície celular, enquanto TLR3, -7, -8, -9 nos compartimentos intracelulares. O reconhecimento de um ligante induz complexos eventos de sinalização intracelular envolvendo o recrutamento do Fator de Diferenciação Mielóide 88 (MyD88), ativação da Cinase de tipo 1 Associada a Receptor de Interleucina1 (IRAK1) e do Fator Associado ao Receptor de TNF 6 (TRAF6), culminando na ativação do Fator de Transcrição NF-kB, Proteína cinase Mitogênica Ativada (MAP) e dos Fatores Reguladores de Interferon (IRF) (APPLEQUIST; WALLIN; LJUNGGREN, 2001; ROACH et al., 2005; GAY; GANGLOFF; WEBER, 2006; O' NEILL, 2006; BOTOS; SEGAL; DAVIES, 2011).

Proteínas citoplasmáticas pertencente à classe denominada NOD (Nucleotide-Oligomerization Domain), também executam importante função no reconhecimento de peptideoglicanos gerados por microorganismos intracelulares e contribuem para a resposta inata, através do aumento da resposta inflamatória via a ativação de NFκB (ALTHMAN; PHILLPOT, 2004; SALEH, 2011; FELDMANN; MONACO, 2012). Além desses, os macrófagos possuem receptores acoplados a proteína G, receptores para Fc de imunoglobulinas, para o fragmento C3 do complemento além de outros receptores para citocinas, principalmente de IFN-γ. (KAWAI; AKIRA; 2011; GUZMAN-BELTRANA et al., 2012).

1.2.3. Mecanismos Efetores dos Macrófagos

Quando os receptores reconhecem a presença de microorganismos invasores e células tumorais ocorre a ativação dos macrófagos. Esses receptores estimularão a produção de enzimas, citocinas, toxinas e espécies reativas, dentre eles o peróxido de hidrogênio (H_2O_2), o

óxido nítrico (NO), radicais de hidrogênio (OH^-), ânion superóxido (O_2^-), e oxigênio (O_2). Essas moléculas podem matar os microorganismos fagocitados criando um ambiente tóxico para os mesmos (NATHAN; SHILOH, 2000; GUZIK et al., 2003; MARTINEZ; HELMING; GORDON, 2009).

- Produção de Espécies Reativas

Durante a formação dos intermediários reativos de oxigênio ocorre o processo conhecido como "burst" respiratório, caracterizado por um acentuado aumento no consumo de oxigênio, resultante da ativação de uma via que reduz o oxigênio a O_2^-, catalisada pelo complexo NADPH oxidase. Logo em seguida, a enzima superóxido dismutase (SOD) converte o O_2^- gerado a peróxido de hidrogênio. Os produtos do "burst" respiratório destroem os patógenos fagocitados ou presentes no meio extracelular (KLEBANOFF, 1992; RADA; LETO, 2008).

A produção de óxido nítrico (NO) é um importante mecanismo efetor de macrófagos ativados indutor de citotoxicidade. NO é um gás permeável a membranas, sendo produzido por diferentes linhagens celulares: neurônios, células epiteliais do trato respiratório, fibroblastos, hepatócitos e macrófagos. Como uma espécie reativa de nitrogênio reage rapidamente com vários compostos, principalmente contendo elétrons desemparelhados, como o oxigênio molecular e metais (MAYER; HEMMENS, 1997). Esta molécula apresenta pequeno tempo de meia vida (alguns segundos), atuando por isso apenas em células próximas do local de produção (VAN DER VEEN et al., 2000). Em soluções aquosas, o NO é rapidamente transformado em nitrito (NO^{2-}) e nitrato (NO^{3-}) (PFEIFFER et al., 2012).

Óxido nítrico é produzido em células de mamíferos a partir de L-arginina e oxigênio por uma família de enzimas conhecidas como Óxido Nítrico Sintetase (NOS), composta de três isoformas: Óxido Nítrico-Sintase neuronal (nNOS), Óxido Nítrico-Sintase induzivel (iNOS) e Óxido Nítrico-Sintase endotelial (eNOS). Essas enzimas são homodímeros contendo FAD e FMN como grupos prostéticos e ainda requerendo tetrahidrobiopterina, NADPH e O_2 para sua atividade. nNOS e eNOS são constitutivamente expressa e dependente de Ca^{2+} para a sua atividade sendo responsáveis pela produção de pequenas quantidades de NO por curtos períodos de tempo. Por outro lado, a que é encontrada nos macrófagos, contém uma molécula de calmodulina fortemente ligada, que a mantém em uma conformação ativa. O aumento na transcrição do gene para esta forma da enzima é observado em resposta a diferentes estímulos,

como produtos bacterianos (Lipopolissacarídeo Bacteriano, LPS), citocinas pró-inflamatórias e quimotoxinas (PELLAT; DRAPIER, 1990; LASKIN, 2012).

O óxido nítrico atua contra células ou patógenos pela inativação de enzimas vitais através da nitrosilação de seus centros ferro-enxofre resultando na citotese de seus alvos (PELLAT; DRAPIER, 1990). Várias pesquisas mostram o papel do óxido nítrico no controle de parasitas, além da ação antitumoral e angiogênica (GOBERT et al., 1998; TAKEDA et al., 2012). Porém, a produção descontrolada de NO pode levar a uma resposta maciça tóxica, implicada em uma série de doenças inflamatórias (BENI et al., 2012).

- Produção de Citocinas

Como citado anteriormente, a ativação dos macrófagos resulta na produção de citocinas pró-inflamatórias como Interferon-gama (IFN-γ), Fator de Necrose Tumoral alfa (TNF-α), Interleucinas (IL) IL-1, IL-6, IL-12, e IL-18, que são derivadas principalmente de células da resposta imune inata e de células Th1 e Th17 (MARTINEZ; HELMING; GORDON, 2009; CLARK et al., 2012).

As citocinas são os principais mediadores da resposta imune e controlam diferentes funções celulares que incluem proliferação, diferenciação, morte celular (apoptose ou necrose), sobrevivência e migração, seja diretamente, por meio de eventos que se seguem ao engajamento de seus receptores específicos na superfície celular, ou indiretamente, pela indução da expressão de numerosos genes (SMITH; HUMPHRIES, 2009).

Diferentes citocinas atuam em seus respectivos receptores e esses receptores utilizam várias combinações de mediadores intracelulares para induzir suas funções, normalmente os da família Janus de proteínas cinases (JAK) e os da família de Ativadores e Tradutores dos Sinais de Transcrição (STAT) (FULOP et al., 2006). TNF-α e IL-1β, por exemplo, atuam sobre o endotélio vascular fazendo com que suas células expressem moléculas de adesão e quimiocinas (como as selectinas, ICAM-11 e VCAM-1) que auxiliarão no recrutamento de leucócitos para o sítio da inflamação (CHEN et al., 2003; TOSI, 2005).

1.3. LECTINAS DE PLANTAS COMO AGENTES IMUNOESTIMULADORES DE MACRÓFAGOS

O termo imunomodulação se refere à regulação positiva ou negativa dos mecanismos efetores do sistema imunológico. Este processo é exercido por substâncias naturais ou

sintéticas (imunomoduladores) capazes de estimular, suprimir ou modular qualquer aspecto do sistema imune, incluindo a defesa inata e adaptativa (KUMAR et al., 2012). As atividades biológicas dos imunomoduladores são influenciadas por diversos parâmetros físico-químicos tais como solubilidade, conformação estrutural, peso molecular e carga (GANGULY; PAUL; MUKHOPADHAYAY, 2010). Os imunomoduladores podem ser classificados em três categorias: adjuvantes imunológicos, imunoestimulates e imunosupressores.

Os adjuvantes imunológicos são substâncias utilizadas para aumentar a eficácia de vacinas devido ao seu potencial de estimular o sistema imune (FLECK et al., 2012). Adjuvantes têm sido tradicionalmente usados para aumentar a magnitude da resposta adaptativa a uma vacina com base na produção de anticorpos ou na capacidade de prevenir a infecção. Outra função relacioanda dos adjuvantes é a de orientar a imunidade adaptativa de forma a aumentar a especificidade a cada tipo de patógeno (COFFMAN; SHER; SEDER, 2010).

Os imunossupressores são grupo heterogêneo de drogas que agem inibindo a ação ou a eficiência do resposta imune. Estes são geralmente administrados para tratar vários tipos de rejeição de transplante de órgãos e em doenças auto-imunes (RATHEE et al., 2012). Dessa forma, os imunossupressores precisam ser utilizados por um longo prazo, causando diversos efeitos colaterais e risco de infecção e câncer (PÜSCHEL et al., 2012).

Imunoestimulantes são compostos que agem de forma não específica na melhoria da resistência do organismo à infecção, podendo atuar através da resposta inata ou adaptativa. Nos indivíduos saudáveis agem como agentes profiláticos e imunopotenciadores, aumentando o nível de base da resposta imune. No indivíduo imunossuprimidos agem como agentes terapêuticos (KUMAR et al., 2012). Os efeitos biológicos dos imunoestimulantes são altamente dependentes dos receptores presentes nas células-alvo (RINGO, 2011).

Vários agentes sintéticos são utilizados como agentes imunoestimuladores tais como levamisol, talidomida, mas induzem vários efeitos colaterais, tais como nefrotoxicidade, hepatotoxicidade, depressão da medula óssea, perturbação gastrointestinal e assim por diante. Devido a esses efeitos colaterais, os compostos naturais, tido como mais seguros, eficazes e baratos, tornaram-se alternativas na busca de novos imunoestimuladores (TAKAOKA; KAWAMURA, 2012; BALEKAR et al., 2013).

Os efeitos biológicos dos imunoestimulantes são altamente dependentes dos receptores presentes nas células-alvo (RINGO, 2011). Muitas lectinas vegetais exercem atividades

imunomoduladoras que são iniciadas pela sua interação com as porções de glicano presentes na superfície das células imunes. Tal interação pode provocar a transdução de sinal e o desencadeamento dos mecanismos efetores, envolvidos na resposta contra tumores ou infecções microbianas. Assim, lectinas imunomoduladoras têm potencial para o desenvolvimento de formas farmacêuticas, além de constituírem ferramentas para a identificação de alvos glicosilados para novas estratégias terapêuticas (UNITT; HORNIGOLD, 2011; SOUZA et al., 2013). O efeito imunoestimulante de lectinas sobre macrófagos têm sido alvo de diversas pesquisas, a abaixo alguns exemplos são discutidos e outros estão listados na tabela 2.

Kesherwani e Sodhi (2007) avaliaram a ação das lectinas Con A, PHA e WGA na sobre ativação de macrófagos peritoneais de camundongos, e observaram que Con A e PHA induziram a produção de NO, de forma dose e tempo dependente. Posteriormente, este mesmo grupo relataram a indução de citocinas pró-inflamatórias (TNF-α ,IL-1β , IL-12, IFN-γ) por Con A e WGA (SODHI; KESHERWANI, 2007a,b) e de receptores TLRs por Con A (SODHI; TARANG; KESHERWANI, 2007).

A ação imunoestimuladora sobre macrófagos da lectina KML-C (Korean mistletoe lectin) também foi analisada utlizando a linhagem RAW264.7. Esta lectina é composta por duas subunidades (A e B), capazes de induzir a produção de citocinas (TNF-α, IFN-γ, IL-1, IL-12 e IL-6) e NO (Hajto et al., 1989; Yoon et al., 2001; KANG et al., 2008) e sua atividade antitumoral é relacionada com a ativação de macrófagos (YOON et al., 2003) que ocorre via ativação do receptor TLR-4 (PARK et al., 2010).

Em outro estudo, Kim e colaboradores (2010) compararam a produção de TNF-α, G-CSF (fator estimulador de colônia de granulócitos) e NO por macrófagos RAW264.7, após o tratamento com as lectinas modeccin e PHA. As duas lectinas induziram a secreção de citocinas, mas com dose e perfis de resposta diferentes. No entanto apenas PHA estimulou a liberação de óxido nítrico.

1.4. RADICAIS LIVRES E DANOS MOLECULARES

Os radicais livres são moléculas ou átomos que possuem a capacidade de existir independente, e podem conter um ou mais elétrons não-pareados em seu orbital. Isto determina uma atração para um campo magnético, o que pode torná-lo altamente reativo,

capaz de reagir com qualquer composto situado próximo à sua órbita externa, passando a ter uma função oxidante ou redutora. Os radicais livres são formados em um cenário de reações de óxido-redução, isto é, ou cedem elétrons, oxidando-se; ou recebem outro elétron, reduzindo-se (HALLIWELL; GUTTERIDGE, 2003; HALLIWELL, 2012).

O processo de oxidação é fundamental para a vida aeróbica. Os radicais livres são formados fisiologicamente pelos organismos, como resultado do processo de respiração celular que ocorre nas mitocôndrias, utilizado para converter em energia os nutrientes absorvidos dos alimentos, a fim de gerar ATP (MURPHY, 2009; KOWALTOWSKIA et al. 2009).

Macrófagos e neutrófilos produzem radicais livres que são cruciais na defesa contra bactérias e fungos invasores. Outra forma de produção é a exposição de organismos a fatores exógenos, como ozônio, radiações gama e ultravioleta, tabaco, substâncias tóxicas presentes em alimentos e bebidas (aditivos químicos, hormônios, aflatoxinas, etc), consumo elevado de gorduras saturadas (frituras, embutidos, etc) e de certos medicamentos (VALKO et al., 2007, HALLIWELL, 2009; RAINS; JAIN, 2011).

Devido à sua capacidade de interação, o excesso de radicais livres e EROs (Espécies Reativas de Oxigênio) ou a deficiência de mecanismos de defesa devido à desnutrição carreta em importantes alterações ao nível molecular, associadas a danos às macromoléculas biológicas como lipídios, proteínas e DNA, prejudicando o equilíbrio e gerando o estresse oxidativo (REUTER et al., 2010; CADET; DOUKI; RAVANAT, 2011).

Tabela 2: Exemplos de lectinas de plantas com ação imunoestimuladora sobre macrófagos

Lectina	Espécie	Carboidrato	Atividade	Referência
Con A	*Canavalia ensiformis*	Manose/Glicose	Indução de óxido nítrico, citocinas (TNF-α ,IL-1β , IL-12, IFN-γ) e TLRs	Kesherwani e Sodhi (2007); Sodhi e Kesherwani (2007a); Sodhi et al. (2007)
PHA	*Phaseolus vulgaris*	Glicoconjugados	Indução de óxido nítrico, TNF-α e G-CSF	Kesherwani e Sodhi (2007); Kim et al. 2010
WGA	*Triticum aestivum*	N-acetilgalactosamina	IL-1β, IL-12, IFN-γ, TNF-α	Kesherwani e Sodhi (2007)
Modeccin	*Modecca digitata*	Galactose/ N-acetilgalactosamina	TNF-α e G-CSF	Kim et AL., 2010
KML -C	*Viscum album* var. *coloratum*	Galactose/ N-acetilgalactosamina	Indução de óxido nítrico, TNF-α, IFN-γ, IL-1, IL-12 e IL-6)	Hajto et al., 1989; Yoon et al., 2001;
EBL	*Musa basjoo*	Glicose	Indução de óxido nítrico	Wong e Ng (2006).
DBL	*Musa acuminate*	Frutose	Indução de óxido nítrico	Cheung et al. (2009).
ArtinM	*Artocarpus heterophyllus*	Manose	IL-12	Panunto, et al., 2001
CCL	*Castanopsis chinensis*	Manose/Glicose	Indução de óxido nítrico	Wong et al. (2008).
MCL	*Momordica Charantia*	Galactose	Indução de óxido nítrico	Fang et al. (2012).
EAPL	*Phaseolus vulgaris* cv. Extralong Autumn Purple Bean	Galactose	Indução de óxido nítrico	Wong e Ng (2010).
BTKL	*Phaseolus vulgaris* cv. Blue tiger king	Ácido poligalacturônico	Indução de óxido nítrico	Fang, E. F., 2010

O dano causado a esses componentes celulares se acumula, com o passar dos anos, e contribui para a degeneração de células somáticas e indução de doenças crônico-degenerativas, especialmente associadas com o envelhecimento, destacando-se câncer, aterosclerose, doenças pulmonares, inflamatórias, mal de Parkinson, mal de Alzheimer e catarata (CIENCEWICKI et al., 2008; MOREIRA, et al. 2008, FEARON et al., 2009; DESAI et al., 2010; LEE et al., 2012; MASGRAS et al., 2012).

