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CARACTERIZAÇÃO ESTRUTURAL E APLICAÇÕES BIOLÓGICAS DA LECTINA COAGULANTE DE SEMENTES DE *Moringa oleifera* (cMoL)

LUCIANA DE ANDRADE LUZ

ORIENTADORA: Prof.ª Dr.ª Luana Cassandra Breitenbach Barroso Coelho
CO-ORIENTADORA: Prof.ª Dr.ª Patrícia Maria Guedes Paiva

RECIFE
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LUCIANA DE ANDRADE LUZ

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco como cumprimento parcial das exigências para obtenção do título de doutor.

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Dedico este trabalho aos meus pais Manoel e Bernadete e a toda minha família pelo amor que sempre recebi. Pelo incentivo constante, exemplo, cuidado, ensinamentos e por terem dado o melhor para mim.

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“O que vale na vida não é o ponto de partida e sim a caminhada. Caminhando e semeando, no fim terás o que colher.”

Cora Coralina

“A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original.”

Albert Einstein

RESUMO

Moringa oleifera é uma planta pantropical cujos tecidos têm sido descritos como fontes de compostos com as mais diversas aplicações. As sementes são consumidas como alimento e fonte de fitoquímicos bastante utilizados na medicina popular em países tropicais e subtropicais. Sementes de *M. oleifera* contêm óleos e proteínas coagulantes naturais, dentre elas as lectinas, uma classe de proteínas que reconhecem e se ligam específica e reversivelmente a carboidratos. Muitas lectinas já foram purificadas e suas especificidades a carboidrato identificadas, permitindo sua utilização como poderosas moléculas de reconhecimento no interior das células, nas superfícies celulares e em fluidos fisiológicos, podendo assim desempenhar diversas atividades biológicas. cMoL (Lectina coagulante de *M. oleifera*) é uma proteína básica, com atividade coagulante para contaminantes da água, a mesma já foi purificada e parcialmente caracterizada anteriormente. Dessa forma, o presente trabalho descreve: a caracterização estrutural de cMoL, a avaliação de seu efeito na coagulação sanguínea *in vitro*; bem como seu efeito citotóxico em células de melanoma B16-F10. A sequência primária revelou que cMoL é uma proteína com 101 aminoácidos, pI teórico de 11.67 e 81% de similaridade com uma proteína floculante de sementes de *M. oleifera* ($MO_{2.1}$). Deconvolução do espectro de dicroísmo circular indicou a presença de 46% de α-hélice, 12% folhas-β, 17% voltas-β e 25% de estruturas desordenadas, pertencendo à classe de estrutura terciária α/ β. cMoL prolongou significativamente o tempo requerido para a coagulação sanguínea, tempo de tromboplastina parcial ativada (TTPa) e tempo de protrombina (TP), mas não foi eficaz em prolongar o TTPa na presença de asialofetuína, glicoproteína que inibe totalmente a atividade da lectina. Dessa forma, cMoL agiu como uma proteína anticoagulante em parâmetros hemostáticos *in vitro* e pelo menos sobre o TTPa agiu potencialmente através do domínio de reconhecimento a carboidratos. A estrutura secundária de cMoL não se alterou em condições ácidas e alcalinas, no entanto quando a lectina foi submetida a aquecimento a 80°C foi observada mudança no conteúdo de α-hélice, seguido por um pequeno aumento de estruturas β. cMoL reduziu a viabilidade e causou morte (47,6%) nas células de melanoma após 48 h de tratamento na concentração de 250 µg/mL. A lectina demonstrou elevada especificidade para células tumorais, uma vez que, fibroblastos humanos (GN) tiveram uma taxa de morte celular em torno de 12,6%. cMoL aumentou a produção de espécies reativas do oxigênio (EROs), principalmente mitocondrial. A lectina também promoveu morte celular por apoptose, detectada pela ativação de caspases 3, 8 e 9. Além disso, a morte celular foi independente de Transição de Permeabilidade Mitocondrial (TPM) em células B16-F10. Esses estudos reportam novas e interessantes abordagens para as sementes de *M. oleifera*, além de fortalecer o entendimento da versatilidade das lectinas em diferentes processos biológicos.

Palavras-chave: *Moringa oleifera*, lectinas, coagulação sanguínea, citotoxicidade