Por isso, em sistemas biológicos existe um equilíbrio entre os fatores que promovem a oxidação e os mecanismos antioxidantes de defesa, incluindo antioxidantes enzimáticos e não enzimáticos (VALKO et al., 2007). No entanto, é valido enfatizar que os radicais livres e espécies reativas são produzidos para ajudar na manutenção da homeostase celular ou regulação de reações de redução e oxidação (redox) em tecidos saudáveis (DEVASAGAYAM et al., 2004; HALLIWELL, 2012).

1.4.1. Principais Radicais Livres e Espécies Reativas de Oxigênio

Os radicais livres derivam majoritariamente do oxigênio, sendo por isso denominados coletivamente de “Espécies Reativas de oxigênio” (EROs). EROs também podem se referir às espécies que não são radicais livres, e sim algumas moléculas derivadas de oxigênio capazes de gerar radical livre, como o peróxido de hidrogênio (H_2O_2). Dentre as EROs, o radical superóxido (O_2^-), radical hidroxila (HO-) e peróxido de hidrogênio (H_2O_2) são formados em todas as células aeróbicas (MURPHY, 2009).

Os radicais livres podem ainda ser formados por reações químicas envolvendo Espécies Reativas de Nitrogênio (ERN), Espécies Reativas de Enxofre (ERS), pelas reações enzimáticas da ciclooxigenase, lipoxigenase e aldeído oxidase, pelas reações catalisadas por metais de transição como o ferro e o cobre, entre outras (KOWALTOWSKI et al., 2009; SUZUKI; MITTLER, 2012).

As mitocôndrias desempenham papel chave na produção de energia, consumindo mais de 90% do oxigênio disponível no organismo, sendo a principal fonte de radicais livres. Durante a obtenção de energia na forma de ATP, ocorre a redução do oxigênio a água, pela adição de quatro elétrons à sua molécula pela enzima citocromo oxidase, nesse processo são gerados diversas espécies reativas: ânion superóxido (O_2^-), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila (OH•) (DESAI et al., 2010).

1.5. MORTE CELULAR INDUZIDA POR PERÓXIDO DE HIDROGÊNIO (H_2O_2)

1.5.1. Visão geral da Morte Celular

Duas formas distintas de morte, necrose e apoptose, têm sido melhores estudados de acordo com as características celulures, morfológicas e bioquímicas (KANDUC et al., 2002; BROKER; FRANK; KRUYT, 2005; LULLI et al., 2010). Na necrose, uma extensa lise celular resulta de lesão aguda, acidental, ou não fisiológica. Este tipo de morte celular está associado a uma lesão grave da membrana, ocasionando liberação de constituintes celulares para o espaço extracelular, que pode conduzir a inflamação local e dano ao tecido circundante (OUYANG et al., 2012).

Por outro lado, a apoptose representa uma forma de morte celular, que requer a ação de proteases e nucleases dentro de uma membrana plasmática intacta (ELMORE, 2007). A disfunção mitocondrial, inibição da cadeia respiratória, perda do potencial de membrana mitocondrial interna ($\Delta\Psi m$), e um aumento da permeabilidade da membrana mitocondrial (MMP) representam características bioquímicas da apoptose. As características morfológicas da apoptose incluem a fragmentação do DNA, retração celular e decomposição celular em corpos apoptóticos ligados à membrana destinados para a fagocitose (BROKER et al., 2005; OUYANG et al., 2012).

A apoptose desempenha uma função fundamental na manutenção da homeostase dos tecidos sob condições fisiológicas, como um componente de programas de desenvolvimento. Contudo também pode contribuir para o desenvolvimento de doenças (LIVSHITS; KOBIELAK; FUCHS, 2012). A apoptose precoce está principalmente associada com dano mitocondrial direto, e em sua fase final, a danos no DNA. Neste caso, a indução da apoptose pode representar um mecanismo de defesa para eliminar células com danos irreparáveis (ROOS; KAINA, 2013; FU; SAMSON, 2012). As células podem iniciar o processo de apoptose por dois caminhos: via intrínseca (dependente da mitocôndria) e via extrínseca (dependente receptor) (ZHOU et al., 2012; RENAULT; CHIPUK, 2013).

Tanto apoptose como necrose podem ocorrer em resposta ao tratamento com diversos estímulos nocivos, geralmente de forma dose-dependente (PIETKIEWICZ et al., 2013; VILLENEUVE et al., 2013). Muitos agentes que causam apoptose em doses baixas a

moderadas podem vir a causar necrose, quando doses relativamente mais altas são aplicadas. Uma série de eventos endógenos governa o equilíbrio entre a morte por apoptose e necrose (KANDUC et al., 2002). Além disso, a apoptose e necrose não são delineados como eventos claramente exclusivos mutualmente. Modos mistos, contendo as características dessas formas de morte celular, foram relatados por diversos pesquisadores. O chamado "aponecrose" pode representar programas apoptóticos abortados ou parcialmente executados, que ocorrem no contexto de um resultado necrótico final (DUMRESE et al., 2005; MARINO et al., 2008).

A existência de vias de morte celular por necrose que são reguladas por um programa de morte intrínseco distinta da apoptose também foi proposto, como a "necroapoptose", aparece morfologicamente semelhantes a necrose (DEMPSEY, 2013). Assim, a terminologia "morte celular programada" (PCD), uma vez reservado estritamente para a apoptose, pode indicar várias formas de morte celular mediada por um programa de morte intracelular (PADMANABHAN et al., 2012;).

1.5.2. H_2O_2 e a indução da morte celular

Embora seja um agente oxidante relativamente fraco em comparação com outro ROS (superóxido, radical hidroxil), o H_2O_2 tem emergido como uma importante molécula de sinalização com base nas suas propriedades bioquímicas únicas: presença em todos os sistemas biológicos, tempo de meia-vida relativamente longo, e mais importante, solubilidade em meios aquosos e lípidicos (VALKO et al., 2007). Desta forma, o H_2O_2 facilmente se difunde aos seus alvos celulares. Pesquisas recentes têm sugerido que o sinal de apoptose possa ser transduzido extracelularmente por fatores solúveis, sugerindo que o H_2O_2 pode atuar como um mediador parácrino de apoptose (PLETJUSHKINA et al., 2006).

O peróxido de hidrogênio tem sido amplamente utilizado como um modelo de estresse oxidativo exógeno em estudos de apoptose (XUE et al., 2012; LIU et al., 2013; ZHOU et al., 2013). O Processo de apoptose induzida por H_2O_2, inclui aumento da proteína apoptótica p53, perda do potencial de membrana, liberação de citocromo c, ativação da caspase-3, e fragmentação de DNA (KITSATI et al., 2012; LIU et al., 2012; RINCHEVAL et al., 2012; DE MARINIS et al., 2013).

Como discutido anteriormente, um mesmo composto pode induzir necrose ou apoptose dependendo do tipo celular e da concentração utilizada. Embora a maioria dos

trabalhos indique um papel pró-apoptótico para o peróxido de hidrogênio, a indução da morte celular pela via necrótica também já foi relatada (XU et al., 1997; TERAMOTO et al., 1998; CHOI et al., 2009). Ali et al. (2013) mostraram a indução de necrose em cardiomiócitos de ratos neonatais submetidos a diferentes concentrações de H_2O_2, sugerindo que a morte celular foi caracterizada por severos danos à membrana plasmática, como evidenciado pela liberação de lactato-desidrogenase, bem como ausência de caspase-3 e da ativação de PARP-1(eventos típicos da apoptose) e independente da ação das metaloproteinases de matriz-2.

Vários estudos têm demonstrado a ação de produtos naturais na inibição da morte celular induzida por H_2O_2, utilizando diferentes linhagens celulares como modelos (tabela 3).

Tabela 3: Exemplos de produtos naturais com ação protetora contra a morte celular induzida por peróxido de hidrogênio (H_2O_2.)

Linhagem Celular	Produto	Espécie	Referência
Cardiomiócitos	Saponina	*Acanthopanax senticosus*	Liang et al. (2010)
Células Vero	loliolide	*Sargassum ringgoldianum*	Yang et al. (2008)
EA.hy926	Extrato de frutas	*Phyllanthus emblica*	Wongpradabchai et al. (2013)
Fibroblasto de pulmão (Hamster)	Hidrolisados enzimáticos	*Vaccinium corymbosum*	Senevirathne et al. (2010).
Eritrócitos humanos	Salidroside	*Rhodiola rosea*	Qian et al. (2012)
Fibroblastos de pele humana	Extrato	*Avena sativa*	Feng et al. (2013)
HeLa	Ácido Gálico	-	Erol-Dayi e Arda; Erdem (2012).
HepG2	Polipeptídeos	*Musca domestica*	Zhu et al. (2013)
HL-7702	Polissacarídeos	*Cordyceps militaris*	Liu et al. (2013)
hMSCs	Curcumina e EGCG (epigallocatechin-3-gallate)	-	Yagi et al. (2013)
HUVEC	Resveratrol	-	Liu et al. (2013)
Macrófagos RAW 264.7	Camomila	*Matricaria chamomilla* L.	Bhaskaran et al. (2013)
Linfócitos humanos	Genisteina	-	Yadav et al. (2012)
Neurônios humanos	Extrato	*Rhodiola rosea*	Palumbo et al. (2012)
Osteoblastos	Tetrahydroxystilbene	*Polygonum multiflorum*	Zhang et al. (2013)
Saccharomyces cerevisiae	Flavanoídes	*Vellozia kolbekii*	Silva et al. (2012)
SH-SY5Y	Quercetina	-	Suematsu et al. (2011).

Legenda: HeLa: células de adenocarcinoma cervical; HepG2 e HL-7702: células hepáticas; hMSCs: Células troncos mesenquimais humanas; HUVEC: células endotelias do cordão umbilical; SH-SY5Y: células de neuroblastoma.,

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar o efeito imunomodulador e citoprotetor das lectinas nativa (pCramoll) e recombinante (rCramoll) de *Cratylia mollis*.

2.2. OBJETIVOS ESPECÍFICOS

- Determinar o efeito das lectinas sobre a viabilidade celular e produção de óxido nítrico de macrófagos peritoneais de camundongos;
- Analisar o efeito das lectinas na produção de citocinas por macrófagos normais e infectados com *Staphylococccus aureus*;
- Avaliar a produção de superóxido mitocondrial e as alterações no potencial da membrana mitocondrial nos macrófagos tratados com as lectinas;
- Investigar a função lisossomal nos macrófagos tratados;
- Analisar a capacidade fagocitar *Staphylococccus aureus* dos macrófagos tratados com as lectinas;
- Avaliar a ação das lectinas sobre a citotoxicidade induzida pelo peróxido de hidrogênio em Células Vero;
- Determinar o efeito das lectinas sobre a inibição da proliferação celular induzida por H_2O_2 em Células Vero;
- Investigar os mecanismos subcelulares involvidos na ação citoprotetora das lectinas contra o H_2O_2 em Células Vero (produção de superóxido mitocondrial, danos lisossomais, perda do potencial de membrana, fragmentação de DNA).

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CAPÍTULO I

Plant Lectins and Toll-like receptors: implications in therapy for microbial infections

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Plant lectins and Toll-like receptor: implications for therapy of microbial infections

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Keywords: TLR receptors, immunomodulation, lectin, microorganisms, infection

Several plant lectins are known as potent immunomodulatory agents, and they are applied in different experimental models of infection (Afonso-Cardoso et al., 2011; Oliveira et al., 2013). Recent research has characterized these proteins as potential agonists of Toll-like receptors (TLRs), a family of mammalian homologs of *Drosophila* Toll protein involved in the detection of microbes and initiating inflammatory responses (O'Neill et al., 2013). In this opinion article, we highlighted some studies of plant lectins as modulators and agonists of TLRs in order to stimulate research interest in this fascinating area and promote knowledge sharing and scientific collaboration.

A SHORT VIEW ON PLANT LECTINS

Lectins are a very large class of carbohydrate-binding proteins of non-immune origin. Through the interaction with sugars, they trigger several important cellular processes. Lectins are universally expressed and have been shown to function in animals, plants, and microorganisms as cell and molecular recognition proteins. However, the carbohydrate-binding domains have been studied most intensively within the plant kingdom (Sharon, 2007; Vandenborre et al., 2011).

The discovery of plant lectins occurred in the 19th century, however, many questions about the biological role of these molecules remain obscure. Lectins may be involved in sugar transport, carbohydrate storage and they are associated as molecular chaperones (Van Damme et al., 2004; Liu and Li, 2013). The adhesion and agglutination properties of lectins have been related in the interaction of both symbiotic and pathogenic interaction of some microorganisms and host (Audfray et al., 2012). Plant lectins represent a group of proteins with obvious differences in their biochemical/physicochemical properties, molecular structure, carbohydrate-binding specificity and biological activities (Liu et al., 2010). Lectins are oligomeric which exhibit a large structural diversity and the molecular size range from 60 to 400 kDa. Each lectin polypeptide contains many molecular domains, one of which is the non-catalytic carbohydrate recognition domain, responsible for their ability to recognize and interact with specific glycoconjugates, without altering their structure. In the past few years, hundreds of plant lectins have been purified and characterized in details with respect to their biochemical properties, carbohydrate-binding specificities, these approaches allowed their classification (Van Dammes et al., 2011).

TOLL-LIKE RECEPTOR

Plant materials represent an excellent source of immune modulators, which have been appointed as a new approach for combating infections caused by resistant microorganisms (Hancock et al., 2012). In this sense, a range of potential compounds have been proposed as agonists and inductors of immune receptors, including TLRs.

The TLR family is the best characterized group of innate immune receptors in terms of known ligands, downstream signaling pathways and functional relevance. They comprise a family of receptors homologous to Toll receptor from Drosophila melanogaster. In humans, the TLR family includes 10 transmembrane proteins that play a crucial role in host defense: they recognize molecular characteristics of microorganisms, known as pathogen-associated molecular patterns (PAMPs) highly conserved between different classes of microorganisms. For example, TLR4 and accessory proteins recognize lipopolysaccharide (LPS), while TLR2 recognizes lipoteichoic acid and various lipopeptides (when in complex with either TLR-1 or TLR-6), and TLR5 recognizes flagellin (Hancock et al., 2012; O'Neill et al., 2013). In this way, TLR receptors represent important therapeutic targets for developing new drugs able to directly modulate the host response against microbial infection. In fact, some TLR agonists and TLR modulators have been investigated as potential drugs on clinical trials and research programmes (Hennessy et al., 2010; Murgueitio et al., 2012).

PLANT LECTINS AND TOLL-LIKE RECEPTOR

Lectins have been extensively used as valuable tools in the biomedical research. The versatility of these biomolecules is due to their interactions with receptor-linked glycans on cell surfaces, which may trigger cell signaling and physiological responses (Lam and Ng, 2011). Plant lectins are characterized as immunomodulator agent, which result in the production of certain cytokines and reactive species and induce efficient immune responses against tumors or microbial infections. A very comprehensive review of immunomodulatory lectins has recently been published by Souza et al. (2013). In order to investigate the mechanism of this action, some

researchers correlated the activation of immune cells by lectins with the expression of TLR receptors.

Sodhi et al. (2007) investigated the expression of different TLRs induced by the famous lectin from Concanavalin A (Con A) using mouse macrophages as a model. Con A enhanced *in vitro* expression of TLRs (2–9) and its action was related with JNK, p38, p42/44, and NF-κB. The authors also showed the heterodimerization of TLR-2 and TLR-6. Additionally, Con A pre-treated-macrophages were more susceptible to induction of proinflammatory cytokines and nitric oxide by different TLR ligands (ZymosanA, PolyI:C, LPS, CpG DNA).

In other study, the Korean mistletoe lectin (KML-C) from *Viscum album coloratum* was shown to be a potent activator of TLR-4. The treatment of mouse peritoneal macrophages with KML-C-induced the upregulation of interleukin-1 receptor-associated kinase-1 (IRAK1) resulting in macrophage activation and TNF-α production, which was not observed when TLR-4 was blocked using a TLR-4-specific neutralizing antibody or TLR-4-deficient macrophages. The expression of TLR-4 was also induced by lectin-like protein from *Anoectochilus formosanus* (IPAF), resulting in the stimulation of TNF-α and IL-1β, CD86, and MHC II and phagocytic activity (Park et al., 2010).

The capacity to modulate the TLR were explored to combat the experimental infection of *Paracoccidiodes brasiliensis* using native and recombinant KM^+, a mannose-binding lectin from *Artocarpus integrifolia*. BALB/c mice were infected with *P. brasiliensis* and after 10 days both proteins were separately administered. KM^+treatment reduced significantly colony-forming unit and induced higher levels of nitric oxide, INF-α, TNF-α, and IL-12, which was dependent on TLR-2 (Coltri et al., 2008).

Recently, in a remarkable paper, phytohaemagglutinin (PHA from *Phaseolus vulgaris*) and its isoforms were showed as a specific human TLR-4 agonist during an initial screening. This result encouraged the authors to examine the effects of this and other lectins on external (-2/6, -4, and -5) and internal (-3, -7, -8, and -9) human TLRs. In this research, SBA (Soybean agglutinin from *Glycine max*), PNA (peanut agglutinin from *Arachis hypogaea*), ConA and PHA only stimulated extracellular TLRs (-2/6, -4, or -5): TLR-4 for SBA and PNA; TLR-2/6 for ConA; TLR-2/6, -4 and for PHA-L. In other hand, WGA (wheat germ agglutinin from *Triticum vulgaris*) was the most promiscuous lectin activating all tested receptors, except TLR-3 and -4. The jacalin (from *Artocarpus integrifolia*) was inactive. This variety of TLR agonist pharmacology is related to different sugar ligand specificity of each plant lectins, suggesting that the action is encoded by the carbohydrate recognition motifs on different TLRs (Unitt and Hornigold, 2011). TLR agonists have been proposed as adjuvants for vaccines against virus (Behzad et al., 2012; Hong et al., 2012), bacteria (Hancock et al., 2012), parasite (Moon et al., 2012) and fungi (LeibundGut-Landmann et al., 2012).

In conclusion, these observations encourage further studies for the characterization of plant lectins as novel agonists and modulators of TLR receptors. These proteins act by increasing the immune response of the host against microbial infections, thus overcoming their immunosuppressive mechanisms and offers an alternative to combat the increasing drug resistance. These approaches may also provide new insights on TLR biology and aid in the discovery of new targets glycosides useful in the therapy based on TLR receptors.

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CAPÍTULO II

Cratylia mollis lectin: a versatile tool for biomedical studies

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Cratylia mollis lectin: a versatile tool for biomedical studies

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Cratylia mollis lectin a versatile biotechnologic tool

Lectins are a heterogeneous group of proteins and glycoproteins that specifically and reversibly bind with high affinity to carbohydrates without altering the covalent structure of any of the recognized ligands. Advances in the plant lectin research have provided many insights into the role of carbohydrate-protein interactions and it has contributed to the development of various others areas (for example: oncology, immunology, microbiology, molecular biology and diagnostic). Isolectins isolated from leguminous forage *Cratylia mollis*, a native plant of Brazilian Caatinga biome, have been demonstrated a remarkable biotechnological aptitude, especially the isoforms called Cramoll 1,4 (pCramoll). We aim to present a comprehensive review of the biological applications of pCramoll, which has been used as immunomodulatory, antitumoral, mitogenic, antiparasitic and healing agent. Others biotechnological applications also involve the characterization of human cancerous tissues, and the development of affinity matrices to purify proteins and molecular biosensors. The present review comprises four main themes: (1) an overview of plant lectins and their biological effects; (2) general characteristics of the pCramoll; (3) biotechnological applications of this lectin; and (4) heterologous expression of Cramoll and folding studies. The process of isolation, structural characterization and biotechnological applications of pCramoll is one of the greatest success stories among Brazilian lectins. Recently, the expression of functional recombinant Cramoll 1 (rCramoll) in *Escherichia coli* was reported by our group. This review will be a valuable resource for more studies in the lectin field, which has a great potential to reveal new targets for therapeutic strategies, molecular diagnosis and biotechnology process.

Keywords: anti-parasitic, biosensor, immunomodulation, lectin, oncology, wound healing.

Introduction

Lectins are known as a large class of carbohydrate-binding proteins which play pivotal role in a range of cellular processes [1, 2]. Lectins usually are oligomeric proteins which exhibit a large structural diversity and the molecular size range from 60 to 400 kDa. Each lectin polypeptide may contain many molecular domains, at least one of which is the non-catalytic carbohydrate recognition domain, responsible for their ability to recognize and interact with

specific glycoconjugates, without altering their structure [3, 4]. Although the carbohydrate-binding domains are best characterized in plants, lectins are found in all domains of life [5, 6, 7]. Plant lectins represent a group of proteins with obvious differences in their biochemical properties, molecular structure, carbohydrate-binding specificity and biological activities [3, 8].

In the past few years, hundreds of plant lectins have been purified and characterized in details with respect to their biochemical properties, carbohydrate-binding specificities, these approaches allowed their classification [4, 9, 10, 11]. According to the overall structures of the mature lectins, they can be divided into ‘merolectins’, ‘hololectins’, ‘chimerolectins’, and‘superlectins’ (Fig. 1).

There are 12 plant lectin families defined according to their different carbohydrate-binding domains (Van Damme et al., 2008), they are: (1) *Agaricus* bisporus agglutinin homologs, (2) Amaranthins, (3) Class V chitinase homologs with lectin activity, (4) Cucurbitaceae phloem lectins (or Nictaba family), (5) Cyanovirin family, (6) EEA family, (7) GNA family, (8) Jacalin-related lectins, (9) LysM domain, (10) proteins with hevein domains, (11) proteins with legume lectin domains, and (12) Ricin-B family (Table 1).

The discovery of lectins occurred in the nineteenth century, however, many questions about the biological role of these molecules remain obscure. Lectins may be involved in sugar transport, carbohydrate storage and to be associated as molecular chaperones [9, 12, 13]. The ability of adhesion and agglutination of lectins has been related in the interaction of both symbiotic and pathogenic microorganisms [14, 15].

Overview of biomedical applications of plant lectins

Plant lectins have been extensively used as valuable tools in the biomedical research [16, 17]. The versatility of these biomolecules is due to their interactions with receptor-linked glycans on cell surfaces, which may trigger cell signaling and physiological responses [18] (table 2).

A number of plant lectins (predominantly galactoside and galNAc specific) have been used in pre-clinical and clinical trials as potential drugs for treatment of cancer [8, 19], usually due the induction of programmed cell death pathways and anti-angiogenesis action [20, 21]. Plant lectins are known as immunomodulator agent, which stimulate the production of cytokines and reactive species producing efficient responses against tumors or microbial [18, 22]. The

plants lectins also have mitogenic [23] anti-asthmatic [24], wound healing [25], anticoagulant activities [26], antioxidant [27], anti-inflammatory [28], analgesic [29], antinoceptive [30] and contraceptive [31] activities.

The search of new antibiotics is important due the growing resistance of microorganisms to convectional antimicrobial agents. In fact, some plant lectins have demonstrated a strong inhibition of various human, animal or vegetal pathogens. The action mechanism may be attributed to indirect effects produced by the binding of lectins to carbohydrates on the surface of the microbial cell wall or bacterial extracellular glycans [32, 33, 34, 35]. Several antiviral lectins have been isolated from plants which can binding to glycoproteins on virus surfaces (such as human immunodeficiency virus and others enveloped viruses) disturbing the interactions between these proteins and host [36, 37, 38]. In addition to these studies, recent works have been investigated the effects of lectins in some parasites [39, 40]. Lectins have been also suggested as one of the most promising agents in opposition to insect pests, which restrain increased crop production [1, 41, 42].

Significant progress has been reached in the last years to explore the lectin-sugar interaction for the development of products (table 3). The plant lectins have historically been used for blood typing, using lectins with different specificities to distinguish between blood types [43]. They also can be used as a tool to identify aberrant glycans expressed by neoplastic cells [44, 45], biosensor [46, 47], drug delivery [48], water treatment [49], affinity matrix to glycoproteins isolation [50, 51]. The global glycan also can be analyzed using a novel platform called lectin microarray, which enables rapid and high-sensitivity profiling of complex glycan features without the need for liberation of glycans of the sample [52, 53]. This method has been applied to discover new glycan specific markers [54, 55].

This overview provides the basic information on plant lectins as biomedical agents, there are numerous examples of them and the text is not exhaustive. In this context, our group has extensively investigated the effects of a plant lectin named Cramoll from *Cratylia mollis*. This subject will be detailed in the subsequent sections of this review.

Isolectins from Cratylia mollis

Cratylia mollis Mart is native forage belongs to Fabaceae family. This plant is endemic from the Semiarid Region of the Northeast of Brazil (Caatinga biome), and is popularly known as camaratu bean. From the seeds of *C. mollis* have been purified four multiple molecular forms

of lectin named Cramoll -1, -2, -3, -4. These isolectins exhibit specificity to different carbohydrates. The isoforms 1, 2 and 4 are non-glycosilated and glucose/mannose specific proteins; and Cramoll 3 is a galactose specific glycoprotein [56, 57]. The localization of this isolectins in *C. mollis* seeds was determinated by ultrastructural and immunocytochemical analysis. This work showed that the *C. mollis* isolectins occurred mainly in the amorphous matrix of protein bodies and in the cellular walls of the embryonic axis, which are consistent with the subcellular localization of several legume lectins. The co-localization of *C. mollis* isolectins whith different carbohydrate specificity and glycosylation suggests different functions inside the same subcellular organelle [58].

Cramoll 1,4 (preparation containing isolectins 1 and 4; pCramoll) is isolated in a similar way to the well-known lectin from *Canavalia ensiformis* seeds, Concanavalin A (Con A) [57]. Cramoll 1 is a major isolectin which consists of 236 residues with 82% identity with ConA, its terciary fold (Figure 2) was determined by X-ray crystallography at 1.77 Å and revealed three β-sheets connected by loops, known as the jellyroll domain (this topological architecture is essentially identical to ConA) [59]. Recently, the expression of functional recombinant Cramoll 1 (rCramoll) in *Escherichia coli* was reported by our group [60].

pCramoll is an imunnomudulatory agent

Immunomodulatory compounds play critical roles in the response against infections and the immunobiological importance of pCramoll has been documented in diverse works (figure 3). The first indication that pCramoll has an immunomodulatory property was its ability to induce *in vitro* mitogenic effect in similar way to the well known T-cell mitogen, concanavalin A (Con A), this effect was inhibited by methyl-α-D-mannoside, indicating the involvement of carbohydrate-lectin binding sites [61]. The mitogenic activity of this lectin was also demonstrated in splenocytes obtained from mice inoculated for 2 days with a single intraperitoneal dose with 100 uL of pCramoll at 100 µg/mL and killed after 72 h. This works showed that pCramoll did not induced apoptosis, stimulated a significant number of cells in the S phase of the cell cycle and high levels of IL-2, IL-6, IFN-γ and nitric oxide production [62]. In a similar research, animals were inoculated with a single intraperitoneal injection of pCramoll (235 µg/mL). Their spleens were removed for immune cell isolation 7 days later and was observed an increase in IL-1β (Th2 response) cytosolic and mitochondrial ROS, which was related to higher cytosolic Ca^{2+} levels [63].

The *in vitro* Th1 cytokine induction pathway was also reported to pCramoll using experimental cultures of mice lymphocytes through IFN-γ production and showed antiinflammatory activity through NO suppression. The induction of IL-10 was minimal and splenocytes treated with pCramoll howed higher IFN-γ levels than PHA and Con A treated-cells [64]. The most recent immunomodulatory work stabilished that pCramoll is capable to trigged the Th17 pathway, provoking the production of IL-6, IL-17A, IL-22, and IL-23 cytokines and to generate immunologic memory in cultured splenocytes. These inductions are more efficient than ConA [65].

pCramoll as anti-parasite agent

The anti-parasite action of pCramoll has already been demonstred against *Trypanosoma cruzi* and *Schistosoma mansoni*. pCramoll has the ability to agglutinate the epimastigotes forms of *T. cruzi* leading to the inhibition of proliferation and the cell death by necrosis. The molecular mechanism is related to permeabilization of the plasma membrane followed by Ca^{2+} influx and mitochondrial Ca^{2+} accumulation, stimulation of mitochondrial ROS, decreased of mitochondrial membrane potential ($\Delta\Psi m$) and impairment of ADP phosphorylation [66].

The effect of pCramoll on experimental schistosomiasis was evaluated in Swiss mice infected subcutaneously and caudally with *Schistosoma mansoni* BH strain (100 cercariae/mouse). Two treatment schedules with an intraperitoneal dose were performed: (1) single dose of pCramoll at 50 mg kg^{-1} after 40 days of infection; and (2) daily dose of pCramoll at 7mg mg kg^{-1} for 7 days after infection). The both treatment were efficient with reduction of: oviposition (79 and 80%), adult worm recovery (71 and 79%) and liver granulomas (40 and 73.5%) in relation to control [67]. These effects of pCramoll in the infection of *S. mansoni* is very exciting and its action mode should be investigated as well the involvement of cytokines.

pCramoll as healing agent

The use of plants material for the treatment of wounds is well known and pCramoll also demonstrated a high pro-healing activity. The effects of pCramoll in the treatment of cutaneous wounds were firstly investigated using normal and immunocompromised mice. The daily topical administration of pCramoll (100 uL at 100 µg/mL) results in the repair of experimental lesions in normal and immunocompromised mice, promoting excellent closing and repair of them. pCramoll treated-wounds showed higher edema, increased polimorphonuclear cells infiltration, higher intensity of granulation tissue and collagen fiber deposition [68].

The second study was desinged to evaluate the action of a hydrogel containing pCramoll in the treatment of second-degree burns in rats. This biopharmaceutical formulation was proposed by Lima-Ribeiro et al. [70] using Carbopol® as a vehicle, the pCramoll-hydrogel was adequate for the maintenance of hemagglutinating activity, suggesting its topical application in vivo. The topical administration of pCramoll-hydrogel occurred 7, 14, 21, 28, and 35 days after the induction of second-degree burns. On the 7th day, the pCramoll-hydrogel group showed intense exudates, wound contraction, necrosis and edema. The tissue reepithelialization and a moderate autolysis were observed on the 14th day. After 21 days of treatment the animal showed intense fibroblastic proliferation, presence of dense collagen, and moderate fibrosis. The complete tissue epithelialization was achieved at day 28 and on the 35th day, the wounds showed modeled dense collagen. The authors also reported that the treatment did not provoke alterations in biochemical and hematological parameters [69].

pCramoll and oncology research

The antitumor activity of pCramoll encapsulated into liposomes was investigated against Sarcoma 180 in Swiss mice. pCramoll-loaded liposomes were produced with an 84% encapsulation ratio (700 µg/mL) and the treatment was performed intraperitoneally (7mg/kg body weight per day) for 7 days. The encapsulation of pCramoll improved its antitumor activity and decreased its tissue toxicity (particularly, in the liver and kidney) [71]. In addition, pCramoll have been used in the diagnosis of malignant tumors, due to its specific binding to glucose/mannose. pCramoll conjugated to horseradish peroxidase were used as histochemical probes in human prostate tissues and it was able to distinguish normal and tumor cells, detecting subtle neoplastic changes of histologically related or similar pathologies [72].

pCramoll and biosensors development

The biomolecular interactions of pCramoll and sugar have been explored in the biosensors development using biomolecule–nanoparticle hybrid systems. In the first approach [73], pCramoll were immobilized on gold nanoparticles (AuNp) with polyvinylbutyral (PVB), and adsorbed on the surface of gold (Au) electrodes. These pCramoll modified electrodes showed an improvement in their sensitivity for sugars detection and may be applicable to construction of a sensitive biosensor to detection of complex glycoconjugates and glycoproteins in solution [73]. This was conducted in a later study, when pCramoll was used to modify gold electrodes were applied in the detection of seric glycoproteins from patients infected with dengue serotypes 1, 2 and 3. The biosensor exhibited a different response according the kind of virus, and also a higher reaction to dengue serotype 2 [74]. Another type of biosensor was employed using pCramoll as the recognition interface to detect the bacterial lipolysaccharide (LPS). In this study, a biosensor based on self-assembled hybrid cystein-gold nanoparticles and pCramoll was selective to the discrimination of LPS types (from *Serratia marcescens*, *Escherichia coli*, *Salmonella enterica* and *Klebsiela pneumonia*) with a high sensitivity [75]. All of these pCramoll based biosensors represent excellent prototypes for application in the molecular diagnostic.

pCramoll for the isolation and characterization of bioactive molecules

Lima et al. [76] prepared an afinity column of pCramoll coupled to Sepharose CL4B to isolate glycoproteins from human plasma and made a comparation with Con A-Sepharose

(commercial available). These authors related differences in the type and amount of serum glycoproteins adsorbed using each lectin and concluded that pCramoll-Sepharose has the potential for isolation and characterization of serum glycoproteins. The same system was used to isolate the Glycine max protein, with in vitro platelet antiaggregation and anticoagulant activities [77]. Finally, pCramoll has also been used in the evaluation of the presence of glucose/mannose in protein structure by gel diffusion assay [78].