ABSTRACT

Moringa oleifera is a pantropical plant whose tissues have been described as sources of compounds with the most diverse applications. The seeds are consumed as food and source of phytochemicals widely used in folk medicine in countries of Asia and Africa. Seeds of *M. oleifera* contain oils and natural coagulant proteins, among them lectins, a class of proteins that recognize and bind specifically and reversibly carbohydrates. A lot of lectins have been purified and their sugar specificities identified, allowing their use as powerful recognition molecules inside the cells, on cell surfaces and in physiological fluids and thus can play different biological activities. cMoL (coagulant *M. oleifera* lectin) is a basic protein with coagulant activity for water contaminants, previously purified and partially characterized. Thus, this present work describes: the structural characterization of cMoL, the evaluation of its *in vitro* effect on blood coagulation as well as its cytotoxic effect on B16-F10 melanoma cell. The primary sequence revealed that cMoL is a protein with 101 amino acids, 11.67 theoretical pI and 81% similarity with a *M. oleifera* flocculent protein ($MO_{2.1}$). Deconvolution of the circular dichroism (CD) spectrum indicated the presence of 46% α-helix, 12% β-sheets, 17% β-turns and 25% unordered structures, belonging to the α/β tertiary structure class. cMoL significantly prolonged the time required for blood coagulation, activated partial thromboplastin (aPTT) and prothrombin times (PT), but was not so effective in prolonging aPTT in asialofetuin presence, glycoprotein that inhibits completely the activity of the lectin. In this way, cMoL acted as an anticoagulant protein on *in vitro* blood coagulation parameters and at least on aPTT, the lectin interacted through the carbohydrate recognition domain. The secondary structure of cMoL was not altered in acidic and alkaline conditions, however when the lectin was subjected to heating at 80 °C it was observed change in α-helix content, followed by a small increase in β-structures. cMoL reduced the cell viability and caused cell death (47.7%) in melanoma cells after 48h of treatment in the concentration of 250 µg/mL. The lectin showed higher specificity for tumor cells, since normal human fibroblasts (GN-13) had a rate of cell death about 12.6%. cMoL increased reactive oxygen species (ROS) production, characterizing the oxidative stress that preceding cell death. cMoL also promoted apoptotic cell death, which could be seen by activation of caspases 3, 8 and 9. Additionally, cell death was independent of Mitochondrial Permeability Transition (MPT) in B16-F10 cells. These studies report new and interesting approaches to *M. oleifera* seeds, and strengthen the understanding of the versatility of lectins in different biological processes.

Keywords: *Moringa oleifera*, lectins, blood coagulation, cytotoxicity.

LISTA DE FIGURAS

FUNDAMENTAÇÃO TEÓRICA **Pág.**

CAPÍTULO 1

(▲) and 100 °C for 30 min (▽). The spectra after heating at 100 ◦C for 1 h is also represented (◁).

Fig. 5. *In vitro* effect of cMoL in hemostatic parameters. Activated partial thrombo-plastin time (aPTT, a) and prothrombin time (PT, b) were determined. The statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. A p-value < 0.05 was considered to indicate significance. *p*-Value < 0.05 (*) and *p*- Value < 0.001 (**). R: ratio of sample coagulation time with control coagulation time. Data represent means ± s.e.m., n = 3. 45

Fig. 6. Blood coagulation assays in the presence of cMoL inhibited by asialofetuin (0.5 mg/mL). Lectin was previously incubated (15 min) with asialofetuin. (a) Activated partial thromboplastin time (aPTT); (b) prothrombin time (PT). The statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. A *p*-value < 0.05 was considered to indicate significance. $p < 0.05$ (*) and $p < 0.001$ (**) represent comparative analysis between cMoL inhibited by asialofetuin and controls. Statistical significance of cMoL in the presence and absence of glycoprotein was also showed. R: ratio of sample coagulation time with control coagulation time. Data represent means ± s.e.m., n = 3. 45

CAPÍTULO 2

Fig. 1. cMoL reduces B16-F10 melanoma cell viability. B16-F10 cells were treated with increasing concentrations of the lectin for 48 h. (A) Cell viability was expressed as the percentage of viable cells related to control. (B) After treatment the number of viable cells were determined using 0.1% trypan blue. The statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. Values are mean ± s.e.m. of at least five independent experiments. A *p*-value < 0.05 was considered to indicate significance. *p*-value < 0.05 (*****) and *p*-value < 0.001 (******). 68

Fig. 2. cMoL induces cell death in B16-F10 melanoma cells. B16-F10 cells were treated with increasing concentrations of cMoL for 48 h and staning with annexin V and propidium iodide. The percentages of necrotic (PI+) or apoptotic (AnxV+) cells were determinate by flow cytometry. The statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. Values are mean ± s.e.m. of at least five independent experiments. A *p*-value < 0.05 (*) was considered to indicate significance. 69

Fig. 3. Morphological alterations of B16-F10 melanoma cells after treatment with cMoL. (A) Control, (B) 50 µg/mL, (C) 100 µg/mL, (D) 250 µg/mL and (E) 500 µg/mL. Images were provided by a Leica DFC360 FX, using the LAS AF software (Leica Microsystems). Bars represent 100 µm. 70

Fig. 4. GN cell lines (human fibroblast) viability after cMoL treatment. After trypsinization, GN (3.5×10^4 cells/mL) were incubated in RPMI-1640 medium 71

LISTA DE TABELAS

CAPÍTULO 1

CAPÍTULO 3

LISTA DE ABREVIATURAS

A_{280}	Absorbância em 280 nm
ADP	Adenosina difosfato
AH	Atividade Hemaglutinante
ATCC	"American Type Culture Collection"
CAT	Carboxiatractilosídeo
CCCP	Carbonyl-cyanide p-trifluoromethoxyphenylhydrazone
DC	Dicroísmo circular
FT	Fator tecidual
FADD	Proteína associada a FAS com um domínio de morte
HPLC	Cromatografia Líquida de Alta Eficiência
PBS	Fosfato dissódico 0,01 M contendo NaCl 0,14 M, KH_2PO_4 0,0017 M e KCl 0,0027 M, pH 7,4
TRADD	Receptor de TNF associado ao domínio de morte
TTPa	Tempo de Tromboplastina Parcial Ativada
TP	Tempo de Protrombina