Studies of pCramoll biodistribuition and allergic potential

Analysis of the biodistribution of a therapeutic agent candidate is essential. The distribuition of pCramoll were performed using Wistar rat which received a dose of pCramoll radiolabeled with technetium-99m (Tc-99m; 3,7 MBq/0,2 mL), in jugular vein. After 1 h, scintigraphy showed that the great part of the lectin was processed by the liver, in a similar way of other injectable compounds. The lectin cannot pass the hematoencephalic barrier. However, an abnormal uptake by the intestine was observed, which the the rate between the intestine and the kidney is over the double. This fact reflects differences on its clearance mechanism, the reabsorption of the drug could be facilitated by the microvilli of the intestine. The major consequence is that the over dose can induce toxic reaction due its accumulation in the bowel [79].

Vaz et al. [80] used pCramoll as a model to evaluate the application of gamma irradiation to reduce or eliminate food allergenicity. For this, albino Swiss mice were previous immunized with pCramoll (10 µg/mL, subcutaneously on days 0, 15 and 30). After three days, the oral treatment started and the animals received an intraperitoneal injection at the same dose. The the animals were subjected to intragastric administration of non-irradiated and irradiated (1, 10 and 25 kGy) pCramoll (27 mg/kg body weight)/day) for 7 days. The authors showed a high relationship between pCramoll intake and body weight loss, eosinophilia, lymphocytic infiltrate in the gut and eotaxin secretion. However, the irradiation significantly reduces these effects, in a dose-dependent way, indicating that high-dose radiation treatment is an alternative to abolish the food allergenicity in a direct and irreversible form.

Heterologous expression of pCramoll

In this revision, we discussed the Cramoll as a valuable biotechnology tool and this potential requires the production of large quantities of protein, and, given the climatic conditions of

Caatinga biome, the quantity of seed can not be optimal. Additionally, the protein expression in seed is also affected by other environmental conditions. All these facts together led to the need for develop a heterologous system to Cramoll, however, the expression of legume lectins is challenge, due to their complex post-translational modifications. The heterologous expression of Cramoll was reported by Varejão et al [60] using a chemically synthesized DNA encoding the mature Cramoll 1 amino acid sequence [59] into a bacterial expression vector (pET-28α) under T7 promoter control. The *E. coli* Rosetta (DE3) was used for expression of recombinant Cramoll 1 (rCramoll). This lysogenic strais contains the T7 RNA polymerase gene under the control of an inducible lacUV5 promoter and pRARE plasmid. This elegant approach resulted in the expression of 6 mg L^{-1} of soluble and active rCramoll. This recombinant proteins share several physicochemical properties such as molecular mass, charge density monosaccharides specificity and tertiary structures, however, most of rCramoll was found in insoluble aggregates (inclusion bodies).

The expression of rCramoll is great step to perform detailed studies of the structure/function relationship. The comparative characterization of the unfolding process of tetrameric and dimeric forms of rCramoll and pCramoll were performed and provided insights in the compreention of the importance of natural fragmatation on the legume lectins stability. Although rCramoll retained several biophysical properties of pCramoll, the tetramers of recombinant lectin, composed of intact monomers, has a little enhancement in stability to acidification, high temperatures or hydrostatic pressure. The results of this work showed that tetrameric pCramoll unfolded through a compact monomeric intermediate, while, dimers of rCramoll tetrameric remarkably showed no evidence of such partially unfolded monomers [81].

Conclusions and future directions

In this review, we have focused on the biomedical properties of pCramoll. Our group has been demonstrated application of this lectin for biomedical studies, diagnostic and therapy. The versatilite of pCramoll makes it a prototype for the study of biological functions of plant lectins. The successful expression of the rCramoll will enable us to study of the structure/function relationship, discovere lectin targets and to perform site-directed mutagenesis. We are currently working on: (i) understanding the molecular action mechanisms involved; (ii) identifying cell-surface receptors; (iii) extending the range of

models to evaluate the healing and immunomudulatory activity and parasite-host interaction; (iv) developing new pharmaceutical forms; (v) determining the properties of rCramoll.

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Table 1: Carbohydrate-binding domains of Plant lectins (adapted from Vandenborre et al. [1])

Domain	***Carbohydrate specificity***	***Exemple***
***Agaricus bisporus* agglutinin domain**	T-antigen	ABA (PDB: 1Y2T)
Amaranthins	T-antigen	Amaranthin (PDB: 1JLY)
Class V chitinase homologs	Blood group B, High-mannose N-glycan	TCLL (PDB: 4B16)
Cyanovirin domain	High-man N-glycans	CV-N (PDB: 2JZJ)
***Euonymus europaeus* agglutinin domain**	Blood group B, High-mannose N-glycan	EEA
***Galanthus nivalis* agglutinin**	Mannose, oligomannosides, high-man N-glycans.	PCL (PDB: 3A0C)
Hevein domain	Chitin, high-man, Man, N-glycans	WGA (PDB: 2UVO)
Jacalins domain	Gal, T-antigen, Man, N-glycans	JAC (PDB: 3P8S)
Legume lectin domain	Man/Glc, Gal/GalNAc, (GlcNAc)n, Fuc, Siaa2,3Gal/GalNAc, complex Nglycans	ConA (PDB: 3D4K)
LysM domain	Chitin-ologosaccharides	LysM
***Nicotiana tabacum* agglutinin domain**	GlcNAc-oligomers, high-man N-glycans	NICTABA,
Ricin-B domain	Gal/GalNAc, Siaa2–6Gal/GalNAc	ML-I (PDB: 1M2T)

Legend: T-antigen: Galβ(1,3)GalNAc; Man: mannose; Gal: galactose; Glc: glucose; GalNAc: N-acetylgalactosamine; GlcNAc: N-acetylglucosamine; Fuc: fucose; Sia: sialic acid; PDB: Protein data bank (http://www.rcsb.org/pdb/home/home.do)

Table 2: Examples of pharmacologic activities of Plant lectins

Action	Lectin	Source	Reference
Analgesic	CrataBL	*Crataeva tapia*	30
Anthelmintics	PHA-L	*Phaseolus vulgaris*	83, 84
Anti-angiogenesis	ConA	*Canavalia ensiformis*	82
Anti-asthmatic	ScLL	*Synadenium carinatum*	24
Antibacterial	EuniSL	*Eugenia uniflora*	34
Anticoagulant	BfL	*Bauhinia forficata*	56
Antifungic	SejaBL	*Sebastiania jacobinensis*	35
Anti-inflammatory	LAL	*Luetzelburgia auriculata*	28
Antinociceptive	ConBr	*Canavalia brasiliensis*	30
Anti-oxidant	KM	*Viscum album coloratum*	27
Antiprotozoal	ScLL	*Synadenium carinatum*	39
Antitumoral,	PTL	*Pinellia ternata*	85
Anti-viral	GNA	*Galanthus nivalis*	37
Bioinsecticidal	cMoL	*Moringa oleifera*	41
Contraceptive	PHA-L	*Phaseolus vulgaris*	31
Healing	EmaL	*Eugenia malaccensis*	25
Imunomoduladory	ArtM	*Artocarpus heterophyllus*	18
Mitogenic	BKBL	*Phaseolus vulgaris* cv. brown kidney bean.	23
Vasodilatation	DLL	*Dioclea lasiocarpa*	86

Table 3: Pharmacologic potential of pCramoll

Action	Reference
Anthelmintic	67
Antiprotozoal	66
Antitumoral	71
Healing	68, 69
Imunomoduladory	62-65
Mitogenic	61

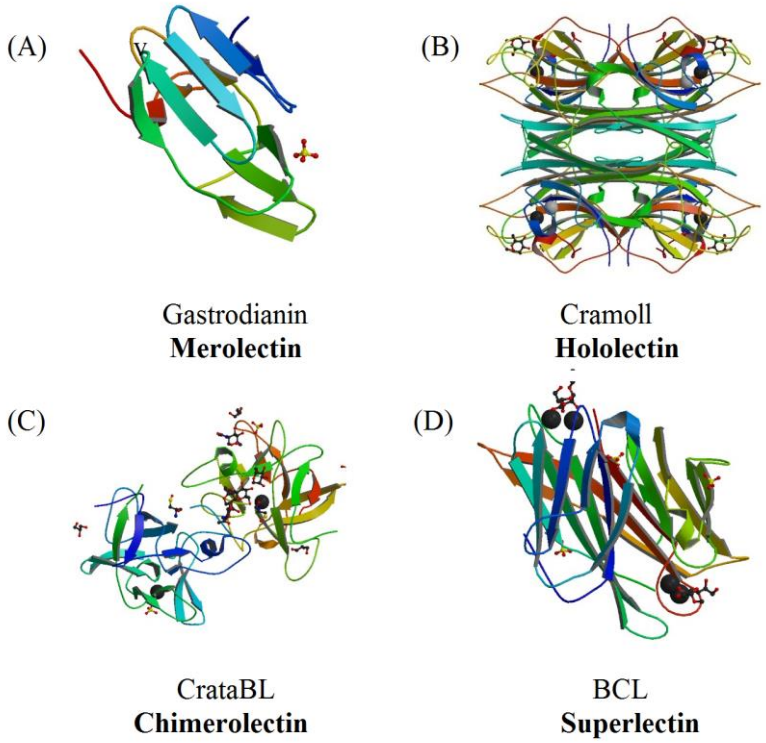

Figure 1: Representative structures of merolectins, hololectins, chimerolectins, superlectins. (A) Gratrodianin from *Gastrodia elata* (PDB: 1XD6); (B) Cramoll from *Cratylia mollis* (1MVQ); (C) CrataBL from *Crataeva tapia* (4IHZ); (D) BCL-A from *Burkholderia cenocepacia* (2XR4);

(A) (B)

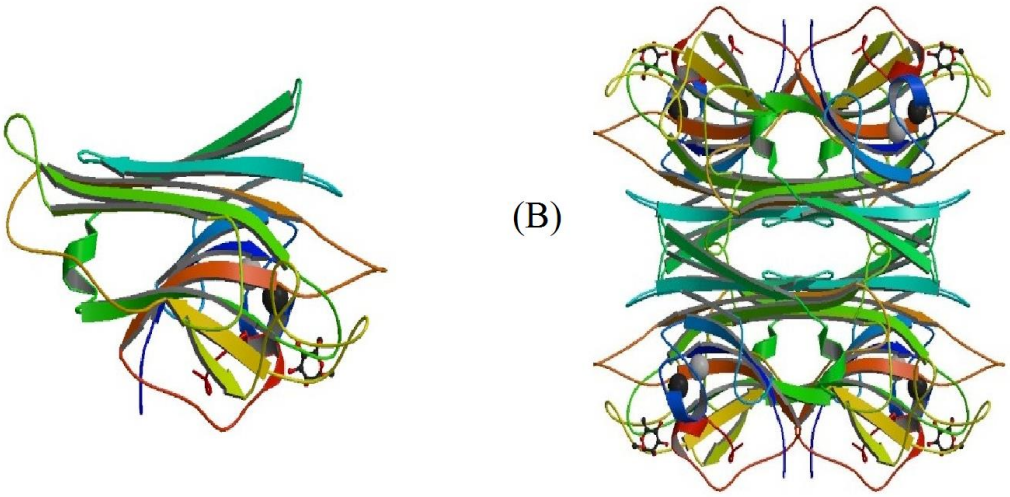

Figure 2: Tertiary structure of Cramoll 1 (PDB ID: 1MVQ). (A) Monomer and (B) Tetramer.

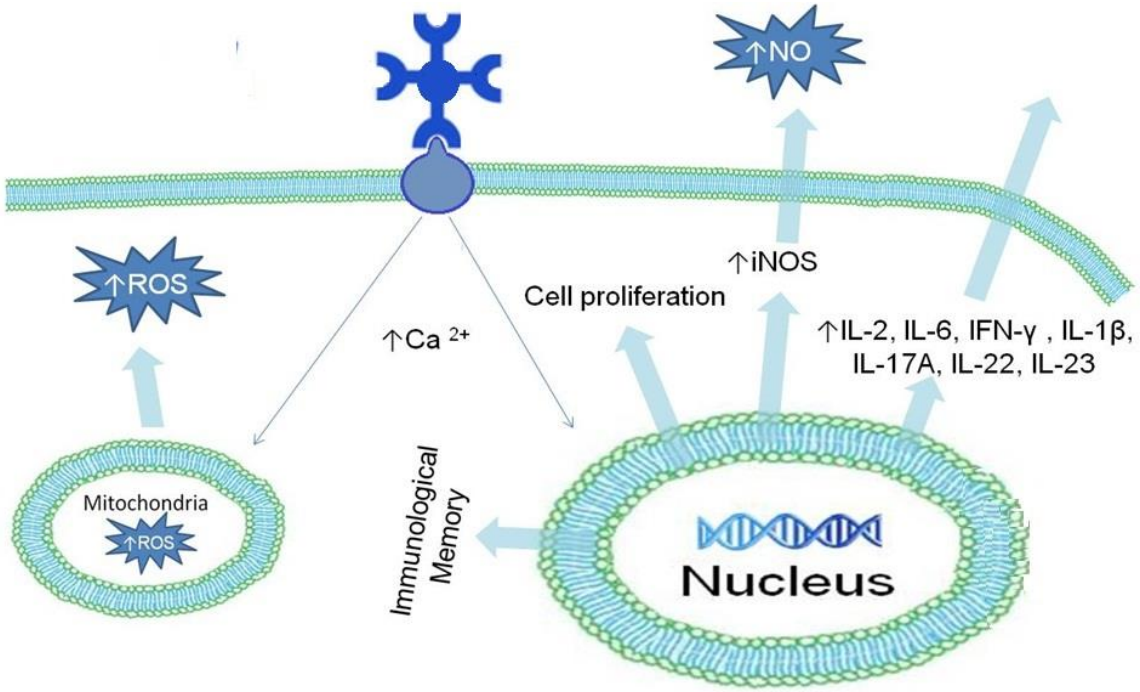

Figure 3: pCramoll as an imunnomudulatory agent

CAPÍTULO III

Immunomodulatory activity of pCramoll and rCramoll in *S. aureus*-infected and non-infected mice peritoneal macrophages.

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Immunomodulatory activity of pCramoll and rCramoll in *S. aureus*-infected and non-infected mice peritoneal macrophages.

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Immunomodulatory activity of pCramoll and rCramoll in *S. aureus*-infected and non-infected mice peritoneal macrophages.

In this study we evaluated the *in vitro* effects of pCramoll and rCramoll in the functions of mice peritoneal macrophages. The peritoneal macrophages from male Balb/c mice were cultivated (10^6 cells/mL) in 96 well plates and placed in CO_2 incubator for 24h. RPMI medium was removed and the macrophages were cultivated in the presence of lectins (0.625-10 µM), LPS (10 µg/mL) or without stimuli. After 24h, the nitric oxide (NO) production and cytotoxicity were checked by Griess and MTT assays, respectively. The Live/dead assay, Lysosomal activity (acridine orange), Superoxide production (Mitosox kit), Mitochondrial membrane potential (ΔΨm) (Rhodamine 123) and Phagocytosis ability (using live CFSE-stained *Staphylococcus aureus*) were also investigated by flow cytometry. Both lectins significantly enhanced NO and cytokines (IL-1β, IL-6, IFN-γ and TNF-α) production with the maximum expressions observed at 2.5 µM. These lectins were not cytotoxic as determined by MTT assay. However Live/Dead analysis revealed an increase of cell death and reduction of lysosomal activity in treated cells. The superoxide production was stimulated by both lectins, which was confirmed with loss of ΔΨm. pCramoll and rCramoll downregulated the induction of TNF-α and IL-6 and upregulated the expression of IL-1β, IFN-γ by *S. aureus* treated macrophages. *S. aureus* phagocytic activity of macrophages were enhanced in 27.1% and 22.47% by pCramoll and rCramoll, respectively. Our results showed that pCramoll and rCramoll have a great capacity to modulate immune response of macrophages, but the molecular mechanisms involved has yet to be determined.