AMINOÁCIDOS

Abreviação de três letras	Abreviação de uma letra	Aminoácido
Ala	A	Alanina
Arg	R	Arginina
Asn	N	Asparagina
Asp	D	Ácido Aspártico
Cys	C	Cisteína
Gln	Q	Glutamina
Glu	E	Ácido Glutâmico
Gly	G	Glicina
His	H	Histidina
Ile	I	Isoleucina
Leu	L	Leucina
Lys	K	Lisina
Met	M	Metionina
Phe	F	Fenilalanina
Pro	P	Prolina
Ser	S	Serina
Thr	T	Treonina
Trp	W	Triptofano
Tyr	Y	Tirosina
Val	V	Valina

SUMÁRIO

INTRODUÇÃO

Moringa oleifera é uma planta multi-uso, com suas folhas, sementes e flores, sendo utilizadas como alimento e fonte de compostos antioxidantes (Mendieta-Araica *et al*., 2011; Santos *et al*., 2012). As sementes são bastante conhecidas e utilizadas no tratamento de água (Gassenschmidt *et al*., 1995; Ghebremichael *et al*., 2005); o óleo presente nelas é empregado em cosméticos (Kleiman *et al*., 2008) e lubrificantes (Mani *et al*., 2007); as sementes igualmente constituem rica fonte de fitoquímicos como saponinas, taninos, terpenos, alcalóides e flavonóides (Ajibade *et al*., 2013). Também foram indicadas como boa matéria-prima para produção de biodiesel (Rashid *et al*., 2008).

Nas sementes de *M. oleifera* já foram identificadas e purificadas 3 lectinas (Santos *et al*., 2005; Katre *et al*., 2008; Santos *et al*., 2009). Lectinas são proteínas de origem não imune que ligam a várias estruturas de carboidratos desencadeando assim distintos processos celulares (Sharon, 2007). Apresentam distribuição ubíqua em uma variedade de espécies de planta (Peumans e Van Damme, 1995). Nos últimos anos, centenas de lectinas de planta foram purificadas e detalhadamente caracterizadas com relação a suas propriedades bioquímicas, especificidade de ligação a carboidrato e função biológica (Lam e Ng, 2011). Santos *et al*. (2005) reportaram a presença de uma lectina de caráter ácido, WSMoL (Lectina de *M. oleifera* solúvel em água). Katre *et al*. (2008) relataram a presença de um homodímero com massa molecular de 14 kDa e subunidades (7,1 kDa) ligadas por pontes dissulfeto, chamado MoL (Lectina de *M. oleifera*). Santos *et al*. (2009), por um novo protocolo, purificaram uma lectina diferente de outras já reportadas, cMoL (Lectina coagulante de *M. oleifera*), uma proteína básica, ativa em ampla faixa de pH, termoestável e com atividade coagulante para

contaminantes da água (Santos *et al*., 2009). Adicionalmente, cMoL demonstra atividade inseticida contra a espécie *Anagasta kuehniella* (Oliveira *et al.*, 2011).

A hemostasia é uma série complexa de fenômenos biológicos que ocorre em imediata resposta à lesão de um vaso sanguíneo, evitando assim a hemorragia. Problemas nesse processo podem levar a extravasamento de sangue pelos vasos ou obstrução do fluxo pela presença de trombos. A coagulação constitui uma série de reações químicas, entre várias proteínas que convertem pró-enzimas (zimogênios) em enzimas (proteases). Essas pró-enzimas e enzimas são denominadas fatores de coagulação (Silva & Hashimoto, 2006). A coagulação é iniciada, na via extrínseca, pela interação do Fator Tecidual (TF) exposto por lesão vascular com o Fator VIIa que, por sua vez, ativa os fatores IX e X, o que resulta na formação de pequena quantidade de trombina. A trombina, por sua vez, atua sobre o fibrinogênio formando o coágulo de fibrina. Essas reações ocorrem em superfícies fosfolipídicas, geralmente superfície ativada de plaquetas (Goodnight e Hathaway, 2001).

A terapia anticoagulante envolve a utilização de drogas, tais como heparina de baixa massa molecular, heparina não-fracionada, anticoagulantes que afetam as vias extrínseca e intrínseca da cascata da coagulação e prolongam os tempos de coagulação do sangue (Pedersen *et al*., 2005).

Câncer é uma doença que resulta da mutação de oncogenes e/ou genes supressores de tumor, que pode evoluir para a alteração das vias de sinalização, incluindo algumas vias envolvidas na proliferação das células tumorais e/ou em sua morte (Liu *et al*., 2011). Um dos processos mais importantes que regula o balanço entre o crescimento e a morte celular é a Morte Celular Programada (MCP) (Hanahan e Weinberg, 2000; Beth, 2007). Existem duas formas de MCP, apoptose e autofagia, e diferenças morfológicas são evidentes entre elas. Muitas lectinas estão sendo descritas

por induzirem células a MCP por apoptose, caracterizando assim, suas potenciais atividades citotóxicas e antiproliferativa (Peng *et al*., 2009; Liu *et al*., 2009f; Yao *et al*., 2010; Fu *et al*., 2011; Chan e Ng, 2013).

As várias interações químicas e a versatilidade de atividades biológicas desenvolvidas pelas lectinas estimulam o desenvolvimento das mesmas como ferramentas para uso clínico, diagnóstico e na pesquisa. Portanto, o objetivo deste trabalho foi: 1-investigar e desvendar a estrutura de cMoL através da obtenção de sua estrutura primária e análise do conteúdo de estrutura secundária; 2-avaliar o comportamento de cMoL, uma proteína coagulante, em processos de coagulação sanguínea humana, uma abordagem inédita para proteínas de sementes de *M. oleifera*; 3- Investigar a citotoxicidade da lectina sobre linhagem tumoral e os mecanismos envolvidos no processo; 4- Revisar os conceitos dos processos de coagulação, floculação, aglutinação, hemaglutinação e propor um mecanismo de ação em água para a lectina coagulante de *M. oleifera*.

1. FUNDAMENTAÇÃO TEÓRICA

1.1 Lectinas

1.1.1 Breve histórico, definição e distribuição na natureza

O primeiro relato sobre lectinas foi descrito por Peter Herman Stillmark em 1888, a partir de uma preparação protéica parcialmente purificada, obtida de *Ricinus communis* (mamona), a qual denominou ricina; a preparação continha uma proteína tóxica que aglutinava eritrócitos (Peumans e Van Damme, 1998a). Em 1889, H. Hellin demonstrou a presenca de uma hemaglutinina tóxica em extrato de sementes de feijão jequiriti (*Abrus precatorius*), a qual chamou abrina. Em 1891, Paul Ehrlich introduziu as lectinas na pesquisa imunológica usando ricina e abrina (Kennedy *et al*., 1995).

A concanavalina A (Con A) foi a primeira aglutinina de planta obtida na forma pura a partir de sementes de *Canavalia ensiformis*. Sumner e Howell (1936) demonstraram que além de aglutinar células, a Con A precipitava glicogênio e amido e sua atividade hemaglutinante podia ser inibida pelo açúcar da cana (sacarose), sugerindo que a aglutinação se dava através de uma reação da proteína com carboidratos presentes na superfície dos eritrócitos, demonstrando pela primeira vez a especificidade de ligação a açúcares das lectinas (Sharon e Lis, 2004).

Dessa maneira, em 1954, Boyd e Shapleigh propuseram o termo lectina (do latim *lectus*, que significa selecionado, escolhido) para designar o grupo de proteínas que apresenta a característica comum de seletividade na interação com carboidratos. As lectinas são uma classe de proteínas ou glicoproteínas hemaglutinantes estruturalmente diversa e contêm pelo menos um domínio de ligação a carboidratos, tais como monossacarídeos e oligossacarídeos que se ligam com elevada especificidade e de forma reversível (Peumans e Van Damme, 1995; Sharon e Lis, 2004; Correia *et al*.,

2008), aglutinam células vegetais ou animais, bem como precipitam polissacarídeos, glicoproteínas ou glicolipídeos (Goldstein *et al.*, 1980).

As lectinas são proteínas hemaglutinantes que, embora tenham sido primeiramente identificadas em plantas, sabe-se, hoje, que estão amplamente distribuídas na natureza, incluindo organismos eucariontes e procariontes (Correia *et al*., 2008). Podem ser encontradas em venenos de animais (Nunes *et al*., 2011), plantas (Bhat *et al*., 2010), bactérias (Imberty *et al*., 2004), vírus (Song *et al*., 2005) e fungos (Bovi *et al*., 2011). Sua distribuição generalizada no Reino *Plantae* sugere uma função fisiologicamente importante (Sharon, 2007). Em plantas, as lectinas têm sido isoladas de sementes (Santos *et al*., 2009), folhas (Costa *et al*., 2010), casca (Vaz *et al*., 2010), entrecascas (Napoleão *et al*., 2011) e raízes (Souza *et al*., 2011).

Baseado na estrutura global, lectinas de plantas são também classificadas em merolectinas, hololectinas, quimerolectinas e superlectinas (Peumans e Van Damme, 1998a), ou ser agrupadas em diferentes famílias como lectinas de leguminosas, proteínas inibidoras de ribossomos tipo II, lectinas de monocotiledôneas ligadoras de manose e outras lectinas (Lam & Ng, 2011). Merolectinas são pequenas e simples; devido à sua natureza monovalente são incapazes de precipitar glicoconjugados ou aglutinar células. Hololectinas contêm dois ou mais sítios de ligação para carboidratos, idênticos ou homólogos; devido à sua natureza di ou multivalente aglutinam células e ou precipitam glicoconjugados. A maioria das lectinas isoladas de plantas pertence ao grupo das hololectinas. Quimerolectinas são proteínas que possuem um ou mais sítios de ligação para carboidratos e outro sítio com atividade catalítica (ou outra atividade biológica) que funciona independentemente daquele de ligação para carboidratos. Dependendo do número de sítios de ligação para carboidratos, quimerolectinas agem como merolectinas ou hololectinas. Superlectinas consistem de pelo menos dois sítios

de ligação para carboidratos diferentes e podem ser consideradas como um grupo especial de quimerolectinas (Figura 1).