Keywords: macrophages activation, innate immunity, phagocytic capacity.

Supported by: FACEPE, CNPq and CAPES.

1. Introduction

Dysfunctions of immune system are related to the pathologic mechanism of diverse disease such as cancer, burns, diabetes mellitus, infections and sepsis [1,2,3], which usually result in

slower wound healing and transient suppression of both innate and adaptive immune system [4,5], and a therapeutic intervention using immunostimulatory agents is essential [1,2,3]. Several medicines, chemical and natural products have been introduced as immunostimulants of both non-specific and specific immune responses. However, the side effects related to synthetic and their high cost have required the search for new bioactive compounds with lower or no side effects [6].

Lectins are proteins or glycoproteins that selectively recognize and reversibly bind to carbohydrates, without altering the covalent structure of binding-sites, through hydrogen bonds and hydrophobic interactions in the carbohydrate recognition domains [7]. They are found in of organisms from all kingdoms and due the specific recognition ability, they play a important role in the mediation of crucial cellular activities [8,9]. Several plant lectins have been demonstrated a high potential as immunomodulatory agents, improving activity of macrophages and others immune cells [10].

The glucose/mannose lectin isolated from *Cratylia mollis* seeds (Cramoll 1,4 or pCramoll) [11], a native plant of Brazilian Caatinga, have been demonstrated a remarkable biotechnological potenial [12,13,14,15]. Recently, the expression of functional recombinant Cramoll 1 (rCramoll) in *Escherichia coli* was reported by our group [16]. The reported antitumoral and healing activities of pCramoll may be due its *in viv*o interaction with macrophages [17,18,19]. In this context, the aim of this study was to evaluate the in vitro effects of pCramoll and rCramoll on *S. aureus*-infected and non-infected mice peritoneal macrophages.

2. Materials and methods

2.1. Lectins purification

pCramol and rCramoll1 were purified using the previously established protocols [15,16].

2.2. Mice and peritoneal macrophages obtaintion

Peritoneal macrophages were obtained from inbred strains of Balb/c mice of either sex at 8–10 weeks of age. Exudates cells were harvested from peritoneal lavage using 10 mL of ice-cold sterile phosphate-buffered saline (PBS) (pH 7.2). After centrifugation at 120 g for 5 minutes, the cell pellets were suspended in RPMI-1640 medium supplemented with bovine calf serum (10%; v/v), penicillin and streptomycin (100 U/mL) (all from Sigma-Aldrich).

2.3. Determination of Oxide Nitric Production and Cell Viability

For both assays, the macrophages ($1x10^6$ cells/mL) were seeded in a 96-well plate for 24 h. The cells were then treated with lectins (0.625–10 μM). After 24 h, the supernatant was used to determination of oxide nitric (NO) production, and the adherents cells to MTT assay, following the protocols in quadruplicates with two independents experiments, and results are expressed as mean ± standard deviation (S.D.).

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- **NO production:** the concentration of stable nitrite, the end product from NO generation by peritoneal macrophages was determined using Griess reaction. Briefly, the supernatant (100 μL) from each well was mixed with 100 μL of Griess reagent in a 96-well plate. After an incubation of 15 min at room temperature, the optical density was determined at 540 nm with a microplate reader (Benchmark plus, Bio-Rad, Califórnia, EUA). Nitrite content (μmol/10^6 cells) was quantified by extrapolation from sodium nitrite standard curve in each experiment.

- **MTT assay:** cell viability was evaluated using the MTT assay which measures the metabolic conversion of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt to the colored formazan dye. At the end of the incubation, the medium was removed and a MTT solution (5 mg/mL in RPMI) was added to the cells that were further incubated for 3 h. Afterwards the medium was removed and the intracellular formed formazan product was dissolved in DMSO. The optical density (OD) was measured at 595 nm. Cell viability was calculated in comparison to the OD obtained by untreated cells, considered as 100%. LPS (10 µg/mL) was used as positive control.

2.4. Multiplex cytokine analysis

Macrophages ($1x10^6$ cells/mL) were seeded in a 96-well plate for 24 h and treated with lectins (0.625–10 µM). After 24 h, the supernatant was used to quantification of inflammatory cytokines (IL-1β, IL-6, TNF-α and IFN-γ) using Millipore multiscreen 96-well filter plates (Bedford, MA, USA). The Multiple kit was obtained from Genese Produtos Diagnósticos Ltda (São Paulo, SP, Brazil). Assays were run according to the manufacturers' protocol. Data were collected using the Milliplex Analyser 200 version 2.3 (Luminex, Austin, USA).

2.5. Viability/Cytotoxicity assay

The effects of lectins on cell viability was confirmed using the LIVE/DEAD® Viability/Cytotoxicity kit for mammalian cells (Molecular probes) following manufacturer's instructions. Briefly, peritoneal macrophages ($1×10^6$/mL) were seeded on a 24-well plate for 24 h, and then were treated with both lectins (2.5 µM). H_2O_2 was used as positive control. After 24 h, the cells were trypsinized and washed with PBS (Phosphate buffered saline, pH 7.4). The pellet containing the cells was resuspended in 500 µL of PBS, mixed with 2 µL of

calcein AM (50 µM) and 4 µL of ethidium homodimer, and then incubated for 20 minutes at room temperature, protected from light. Afterward, the samples were analyzed by flow cytometry (FACSCalibur-BD, San Jose, CA) using the software CELLQuestProTM (BD Bioscience, San Jose, CA) for acquisition and analysis of data.

2.6. Acridine orange staining

After the treatment (as described above), the cells were incubated at 37 ºC in the presence of 1 mM acridine orange (Sigma) and washed in PBS (2X) after 30 min. The pellet containing the cells was resuspended in PBS and analyzed by flow cytometry for acquisition and analysis of data.

2.7. Mitochondrial superoxide production

The production of superoxide anion by mitochondria was measured with the probe MitoSOX^{TM} Red mitochondrial superoxide indicator (Molecular Probes). The macrophages were treated with both lectins (2.5 µM) for 30 minutes. After the trypsinization and washing (PBS), the MitoSOX™ reagent (1 mL/5 µM) was added and the samples were incubated for 10 minutes at 37 ˚C, protected from light. The cells were washed with warm PBS (three times) and analyzed by flow cytometry.

2.8. Determination of mitochondrial membrane potential (ΔΨm)

The uptake and retention of rhodamine123 (cationic fluorescent dye) was used to measure mitochondrial membrane potential (ΔΨm). The macrophages were prepared as described above. After 24 h of lectin treatment (at 2.5 µM), the cells was resuspended in 1 mL of fresh RPMI containing 2 µM rhodamine123 and incubated at 37 °C in a thermostatic bath for 20

min with gentle shaking. The stained cells were washed and then resuspended in 1 mL of PBS, and then analyzed by flow cytometry.

2.9. Phagocytosis of CFSE-staned *Staphylococcus aureus*

The overnight culture of *S. aureus* (UFPEDA 02) were centrifuged at 10,000 × g for 10 minutes, washed twice with PBS, and resuspended in the same buffer (optical density of 1.0 at 600 nm). This suspension was incubated at 37 °C for 10 min in the dark with 2.5 μM of 5(-and 6) carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, Oregon), diluted in the prewarmed PBS. After washing three times with PBS to remove excess probe, the bacteria were add to a 24-well plate containing peritoneal macrophages ($1x10^6$/mL) treated with both lectins (2.5 μM). The ratio of infection was 10:1 bacteria/macrophages. After 2 h, the adherent cells were trypsinized and then analyzed (minimum of 10.000 events/tube) through flow cytometry.

2.10. Cytokine release by *S. aureus* infected macrophages

The macrophages ($1x10^6$ cells/mL) were seeded in a 96-well plate for 24 h and infected with *S. aureus* at a ratio of 10:1 (bacteria/macrophages) in the presence or absence of both lectins (0.625–10 μM). After 24 h, the supernatant was used to quantification of inflammatory cytokines (IL-1β, IL-6, TNF-α and IFN-γ) (as described above).

2.11. Statistical analysis

Data is analyzed by one-way analysis of variance (ANOVA) and Turkey to determine the statistical significance using GraphPad Prism. A p-value of <0.05 was considered to be statistically significant. In all graphs, bars represent mean value ± standard deviation.

3. Results

3.1. Effects of pCramoll and rCramoll on cell viability and NO production

As shown in figure 1A, the viability of treated macrophages was similar to control cells at 0.625 μM of both lectins. However, the other concentrations were found to increase the viability ($p < 0.05$), as observed by enhance in MTT reduction (Fig. 1A). Additionally, treatment of Balb/c mouse peritoneal macrophages with different concentrations of pCramoll and rCramoll resulted in significant production of NO (Fig. 1B). Maximum NO release was observed with 1.25 and 2.5 μM of lectins ($p> 0.05$). pCramoll was able to enhance significantly the NO production in all tested concentration in relation to untreated cells. The effects of rCramoll were only significant at 1.25 and 2.5 μM.

3.2. pCramoll and rCramoll induce the production of IL-1β, IL-6, TNF-α and IFN-γ in BALB/c peritoneal macrophages

Treatment of BALB/c peritoneal macrophages with different doses of pCramoll and rCramoll resulted in a significant enhanced production of IL-1β, IL-6, TNF-α and IFN-γ in relation to untreated cells. The lectins did not display a dose-dependent effect. The most effective concentration to stimulate all tested cytokine was 2.5 μM for both lectins. In this concentration, production of IL-1β induced by pCramoll (148.42 ± 11.43 pg/mL) was 1.9 higher than rCramoll (77.58 ± 3.64 pg/mL) and 3.5 times higher than LPS (42.41 ± 3.66 pg/mL) ($p < 0.05$) (Fig. 2A). The stimulation of IL-6 induced by pCramoll (1495.18 ± 49.31 pg/mL) and LPS (1531.20 ± 108.62 pg/mL) were similar ($p > 0.05$), however, they induced 2 folds more production than rCramoll (737.58 ± 99.53 pg/mL) (Fig. 2B). On the other hand,

the supernatant levels of IFN-γ in the cells treated with pCramoll (121.69 ± 12.18 pg/mL) and rCramoll (77.31 ± 5.5 pg/mL) were 4.7 and 3.0 times higher than LPS (26 ± 5.66 pg/mL) ($p < 0.05$) (Fig. 2C). In relation to TNF-α, pCramoll showed the best induction power (199.69 ± 113.29 pg/mL) ($p < 0.05$), which was 2.8 and 1.6 more efficient than rCramoll (679.48 ± 26.55 pg/mL) and LPS (1214.60 ± 50.07 pg/mL) (Fig. 2D). We used the dose of 2.5 2.5 µM for adtionals test with both lectins.

3.3. The activation of macrophage by pCramoll and rCramoll is related with cell death.

The effects of pCramoll and rCramoll in the induction of macrophage cell death were confirmed using Live/Dead kit and acridine orange staining. These analyses revealed that both lectins induced a significant increment of cell death at 2.5 µM. In the Live/Dead assay the percentege of live cells was reduced to 59.46% and 63.30% for pCramoll and 28.46% to rCramoll (Fig 3). The intensity of acridine orange fluorescence was also attenuated in both treatments. The reductions were 31.24% to pCramoll and 28.46% to rCramoll (Fig 4).

3.4. pCramoll and rCramoll induced Superoxide Production and loss of mitochondrial membrane potential (ΔΨm)

The induction of Mitochondrial Superoxide by pCramoll and rCramoll was evaluated using MitoSOX fluorescence probe. As shown in figure 3, both lectins (at 2.5 µM) enhanced the superoxide production by the macrophages. pCramoll activated the process of ROS formation 3.6 folds more than the control cells, while rCramoll enhanced 2.2 folds (Fig 5). The high ROS production was confirmed by the detection of changes in ΔΨm induced by lectins of *C. mollis* (Fig 6). Treatment of macrophages with both lectins led to lowering of ΔΨm.

3.5. pCramoll and rCramoll improve *S. aureus* phagocytosis

After co-cultures of treated and untreated peritoneal macrophages with *S. aureus*, we performed a phagocytosis assay using flow cytometry. As can be seen in figure 7, both lectins (at 2.5 µM) stimulate the phagocytosis of *S. aureus*. pCramoll enhanced in 25.38% the phagocytic action, while rCramoll enhanced in 22.47%.

3.6. pCramoll and rCramoll exert immunomodulatory action on macrophages infected with *S. aureus*

Treatment of BALB/c peritoneal macrophages with pCramoll and rCramoll successfully reduced the release of TNF-α by *S. aureus* (592.39 ± 39.01 pg/mL), in all concentrations ($p < 0.05$) (Figure 8A). In relation to pCramoll, the best concentrations were 1.25 µM (192.96 ± 11.87 pg/mL), 2.5 µM (174.96 ± 10.74 pg/mL) and 5.0 µM (145.76 ± 8.97 pg/mL) ($p < 0.05$), which showed reductions of 67.48%, 70.59% and 75.44%, respectively. For rCramoll the best results were at 2.5 µM (194.40 ± 10.55 pg/mL; 67.24%) and 5.0 µM (182.82 ± 26.60 pg/m; 69.19%) ($p < 0.05$). In a similar way, pCramoll and rCramoll inhibited the release of IL-6 in all concentration (except to rCramoll at 10 µM) (Figure 8B). The maximum effects occurred at 2.5 µM for pCramoll (69.22± 3.27 pg/mL) and rCramoll (69.71 ± 3.30 pg/mL), where an inhibition of 60.01% and 59.7% were observed (respectively), although no statistical difference was found between these and the other concentrations ($p > 0.05$).

Conversely, the lectins stimulated the production of IL-1β and IFN-γ (Figures 8C e 8D). The stimulation of IL-1β increased significantly when *S. aureus* infected cells were treated with pCramoll at 5 µM (601.48± 28.29 pg/mL; 207.86%) and 10 µM (427.78 ± 164.60 pg/mg; 118.96%) and rCramoll at 5 µM (269.05 ± 31.89 pg/mL; 37.71%). In relation to IFN-γ, its expression was significantly stimulated with all doses of pCramoll and the higher

concentrations of rCramoll. For both lectins the best results were 51.69% (11.06 ± 0.48 pg/mL) and 31.34% (7.78 ± 0.33 pg/mL) when *S. aureus* treated macrophages were co-incubated with pCramoll and rCramoll at 2.5 5 µM, respectively.

3.7. Discussion

This study evaluated the effects of pCramoll and rCramoll on the viability and functions of mouse peritoneal macrophages. pCramoll has been showed a high biotechnology potential as antitumor, healing and immunomodulatory agent [11,12,14,17,18,19]. However, its effects on macrophages had not been studied.

Macrophages have been considered to be important effector cells that play crucial roles in host defense, healing and immune regulation [20,21]. Macrophages are phagocytic cells which are widely distributed throughout the body compartments, coordinating innate immunity and inflammatory responses [22].

The results presented in this paper clearly indicated that pCramoll and rCramoll exert significant immunomodulatory effects on macrophage functions. These lectins are effective activators of NO which is produced by arginine pathway and is a marker of macrophage activation [23]. Activated macrophages produce oxygen radicals and NO which have potent cytolytic action of macrophages against a range of tumors and microorganisms [24,25].

Cytokines are local mediators involved in almost all important biological processes including inflammation and cell growth, differentiation and activation [26,27]. Secretion of cytokines by activated macrophages is central to their immunoregulatory role and the orchestration of a robust immune response by macrophages [20]. In this study we showed that pCramoll and rCramoll enhanced IL-1β, IL-6, IFN-γ and TNF-α production in peritoneal macrophages.

These cytokines are closed related with the Th1 and Th17 pathway. The induction of these response has already established for pCramoll using splenocytes [11,12].

The oxidative burst is related to the generation of several reactive species, derived from oxygen and nitrogen, named ROS and RNS, respectively. The mitochondria constantly produce ROS when oxygen and oxidative substrates are available [28]. The superoxide anion is one of the main ROS and is very cytotoxic, playing a pivotal role for killing, oxidation and disintegration of infectious pathogens and tumors [29]. We found that the production of superoxide anion was improved by lectins, which may increase the ability of the phagocyte to destroy engulfed particles.

The effects of *C. mollis* isolectins in macrophages viability were evaluated primarily by MTT assay. The reduction of MTT to formazan is considered as index of metabolic viability [30]. Our results demonstrated that both lectins stimulate an increased reduction of MTT. However, the increment in MTT reduction is not a safe indicator of viability because the reduction may be caused by the action of superoxide anions produced in the mitochondria or in intracellular vesicles (endosomes and lysosomes) [31]. The high levels of reduced MTT could be explained by the increase of superoxide anion production induced by lectins. To preserve suitable function, cells should to stabilize their intracellular redox environment. Overproduction of ROS may lead to cell stimulation or death depending on its concentration [40]. In fact, we found that lectins at 2.5 µM induced macrophage cell death as revealed by analysis using LIVE/DEAD kit and acridine orange. Several studies have been correlated the process of macrophage activation with cell death through apoptosis pathway [33,34].

The ability to phagocytose *S. aureus* was also enhanced in macrophages treated with both lectins. In mammals, phagocytosis is a crucial defense mechanism for protection against pathogen invasions, and apoptotic cell scavenging. Phagocytosis is generally mediated by

membrane receptors present on immune cells. These receptors bind ligands present on the particle surface, promoting the cytoskeletal and membrane remodeling necessary to internalize the particle [35]. The lectins may act increasing the expression of these receptors.

Finally, we showed the effects of pCramoll and rCramoll on the cytokine profile of macrophages infected by *S. aureus*. This bacterium has been considered the most important human pathogen in the twenty-first century, due to its exceptional capacity to acquire resistance to antibiotics [36, 37]. The pro-inflammatory cytokines (including IL-1β, IL-6, IFN-γ and TNF-α) is secreted by macrophages in response to *S. aureus* and they play dual role in its pathogenicity [37,38]. *S. aureus* infections often are associated with severe and prolonged host inflammatory responses [39]. pCramoll and rCramoll downregulated the induction of TNF-α and IL-6 and upregulated the expression of IL-1β, IFN-γ by *S. aureus* treated macrophages. These cytokines are strongly related with severity of *S. aureus* sepsis [40, 41, 42, 43]. Although IL-1β and IFN-γ are pro-inflamatory cytokines, their suppression by *S. aureus* has been described as a mechanism to evade the host defense [37, 44, 45].

In summary, the findings of the present study suggest that pCramoll and rCramoll are potent immunomodulatory proteins on macrophages through an enhancement of reactive species production and cytokine release. Together with the others finding of our group, the results suggest that these lectins be useful as a therapeutic agent for the treatment of the immune deficiency diseases.

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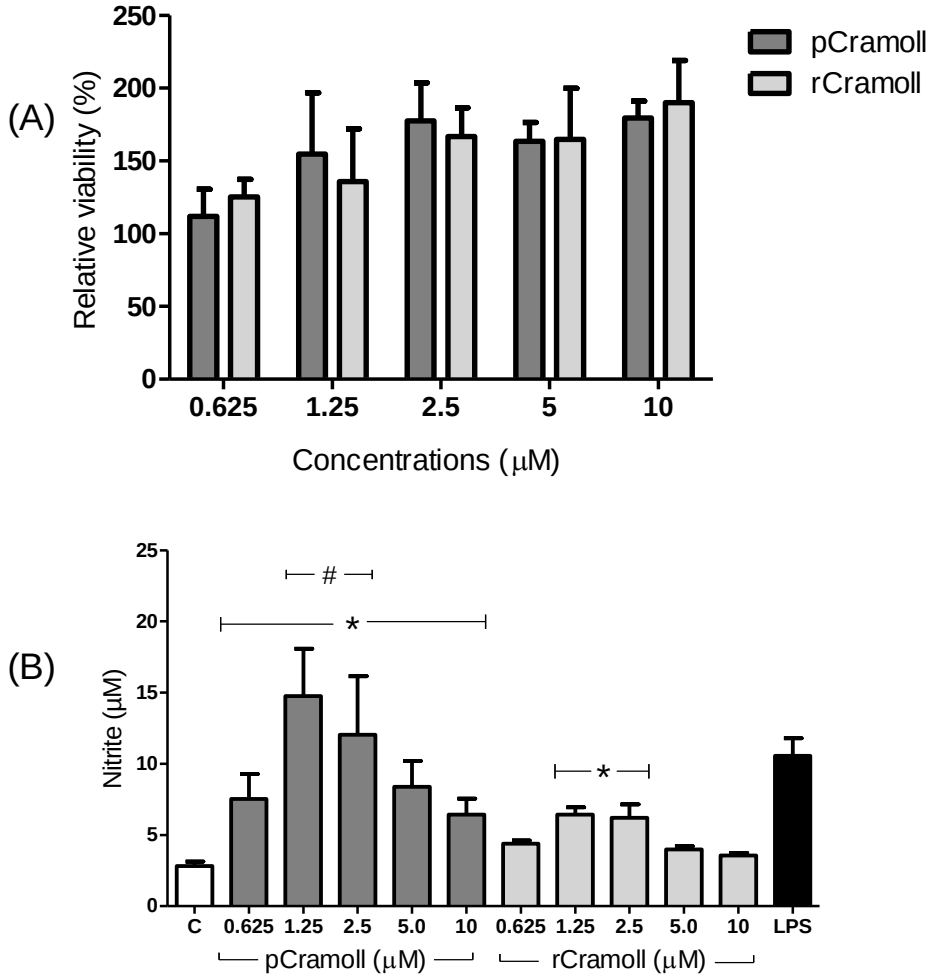

Figure 1: Effect of pCramoll and rCramoll on macrophages viability (A) and NO production. (*) Significant differences in relation to control; (#) Significant differences between others concentrations ($p<0.05$).

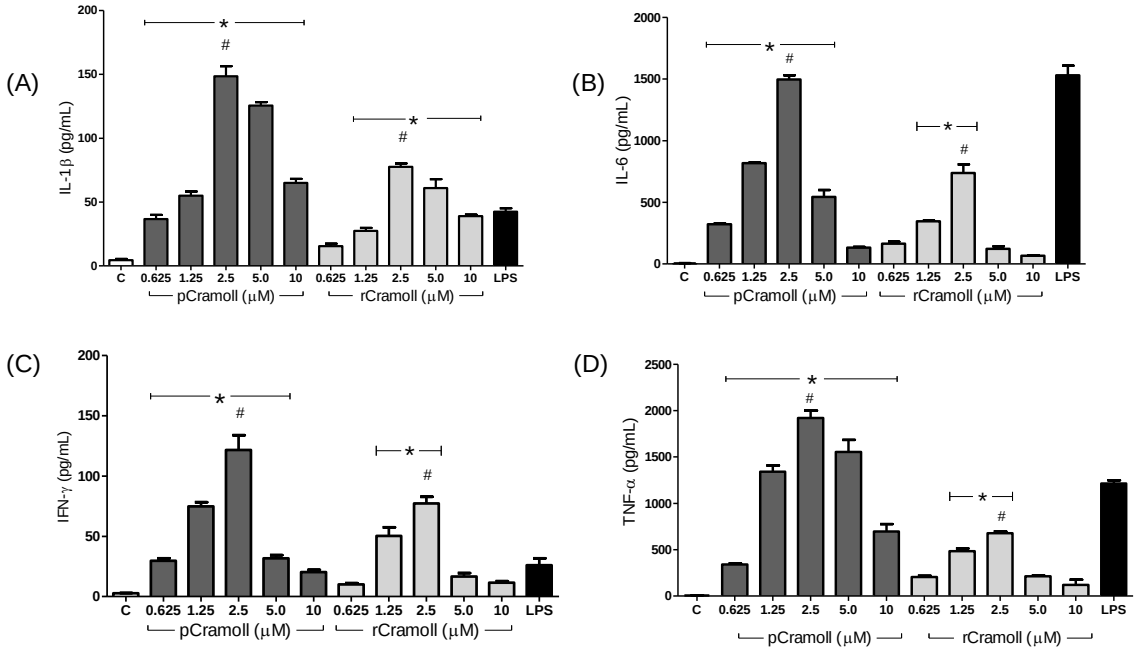

Figure 2: Effect of pCramoll and rCramoll on production of IL-1β (A), IL-6 (B), IFN-γ (C) and TNF-α (D) cytokines. (*) Significant differences in relation to control; (#) Significant differences between others concentrations.

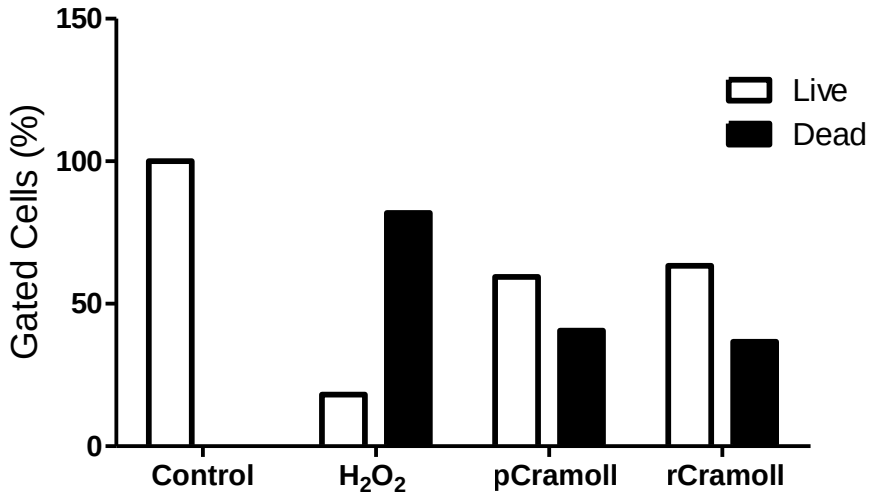

Figure 3: Effect of pCramoll and rCramoll on macrophages viability using Live/Dead kit.

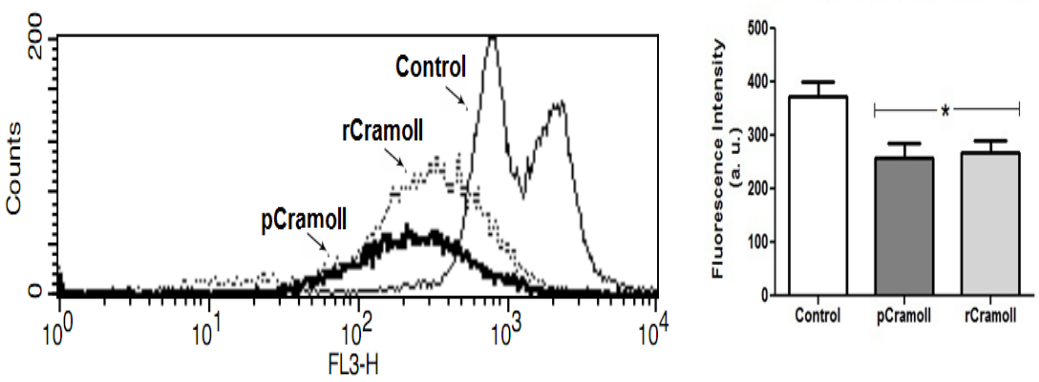

Figure 4: Effect of pCramoll and rCramoll on Lysosomal viability of peritoneal macrophages. (*) Significant differences in relation to control.

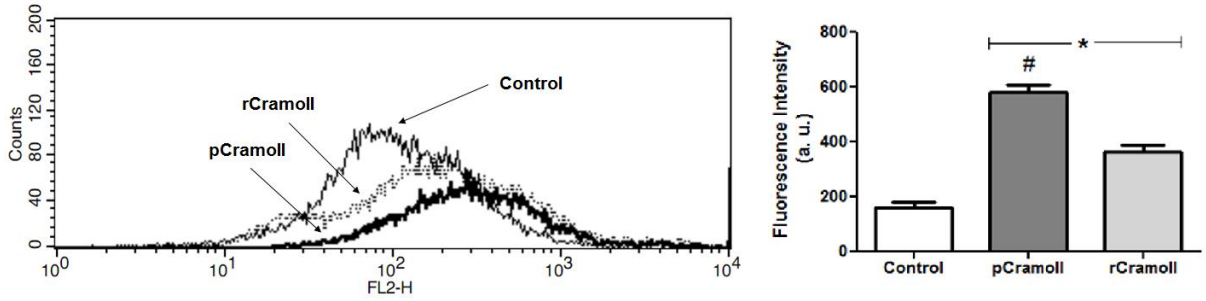

Figure 5: Effect of pCramoll and rCramoll on the accumulation of mitochondrial superoxide anion. (*) Significant differences in relation to control; (#) Significant differences between others concentrations.

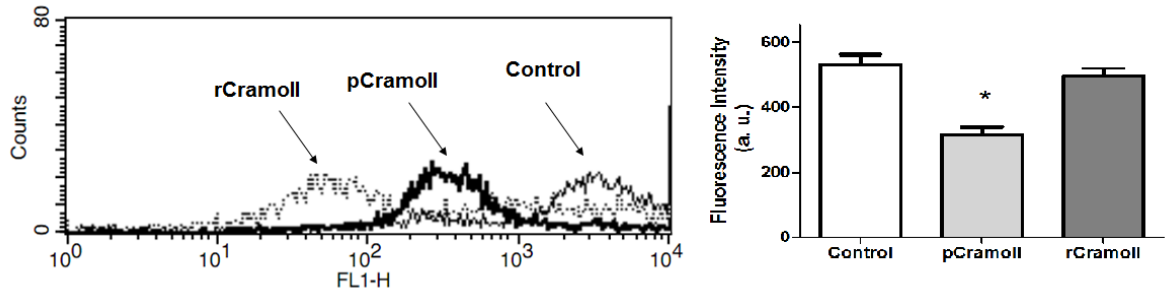

Figure 6: Effect of pCramoll and rCramoll on ΔΨm of peritoneal mouse macrophages. (*) Significant differences in relation to control.

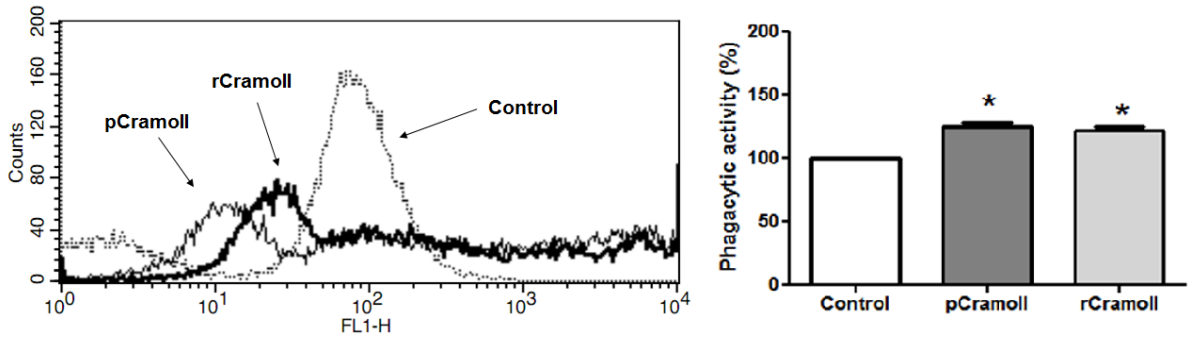

Figure 7: Effect of pCramoll and rCramoll on phagocytic ability of peritoneal mouse macrophages. (*) Significant differences in relation to control.