As lectinas diferem entre si pela composição e sequência de aminoácidos na cadeia polipeptídica, quanto ao número de subunidades na estrutura protéica, quanto à necessidade de presença de metais para a AH, bem como especificidade do sítio de ligação a carboidratos. O conhecimento das suas características estruturais possibilitam seu uso para aplicações terapêuticas e fins biotecnológicos.

Numerosos estudos mostraram que lectinas ligadas a carboidratos da superfície celular podem promovem vários efeitos biológicos (Gastman *et al*., 2004). Lectinas já demonstraram atividade inibitória contra fungos e bactérias (Vaz *et al*., 2010; Charungchitrak *et al*., 2011), inseticida (Oliveira *et al*., 2011), contra vírus (Sato *et al*., 2011) e citotóxica para células tumorais (Fu *et al*., 2011). Estudos também já demonstraram a baixa toxicidade e genotoxicidade de lectinas de plantas bastante utilizadas na medicina popular, como a lectina de *Sebastiania jacobinensis* (SejaBL) e a lectina de folhas de *Bauhinia monandra* (BmoLL) (Vaz *et al*., 2010; Sisenando *et al.*, 2009).

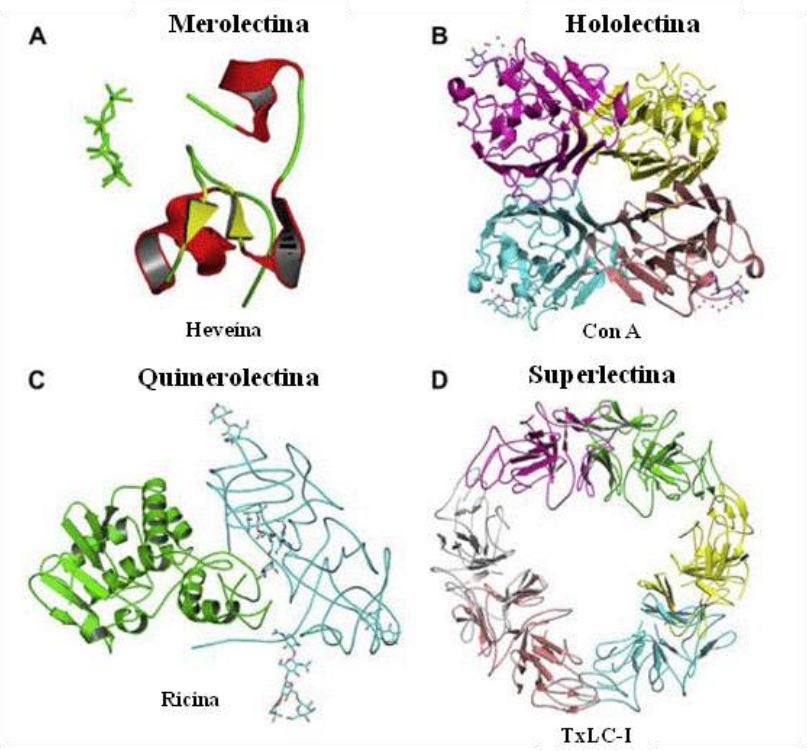

Figura 1. Classificação de lectinas de plantas de acordo com a estrutura global em merolectinas, hololectinas, quimerolectinas e superlectinas e seus respectivos exemplos (Liu *et al*., 2010a).

1.1.2 Detecção e especificidade

Lectinas possuem a habilidade de induzir o fenômeno de aglutinação celular e a presença dessas proteínas é detectada através de um ensaio de hemaglutinação (Kennedy *et al*., 1995). Nesse ensaio é feita uma diluição serial da lectina e incubação com eritrócitos humanos ou outras espécies animais (Figura 2). Os eritrócitos podem ou não ser submetidos a tratamentos com enzimas ou com soluções químicas (glutaraldeído ou formaldeído) para que haja um aumento na sensibilidade de aglutinação por lectinas devido à estabilização das células, promovendo uma preparação padrão de eritrócitos, além de aumentar o tempo de armazenamento (Coelho e Silva, 2000). A atividade das lectinas é usualmente medida pela técnica de diluições sucessivas (Guimarães-Gomes *et al*., 2004). A definição da especificidade da lectina pode ser feita por ensaios de inibição

da atividade hemaglutinante (AH) com diferentes monossacarídeos, oligossacarídeos ou glicoproteínas ou por ensaios de precipitação de moléculas glicídicas (Sharon e Lis, 1990).

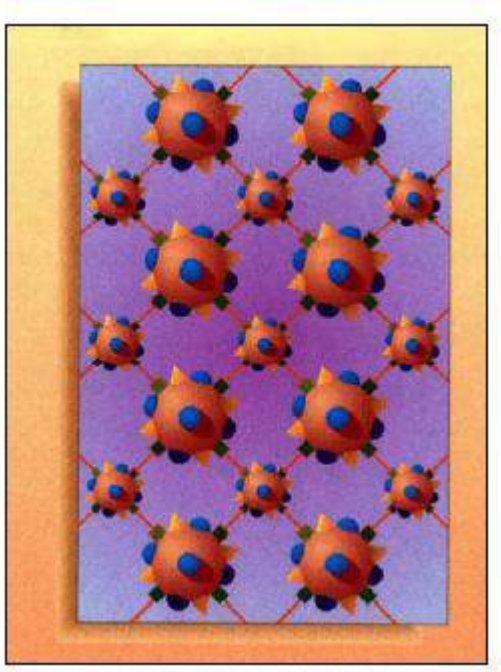

Figura 2. Representação esquemática da rede de eritrócitos promovida pela ligação da lectina à superfície de carboidratos (Correia *et al*., 2008).

1.1.3. Caracterização estrutural

A caracterização é realizada por meio da determinação de diferentes propriedades físico-químicas da lectina e envolve métodos diversos como inibição da AH por carboidratos e/ou glicoconjugados (Yang *et al.*, 2007), avaliação da AH com eritrócitos de diferentes espécies de animais (por exemplo: coelho, galinha, sistema sanguíneo humano A, B, AB e O), em presença de íons e em diferentes valores de pH e temperatura (Santos *et al*., 2009). Técnicas eletroforéticas, mono ou bidimensional, são eficientes para definir a natureza da carga líquida da proteína e o peso molecular das subunidades, bem como para avaliar a pureza da preparação obtida (Nasi *et al*., 2009).

A combinação de análises cromatográficas com espectrometria de massas (EM) tem revelado uma definição estrutural dessas proteínas, principalmente de glicoproteínas por superar as limitações decorrentes da heterogeneidade dos açúcares (Kubota *et al*., 2008).

Cromatografia Líquida de Alta Eficiência (do inglês, *High performance liquid chromatografy*, HPLC), está agora firmemente estabelecida como a técnica principal para a análise e purificação de uma ampla gama de moléculas. HPLC em suas várias modalidades tornou-se a técnica central na caracterização de peptídeos e proteínas e, portanto, tem desempenhado um papel crítico no avanço nas Ciências Biológicas e Biomédicas nos últimos 10 anos. O sucesso da técnica pode ser atribuído a uma série de características inerentes associadas com reprodutibilidade, facilidade de manipulação, seletividade e recuperação geralmente elevados. A característica mais significativa é a excelente resolução que pode ser alcançada sob uma ampla gama de condições para moléculas intimamente relacionadas, bem como para moléculas estruturalmente distintas (Aguilar, 2004).

O crescimento em biologia estrutural também tem sido dirigido pelo desenvolvimento da tecnologia do DNA recombinante que permite produzir proteínas em quantidades requisitadas, bem como o avanço da análise de dados com bioinformática. No entanto, há uma necessidade de se elucidar estudos estruturais nas condições em que as proteínas normalmente atuam (geralmente em solução), bem como sob outras condições e fornecer medidas das taxas de mudanças estruturais das proteínas que em geral são essenciais para suas funções biológicas. Neste sentido as análises espectroscópicas têm se mostrado ferramentas úteis, dentre elas o dicroísmo circular (Kelly *et al*., 2005). O dicroísmo circular (DC) é observado quando uma molécula opticamente ativa, denominada cromóforo, absorve de forma diferente as componentes opostas de luz circularmente polarizada, à esquerda e à direita. Esta atividade ótica é

causada pela assimetria do cromóforo. A conformação de uma molécula está intimamente relacionada à sua atividade ótica e, por isto, esta técnica é muito utilizada para monitorar mudanças conformacionais bem como para estimar o conteúdo de estrutura secundária de proteínas (Silva-de-Lucca *et al*., 2006). Em proteínas, os principais grupos opticamente ativos são as ligações amida da cadeia peptídica, monitoradas na região do ultravioleta (UV) distante [190-250 nm]; bem como as cadeias laterais aromáticas e as ligações dissulfeto, monitoradas no UV próximo [250-360 nm]. Portanto, o espectro de CD entre 190 e 250 nm (região ultravioleta distante) pode identificar diferentes tipos de estrutura secundária como α-hélices, folhas-β, voltas-β e estruturas desordenadas (Woody, 1994; Venyaninov e Yang, 1996). Para proteínas α+β a banda em 208-220 nm geralmente apresenta uma intensidade maior do que em 222 nm, enquanto que para proteínas α/β o inverso é observado (Venyaninov e Yang, 1996). A caracterização estrutural de proteínas constitui hoje uma importante área de estudo uma vez que permite a determinação de sua estrutura tridimensional, para que seja possível a correlação entre a estrutura e sua função biológica.