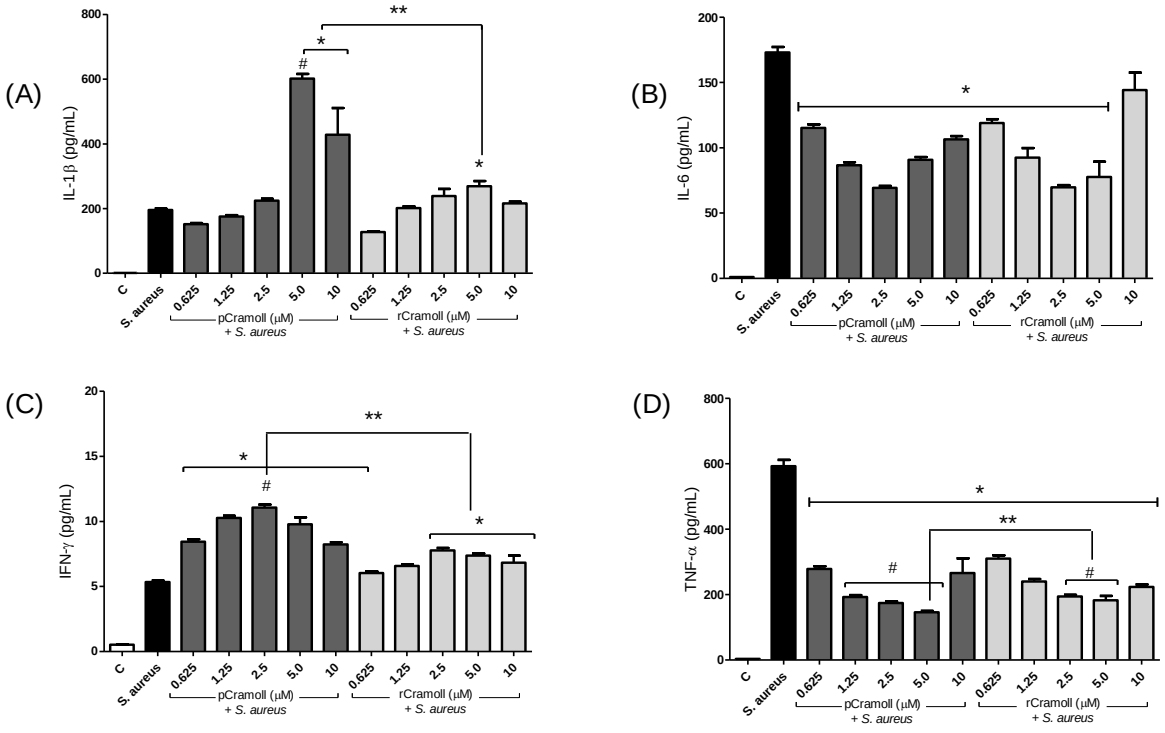

Figure 8: Effect of pCramoll and rCramoll on cytokine release by peritoneal macrophages infected with *S. aureus*. (A) IL-1 β, (B) IL-6, (C) IFN-γ and (D) TNF-α.

CAPÍTULO IV

Protective effects of pCramoll and rCramoll against hydrogen peroxide induced apoptosis in Vero cells

(Artigo a ser submetido à Revista "Phytomedicine")

Protective effects of pCramoll and rCramoll against hydrogen peroxide induced apoptosis in Vero cells

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Protective effects of pCramoll and rCramoll against hydrogen peroxide induced apoptosis in Vero cells

Abstract

Oxidative stress plays an important role in the induction of apoptosis and is related with various pathologic disorders. We evaluated the protective effect of native Cramoll 1,4 (pCramoll) and recombinant Cramoll 1 (rCramoll) against H_2O_2-induced dysfunction in Vero cells (monkey fibroblast). Both lectins significantly attenuated the H_2O_2-induced cytotoxicity in a dose-dependent way, the maximum protective effects were 96.85 ± 15.59% (rCramoll) and 59.48 ± 23.44% (pCramoll). The Live/Dead analysis showed a reduction in apoptotic cells from 65.04 ± 3.29% (H_2O_2) to 39.77 ± 2.93% (pCramoll) and 13.90 ± 9.01% (rCramoll). The deleterious effects of H_2O_2 on cell proliferation were reduced in 10.83% (pCramoll) and 24.17% (rCramoll). The lectins attenuated the excessive superoxide production, the collapse of ΔΨm, lysosomal and DNA damage that occurred in Vero cells exposed to H_2O_2. In conclusion, the results suggested that pCramoll and rCramoll blocked H_2O_2-induced apoptosis through decreasing reactive oxygen species, restoring ΔΨm, preventing lysosomal damage and DNA fragmentation, and thus promoting cell survival and proliferation.

Keywords: cell death, oxidative stress, lectins, protective effects.

1. Introduction

Oxidative stress is characterized as an imbalance in the redox status of the cell, and has been implicated in a range of age-associated and neurodegenerative diseases, such as aging, cancer, diabetes, Alzheimer's disease and Parkinson's disease (Valko et al., 2007; Rains and Jain, 2011). The reactive oxygen species (ROS) are oxygen-containing molecules that are constantly produced in cells through normal metabolic processes. ROS are known to be responsible for cell toxicity when the generation of ROS exceeds the clearance capacity of the cellular antioxidants system (Halliwell, 2012). They include superoxide anions (O_2^-) and hydroxyl radicals ($OH^\bullet$) and H_2O_2 (nonradical derivative of oxygen). H_2O_2 is thought to be the major precursor of highly reactive free radicals, such as hydroxyl radicals via Fenton's reaction (Halliwell, 2011).

ROS may damage all of the major classes of biological macromolecules in the cells through direct oxidation of lipids, proteins, and nucleic acids, thereby disrupting cellular function and integrity, which leads to cell death (Valko et al., 2007; Halliwell, 2011). Nowadays, the search for natural products that attenuate the effects produced by oxidant agents is greatly increased (Godkar et al., 2006; Da Silva et al., 2011).

Lectins are a heterogeneous group of non-immune proteins and glycoproteins that specifically and reversibly bind with high affinity to carbohydrates without altering the covalent structure of any of the recognized ligands. Lectins can agglutinate various cells through binding to cell surface glycoconjugates. They are distributed in plants, animals and microorganisms (Sharon, 2007; Correia and Coelho, 1995).

Cratylia mollis Mart (Fabaceae family) is native leguminous forage from the Semiarid Region of the Northeast of Brazil (Caatinga biome). This plant is popularly known as camaratu bean. Four multiple molecular forms of lectin have been purified from this plant: Cramoll -1, -2, -3, -4. They exhibit specificity to different carbohydrates. The isoforms 1, 2 and 4 are non-glycosilated and glucose/mannose specific proteins; and Cramoll 3 is a galactose specific glycoprotein (Paiva and Coelho, 1992; Correia and Coelho, 1995).

Cramoll 1,4 (preparation containing isolectins 1 and 4; pCramoll) is isolated in a similar way to Concanavalin A (Con A), a well-known lectin from *Canavalia ensiformis* seeds (Correia and Coelho, 1995). This preparation has been used as immunomodulatory (Melo et al., 2010), antitumoral (Andrade et al., 2004), antiparasitic (Fernandes et al., 2010) and healing agent (Melo et al., 2011; Pereira et al., 2012). Biotechnological applications also involve the characterization of human cancerous tissues (Lima et al., 2010), affinity matrix to purify proteins (Silva et al., 2011), sensor to detection of dengue virus serotypes (Oliveira et al., 2011).

Cramoll 1 is a major isolectin which consists of 236 residues with 82% identity with Con A. Cramoll 1 terciary fold was determined by X-ray crystallography at 1.77 Å and revealed three β-sheets connected by loops, known as the jellyroll domain (this topological architecture is essentially identical to Con A) (Souza et al., 2003). Recently, the expression of functional recombinant Cramoll 1 (rCramoll) in *Escherichia coli* was reported by our group (Varejão et al., 2010).

In this study the cytoprotective action of pCramoll and rCramoll against H_2O_2-induced dysfunction in Vero cells (monkey fibroblast) were investigated. We found that the anti-

apoptotic effects are mediated by altering the superoxide species production, inhibiting the mitochondrial, lysosomal dysfunction and DNA damage.

2. Materials and methods

2.1. Lectins purification

Cramol 1,4 (pCramoll) and rCramoll were purified using the previously established protocols (Correia and Coelho, 1995; Varejão et al., 2010).

2.2. Cell culture

The monkey kidney fibroblast line (Vero) was maintained at 37 ºC in an incubator with humidified atmosphere of 5% CO_2. Cells were cultured in RPMI medium containing 10% heat-inactivated fetal calf serum, penicillin and streptomycin (100 U/mL), all from Sigma-Aldrich.

2.3. MTT assay

Cell viability was evaluated using the MTT assay which measures the metabolic conversion of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) salt to the colored formazan dye. Vero cells (1×10^5/mL) were incubated in a 96-well plate in quadruplicate for 24 h. Cells were then treated with lectins (0.625–10 μM) for 30 min and subsequently with H_2O_2 (1 mM) for 24 h. At the end of the incubation, the medium was removed and a MTT solution (5 mg/mL in RPMI) was added to the cells that were further incubated for 3 h. Afterwards the medium was removed and the intracellular formed formazan product was dissolved in DMSO. The optical density (OD) was measured at 595 nm in a microplate reader (Benchmark plus, Bio-Rad, Califórnia, EUA). Cell viability was calculated in comparison to the OD obtained by control cell, considered as 100%.

2.4. Viability/Cytotoxicity assay

The cytoprotective effect of lectins was confirmed using the LIVE/DEAD® Viability/Cytotoxicity kit for mammalian cells (Molecular probes) following manufacturer's instructions. Briefly, the Vero cells (1×10^5/mL) were incubated on a 24-well plate for 24 h , and then were pretreated with both lectins (10 μM) for 30 min, with the oxidative stress being

induced by the addition of H_2O_2 (100 µL at 1 mM). After 24 h the cells were trypsinized and centrifuged at 3000 g for 5 min, and washed with PBS. The pellet containing the cells was resuspended in 500 µL of PBS, mixed with 2 µL of calcein AM (50 µM) and 4 µL of ethidium homodimer, and then incubated for 20 minutes at room temperature, protected from light. Afterward, the samples were then analyzed (minimum of 10.000 events/tube) through flow cytometry (FACSCalibur-BD, San Jose, CA) using the software CELLQuestPro™ (BD Bioscience, San Jose, CA) for acquisition and analysis of data.

2.5. Mitochondrial superoxide production

The production of superoxide anion inside the mitochondria was measured with the probe MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes). The cells were pretreated with lectins and treated with H_2O_2 for 30 min. After the trypsinization and washing, the MitoSOX™ reagent (1 mL/5 µM) was added and the samples were incubated for 10 minutes at 37 ˚C, protected from light. The cells were washed with warm buffer (three times) and analyzed by flow cytometry (FACSCalibur-BD, San Jose, CA) using the software CELLQuestPro™ (BD Bioscience, San Jose, CA) for acquisition and analysis of data.

2.6. Acridine orange staining

The Cells were prepared as described above and incubated at 37 ºC in the presence of 1 mM acridine orange (Sigma) and washed in PBS (2X) after 30 min. The pellet containing the cells was resuspended in PBS and analyzed by flow cytometry (FACSCalibur-BD, San Jose, CA) using the software CELLQuestPro™ (BD Bioscience, San Jose, CA) for acquisition and analysis of data.

2.7. Determination of mitochondrial membrane potential (ΔΨm)

The uptake and retention of rhodamine123 (cationic fluorescent dye) was used to measure mitochondrial membrane potential (ΔΨm). Treated cells were trypsinized and washed twice with PBS, and centrifuged at 300 ×g for 10 min. The cell pellet was then resuspended in 1 ml of fresh incubation medium containing 2 µM rhodamine123 and incubated at 37 °C in a thermostatic bath for 20 min with gentle shaking. The stained Vero Cells were washed and then resuspended in 1 mL of PBS. The variation index (VI) was calculated following this formula:

Variation Index (VI) = $(FI_c - FI_s)/FI_c$

Where FI_c is the mean of fluorescent intensity of control and FI_s the mean of treated cells.

2.8. CFSE proliferation assay

The carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assay is based in the ability of CFSE probes to bind to lipids within the cell membrane. After each mitosis, the fluorescence intensity decreases to approximately half. In this assay the Vero Cells (1×10^5/mL) were stained with CFSE (2.5 μM) and incubated at 37 ºC for 10 min. The reaction was stopped by the addition of cold RPMI. The cells were centrifuged (300 g for 10 min) and the cell pellet was washed with PBS in the same conditions. Afterwards, the cells were resuspended in RPMI and pretreated with lectins, following the addition of H_2O_2 as described. The proliferation indexes were determined after 72 h by flow cytometry (FACSCalibur-BD, San Jose, CA) using the software CELLQuestPro™ (BD Bioscience, San Jose, CA) for acquisition and the FlowJo software (Tree Star, Ashland OR) for analysis of data.

2.9. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Click-iT assay

TUNEL assay was performed using the Click-iT TUNEL Alexa Fluor 488 (Invitrogen) following the manufacturer's protocol. Briefly, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized (0.25% Triton® X-100 in PBS) for 20 minutes at room temperature. Then they were washed twice with deionized water. The TdT reaction cocktail was added and incubated for 1 h, followed by 30 min incubation with the Click-iT reaction cocktail. The stained Vero Cells were washed, resuspended and the fluorescence intensity was analyzed by flow cytometry (FACSCalibur-BD, San Jose, CA) using the software CELLQuestPro™ (BD Bioscience, San Jose, CA) for acquisition and analysis of data.

2.10. DNA fragmentation analysis

After the cells were treated as described for flow cytometry experiments (section 2.4), the genomic DNA was isolated from each sample using the AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, Inc., Union City, USA) following the

manufacturer's instruction. The DNA samples analyzed by electrophoresis on a 1.2% agarose gel and stained with SYBR® Green.

2.11. Spectroscopic Measurements

The effects of H_2O_2 treatment on lectin tertiary structure were evaluated by intrinsic fluorescence using a Jasco FP-6300 spectrofluorometer (Jasco, Tokyo, Japan). Both lectins (10 μM) were incubated with H_2O_2 (1 mM). The fluorescence emission of tryptophan was measured at 25 ºC in a rectangular quartz cuvette with a 1 cm path length, the excitation was at 295 nm and emission was recorded from 305-450 nm using 5 nm band pass filters (for both).

2.12. Statistical analysis

Data is analyzed by one-way analysis of variance (ANOVA) and Turkey test to determine the statistical significance. A p-value of <0.05 was considered to be statistically significant.

3. Results

3.1. pCramoll and rCramoll attenuated the H_2O_2-induced cytotoxicity

The cytoprotective effects of lectins against H_2O_2-induced cell death were evaluated by MTT assay, which measures the loss of metabolic activity of cells and is an early indicator for cell death. Both lectins inhibited the cytotoxicity in a dose-dependent way (Fig. 1). pCramoll showed maximum protective effects at 5 μM (48.36 ± 8.12%) and 10 μM (59.48 ± 23.44%), the difference between these concentrations was not statistically significant ($p>0.05$). rCramoll induced more protection at 10 μM (96.85 ± 15.59%) ($p < 0.05$). It is important to note that rCramoll presented better results than the pCramoll at 5 and 10 μM ($p < 0.05$). The best results were found for 10 μM for both lectins, for this reason we chose this concentration to investigate the signaling involved in the Cramoll-mediated anti-cell death effects.

The anti-cytotoxicity action was confirmed by Live/Dead assay, a flow cytometry analysis that uses the intracellular esterase activity (calcein AM) and plasma membrane integrity (EthD-1) as cell viability markers. The viable cells are positive for calcein and the death cells are positive for ethidium homodimer. The Fig. 2 shows the increase of death in Vero Cells treated with H_2O_2 alone (65.04 ± 3.29%) in relation to control cells. However, two lectins

reduced the rates of death cells to 39.77 ± 2.93% for pCramoll, and 13.90 ± 9.01% for rCramoll.

3.2. pCramoll and rCramoll inhibit the deleterious effects of H_2O_2 on cell proliferation

To evaluated the cell proliferation was used a FACS assay using CFSE staining followed for FlowJo analysis. The H_2O_2-treated cells showed 66.67% of control proliferation index after 72 h. The lectins were able to enhance the proliferation index in the presence of H_2O_2 (10.83% for pCramoll and 24.17% for rCramoll).

3.3. pCramoll and rCramoll blocked the H_2O_2-elicited Mitochondrial ROS formation

As shown in Fig. 3, after H_2O_2 exposition the ROS generation increased more than 52 folds as compared to control. Pretreatment of the cells with 10 μM of lectins for 30 minutes induced significantly attenuation of mitochondrial ROS. The reductions (in relation H_2O_2) were 20.31 ± 7.82% and 39.84 ± 2.36%, to pCramoll and rCramoll, respectively ($p< 0.05$).

3.4. pCramoll and rCramoll restore the mitochondrial membrane potential (ΔΨm)

The alterations on mitochondrial functions were evaluated by the ΔΨm variation using rhodamine123. The H_2O_2-treated cell exhibited a loss of ΔΨm in relation to control cells (depolarization, VI: -1.47 ± 0.18). The lectins pretreatments were able to restore the loss of ΔΨm induced by H_2O_2, inhibiting the depolarization (pCramoll; VI: -0.34 ± 0.03; and rCramoll: -0.05 ± 0.06) ($p < 0.05$) (Fig. 4).