1.2. Hemostasia

No organismo humano, o sangue percorre o sistema circulatório de maneira fluida, ou seja, o sangue não pode coagular, pois levaria à formação de trombos, e não pode extravasar o que acarretaria em uma hemorragia. A manutenção deste equilíbrio, garantido pela ação conjunta de vários fatores, garante a hemostasia. Mecanismos moleculares altamente sofisticados estão envolvidos na manutenção da fluidez sanguínea e no reparo de lesões (Tanaka *et al*., 2009). Estes processos envolvem respostas fisiológicas como vasoconstrição, vasodilatação, respostas celulares (endotélio, plaquetas e hemácias) e interações bioquímicas (fatores da coagulação, da anticoagulação e da fibrinólise). A hemostasia é complexa e dinâmica, podendo ser

dividida em quatro fases: iniciação e formação do tampão plaquetário, propagação da cascata de coagulação, término do processo por mecanismos de controle antitrombóticos e fibrinólise (Moran e Viele, 2005; McMichael *et al*., 2012).

1.2.1. Cascata da coagulação sanguínea

A concomitante ação de substâncias provenientes do tecido lesionado, das plaquetas e do sangue (proteínas que aderem à parede vascular lesionada) conduz a ativação dos fatores de coagulação, os quais são proteases e cofatores plasmáticos, que circulam no plasma na forma de zimogênios (Mann, 1999; Tanaka *et al*., 2009) e que são ativados em uma série de etapas, onde o substrato para cada enzima (ou complexo enzimático) é uma pró-enzima que é ativada para atuar na próxima etapa da reação em um processo denominado "cascata da coagulação" (Macfarlane, 1964). Atualmente, a cascata da coagulação é considerada um modelo celular (Figura 3), onde na fase inicial, o complexo formado entre o fator tecidual (FT), uma proteína de membrana, exposta no sítio da injúria e o FVIIa, uma serinoprotease, ativa o fator FX, direta ou indiretamente, através de fator FIXa (Tanaka *et al*., 2009; McMichael *et al*., 2012). Na fase de propagação, o FXa sob uma superfície fosfolipídica (membrana plaquetária) e na presença de íons cálcio e do fator V, forma o complexo protrombinase (Monteiro, 2005), que reconhece e hidrolisa a protrombina, gerando grandes quantidades de trombina responsável pela amplificação do estímulo pró-coagulante com velocidade cerca de 30.000 vezes superior quando comparado a essa reação na ausência dos componentes do complexo. Por um mecanismo de retroalimentação positiva, a trombina ativa os fatores XI, IX, VIII e V, e estes quando ativos são atraídos para a superfície da plaqueta promovendo a formação dos complexos da coagulação. Além disso, a trombina ativa outras plaquetas, próximas ao local da lesão, via receptores PAR1 E PAR4, (Hirano, 2007; Angiolillo *et al*., 2010), que se ligam ao FXI via receptor GPIb, co-

localizam o fator VIII, componente do complexo tenase e ainda expõem várias moléculas do receptor GPIIb/IIIa, que pode concentrar fibrinogênio suficiente para a formação de fibrina pela trombina. Na fase final, a trombina ativa o FXIII plaquetário e plasmático, que por uma ligação cruzada reforça os monômeros de fibrina, estabilizando o tampão hemostático (Tanaka *et al*., 2009).

Como resultado da ação coordenada de diferentes proteínas no processo de coagulação, ocorre simultaneamente a fibrinólise pela ação da plasmina, uma serinoprotease que degrada a fibrina. Essa enzima circula no plasma como zimogênio, o plasminogênio, fisiologicamente regulado pelo inibidor do ativador de plasminogênio (PAI-1) (Cesarman-Maus e Hajjar, 2005) e tem sua atividade enzimática diretamente inibida pela antiplasmina. A fibrinólise também é regulada positivamente pelos ativadores de plasminogênio do tipo uroquinase (u-PA) e tecidual (t-PA) (Mosnier e Bouma, 2006; Jögi *et al*., 2010).

Existem dois inibidores que regulam a resposta pró-coagulante desencadeada pelo FT, limitando assim a ação de serinoproteases no local da lesão vascular. A via do inibidor do Fator Tecidual (VIFT) neutraliza FXa quando ele está em um complexo com FT-FVIIa. A antitrombina (AT; anteriormente chamada de antitrombina III, um inibidor de serinoprotease; Serpinas) é outro regulador da resposta pró-coagulante do FT, que circula em uma concentração elevada (150 µg/mL) e neutraliza o FXa inicialmente formado e a trombina. Assim, a reação de ativação da coagulação apenas acontece quando o FT é exposto em um nível suficientemente alto capaz de superar a inibição por VIFT e AT (Tanaka *et al*., 2009).