3.5. pCramoll and rCramoll protect the lysosomal damage induced by H_2O_2

In order to evaluated the lysosomal integrity the acridine orange fluorescence was checked by FACS analysis. The control cells showed a very high AO fluorescence intensity, confirm that these cells had intact lysosomes, while as the H_2O_2 reduced 85.66 ± 0.3% of the fluorescence intensity. The lectins showed great potential to inhibit the lysosomal damage, the treated cells showed 64.37 ± 7.98% (pCramoll) and 75.17 ± 7.91% (rCramoll) in relation to the control ($p < 0.05$) (Fig. 5).

3.6. pCramoll and rCramoll prevent the fragmentation of nuclear DNA

The formation of DNA ladders is a consequence of nucleosomal-sized fragmentation and is a conventional event in apoptotic process. The TUNEL assay was carried out to detect the extension of DNA degradation in apoptotic cells. DNA damage was quantified in retaion to control cells. H_2O_2 dramatically induced DNA fragmentation (170.41 ± 42.10%), which could be inhibited by pCramoll (12.01 ± 7.10%) and rCramoll (32.90 ± 18.00%) ($p < 0.05$) (Fig. 6A). The induction of DNA ladders by H_2O_2 was also evaluated by electrophoresis. This effect was partially inhibited by both lectins, revealing that they have ability to protect the apoptotic changes in DNA at the molecular level (Fig 6B). These results are compatible with the TUNEL analysis.

3.7. H_2O_2 did not alter tryptophan fluorescence emission and hemagglutination ability of pCramoll and rCramoll

Conformal stability of pCramoll and rCramoll (10 μM) after 24 hours of H_2O_2 (1 mM) exposition was investigated. Cramoll has four tryptophan residues located in the protein core (Souza et al., 2003). No significant changes in intrinsic fluorescence were detected. Similary, the hemagglutination ability was not modified (Dates not show).

4. Discussion

In this work we reported the protective effects of native and recombinant Cramoll against cell death induced by H_2O_2, which is a representative member of ROS compounds (Halliwell, 2012). The induction of apoptosis by H_2O_2 has been reported in various works with different cell types (Heo et al., 2008; Okello et al., 2011). The severe damage caused by H_2O_2 is related with its capacity to crosses the cellular membranes and react with intracellular metal ions, yielding higly toxic hydroxyl radicals, which is able to cause serious damage in macromolecules, including DNA, proteins, and lipids, and ultimately damage to whole cell (Halliwell, 2011). In fact, several diseases had the oxidative stress as the main trigger (cancer, neurodegenerative, and cardiovascular disorders) (Valko et al., 2007; Rains and Jain, 2011).

H_2O_2 induced significant toxicity, reducing the cell viability to 34.96% (Live/Dead kit). The pretreatment with different doses of lectins showed a significant cytoprotective effect. The cell viability reached the maximum at the concentration of 10 μM (60.23 % for pCramoll and 86.09% for rCramoll) and we used this dose to additional tests. As an apoptotic agent, H_2O_2

also suppress the cell proliferation. The deleterious effects of H_2O_2 on cell proliferation were also attenuated by both forms of Cramoll. We observed no changes in tryptophan fluorescence intensity in both lectins after the H_2O_2 treatment. Other methods should be applied to evaluate the effects of H_2O_2 at structure of pCramoll and rCramoll.

The secondary production of others mitochondrial ROS (such as superoxide) induced by H_2O_2 is one potential mechanism of cell damage (Giorgio et al., 2007). In this study, we measured the superoxide generation using MitoSOX^{TM} probe. The H_2O_2 treated-cells had the superoxide contents increased significantly ($P < 0.05$), which is attenuated in two lectin pretreated groups. These results indicate that the anti-apoptotic effects of both lectins are related with the inhibition of mitochondrial ROS production.

Mitochondria play a critical role in maintaining the physiology of the cell and its dysfunction is an important pathway in the apoptotic cascade (Hamanaka and Chandel et al., 2010). It is well known that several pro-apoptotic mediators (cytochrome c, AIF, Smac/DIABLO, and endoG) are located in the mitochondria. These factors are released into the cytosol and they activate diverse enzymatic reactions that lead to the specific degradation of proteins and DNA during apoptosis (Danial and Korsmeyer, 2004). The conservation of $\Delta\Psi m$ is essential for mitochondrial integrity and functions (Ricci et al., 2004). H_2O_2 induces mitochondrial dysfunction by the loss of $\Delta\Psi m$ (Gong et al., 2008). Our results showed that native and recombinant Cramoll are able to go through the mitochondria pathway to inhibit apoptosis.

Lysosomes have been mainly related with necrotic and autophagic cell death, and their function in apoptosis being restricted to the digestion of engulfed bodies (Repnik et al., 2010). In fact the studies of oxidative-stress-induced apoptosis are focused on alterations of mitochondrial bioenergetic and pro-apoptotic compounds release. However, evidences have been confirmed the role of lysosomes in the initiating phase of apoptosis (Kirkegaard and Jäättelä, 2009). Differents kinds of hydrolytic enzymes (proteases, lipases, nucleases, glycosidases, phospholipases, phosphatases and sulfatases) are present in this organelle and its damage in response to toxic agents causes the release of these enzymes to cytoplasm and reduces the pH of the cytosol and causing acidosis (Schröder et al., 2010). The lysosomal proteases such as Cathepsins family (A, B, D, L) are potent activators of apoptotic effectors, thereby triggering apoptosis (Repnik et al., 2012). The lectins are able to prevent lysosomal damage induced by H_2O_2 and promoting cell survival.

Among apoptotic events, the activation of endonucleases leading to the genomic DNA fragmentation is one of the most characteristic (Yamada et al., 2011). The protective actions of lectins against DNA ladder formation induced by H_2O_2 (a sign of fragmentation of nuclear DNA into oligonucleosomal subunits were confirmed by agarose gel electrophoresis and TUNEL analysis. In both assays, we detect a substantial inhibition DNA laddering in pretreated Vero cells.

In summary, pCramoll and rCramoll could ameliorate H_2O_2 -induced oxidative stress and apoptosis in Vero cells. The protective effects were related to the inhibition of mitochondrial superoxide generation, loss of $\Delta\Psi m$, lysosomal damage and DNA fragmentation, promoting cell survival and proliferation. Further studies are required to investigate the detailed molecular mechanisms involved on preventing oxidative stress.

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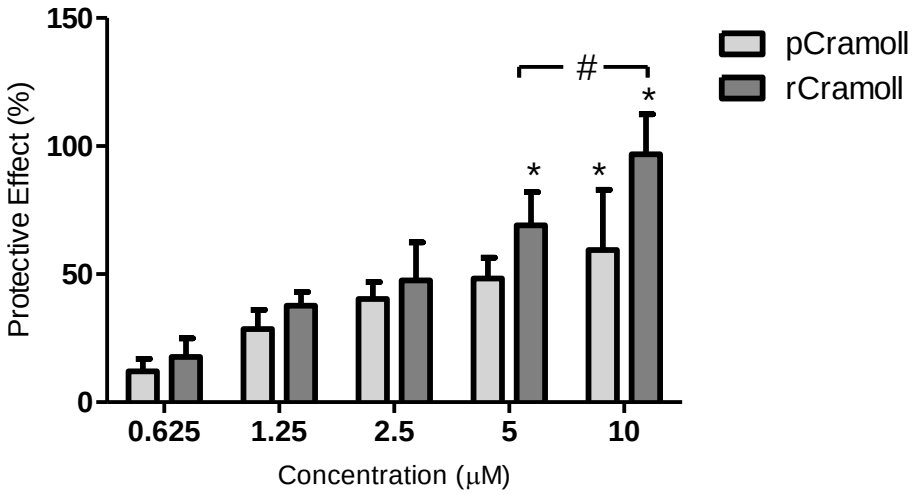

Figure 1: Cytoprotective effects of pCramoll and rCramoll determined by MTT assay. (*) Significant differences between others concentrations. (#) Significant differences between lectins.

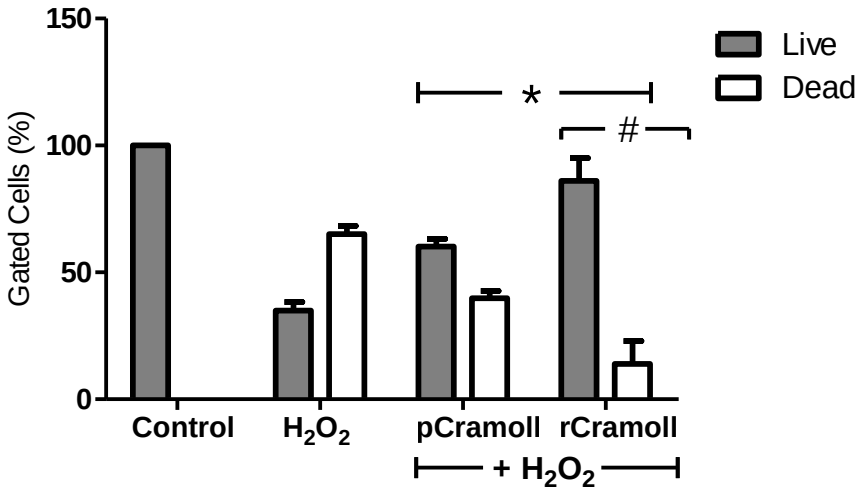

Figure 2: Cytoprotective effects of pCramoll and rCramoll determined by Live/Dead kit using Flow Cytometry. (*) Significant differences in relation to H_2O_2. (#) Significant differences between lectins.

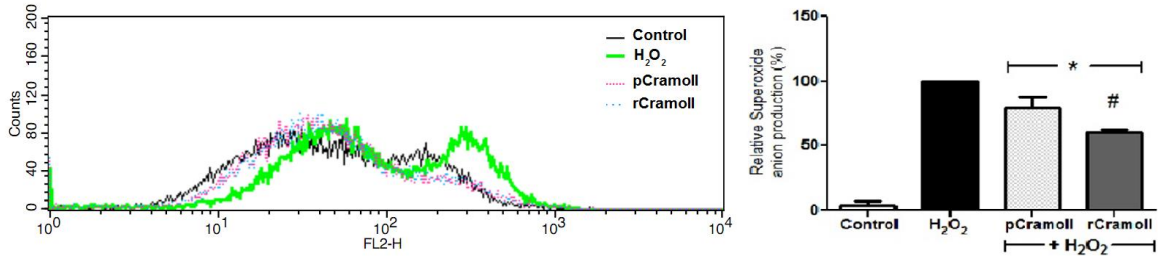

Figure 3: Effect of pCramoll and rCramoll on the H_2O_2-induced accumulation of mitochondrial superoxide anion in Vero Cells. (*) Significant differences in relation to H_2O_2. (#) Significant differences between lectins.

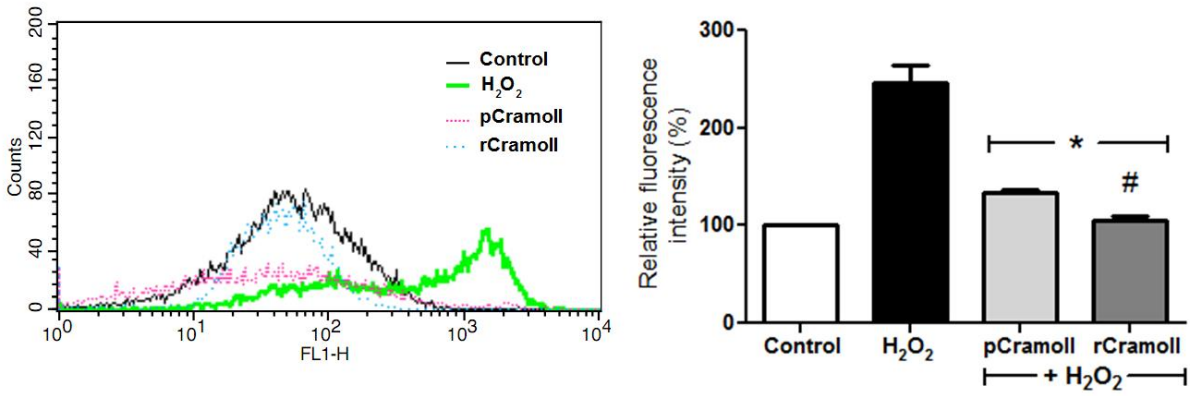

Figure 4: Effect of pCramoll and rCramoll on H_2O_2-induced loss of ΔΨm in Vero Cells. (*) Significant differences in relation to H_2O_2. (#) Significant differences between lectins.

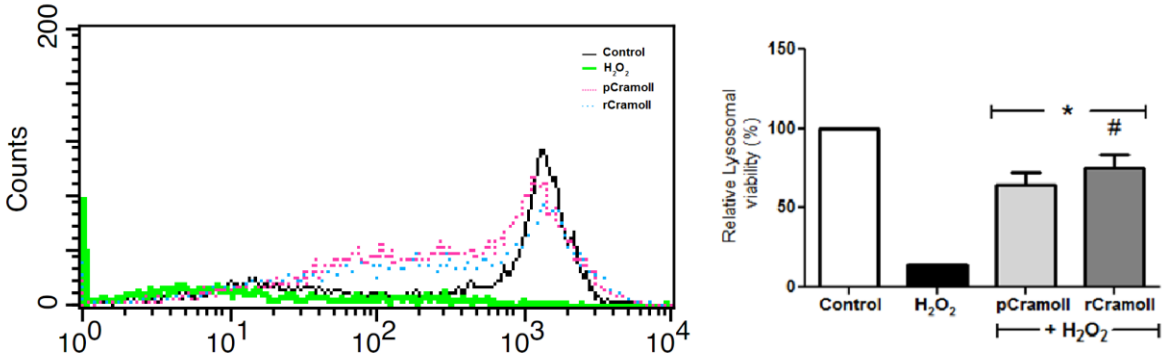

Figure 5: Effect of pCramoll and rCramoll on the Lysosomal damage induced by H_2O_2 in Vero Cells. (*) Significant differences in relation to H_2O_2. (#) Significant differences between lectins.

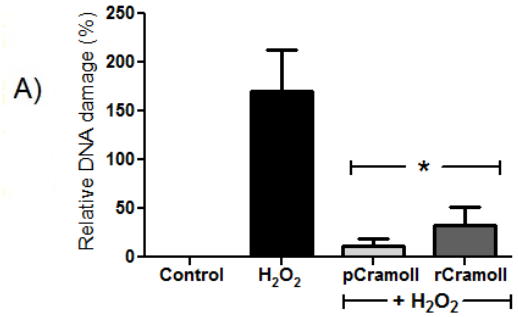

Figure 6: Effect of pCramoll and rCramoll on the DNA damage induced by H_2O_2 in Vero Cells by TUNEL analysis using flow cytometry. (*) Significant differences in relation to H_2O_2. (#) Significant differences between lectins.

PRINCIPAIS ACHADOS E PERSPECTIVAS

Ao fim desta tese podemos destacar os seguintes pontos:

- ✓ pCramoll e rCramoll são capazes de ativar macrófagos peritoneais, induzindo a produção de citocinas pró-inflamatórias (Il-1β, IL-6, IFN-γ e TNF-α), óxido nítrico, ROS e incremento da ação fagocitária;
- ✓ A ativação de macrófagos por pCramoll e rCramoll é relacionada com a indução da morte celular de parte da população de macrófagos;
- ✓ pCramoll e rCramoll têm efeito imunomodulador sobre a infecção de macrófagos por *S. aureus,* inbindo a produção de TNF-α e IL-6 (relacionadas com a sepsis) e estimulando Il-1β e IFN-γ (necessárias para a defesa contra *S. aureus*);
- ✓ pCramoll e rCramoll possuem efeito citoprotetor contra a morte celular induzida pelo H_2O_2 em células vero;
- ✓ A ação protetora dessa lectinas ocorre através da inibição da produção mitocondrial de ROS induzida pelo H_2O_2, previnindo a perda do potencial de membrana mitocondrial, danos lisossomais e ao DNA, promovendo assim a sobrevivência e a proliferação celular.

Todas estas ações estão sendo relatadas pela primeira vez, particularmente a ação de Cramoll em modelos de infecção com bactérias não havia ainda sido demonstrada, tão pouco sua ação como agente inibidor do estresse oxidativo. Adicionalmente este é o primeiro trabalho de aplicação farmacológica da lectina recombinante. Como perspectivas futuras, precisamos avançar na caracterização dos mecanismos moleculares envolvidos nestas ações, detectando os receptores e rotas metabólicas envolvidas. Além de avaliar mais aspectos da ação de Cramoll na relação parasita hospedeiro em modelos celulares e *in vitro.*

ANEXOS