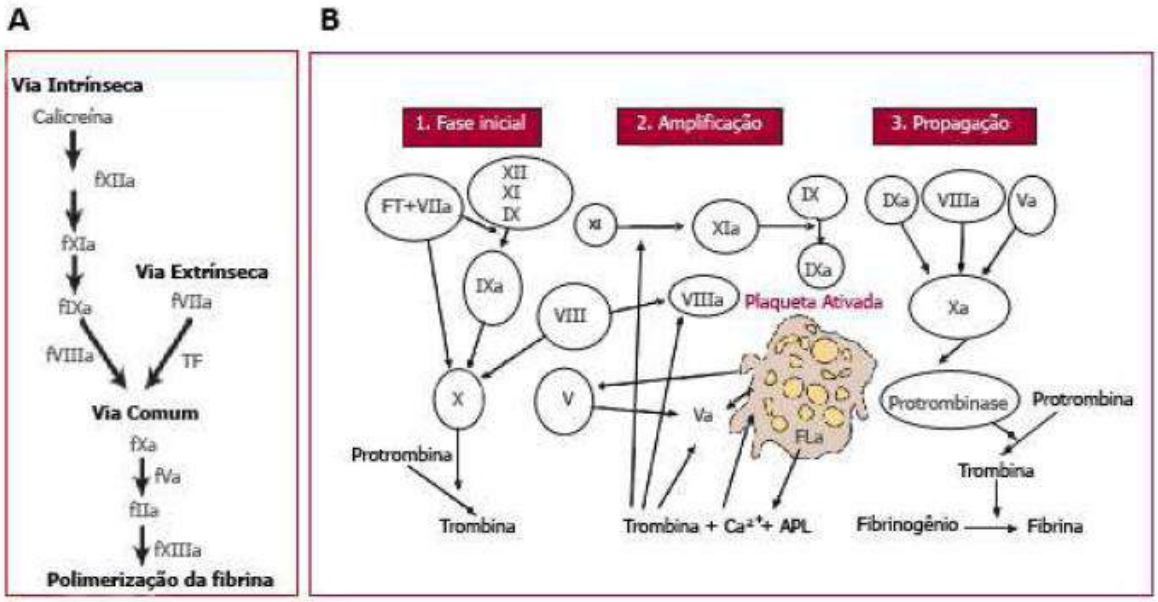

Figura 3. (A) Modelo convencional da cascata da coagulação. Apresentando duas iniciações: via intrínseca e via extrínseca convergindo para a via comum ao nível do fator Xa. (B) Nova cascata da coagulação, também conhecida como modelo celular (Adaptado de Tanaka *et al*., 2009).

1.2.2. Via extrínseca e Tempo de Protrombina (TP)

A via extrínseca é o meio pelo qual a substância ativadora da protrombina é gerada em resposta ao contato do sangue com os tecidos extravasculares (Banks, 1991). Ocorre quando a ativação do fator VII, pelo fator tecidual, produz a ativação do fator X (Bozzini, 2004). O tecido traumatizado libera um complexo de vários fatores, denominado fator tecidual ou tromboplastina tecidual (Guyton e Hall, 2002). O fator III, o cálcio e fator VII formam um complexo que age enzimaticamente na presença de fosfolipídios para converter o fator X para fator Xa (Banks, 1991).

O TP fornece indicação sobre a quantidade total de protrombina presente no sangue (Guyton e Hall, 2002). Este teste é usado para identificar as anormalidades dos fatores envolvidos no sistema extrínseco, a saber, protrombina e fatores V, VII e X. Os testes de rotina laboratoriais, que determinam as concentrações de fibrinogênio no plasma, envolvem a adição de trombina ao plasma para medição da velocidade com que a fibrina é formada (Swenson, 1996). Após recalcificação, o tempo de coagulação é

reduzido, onde, com acréscimo de tromboplastina (um extrato salino que contém fator tecidual e fosfolipídios) é possível determinar o TP (Majerus, 2003).

1.2.3. Via intrínseca e Tempo de Tromboplastina Parcial ativada (TTPa)

A via intrínseca inicia-se pelo contato do sangue com uma superfície diferente do endotélio normal e das células sangüíneas (Bozzini, 2004). A seqüência de reações enzimáticas produz o coágulo sanguíneo nas diferentes etapas: (a) fase de contato; (b) a ativação do fator X; (c) a formação de trombina; (d) a formação de fibrina insolúvel (Swenson, 1996), como descrito no item 1.2.1. O teste mais comumente empregado para verificação do mecanismo intrínseco da coagulação é o tempo de tromboplastina parcial ativada (TTPa) (Swenson, 1996). Após adição de cálcio, fosfolipídios de carga negativa e de uma substância particulada, como caulim (silicato de alumínio), ocorre a ativação dos fatores XII e XI por essas substâncias, sendo possível determinar o TTPA (Majerus, 2003). Esse teste é utilizado para o diagnóstico de anomalias dos fatores da coagulação XII, XI, IX, VIII, X, protrombina e fibrinogênio.

1.2.4. Via comum

A via comum se inicia com ativação do fator X, pela combinação de várias substâncias, fator III, cálcio, fator VII e fosfolipídios teciduais na via extrínseca e, da mesma forma, o FP3, fator IX e o fator VII na via intrínseca (Banks, 1991). Ver item 1.2.1.

1.2.5. Atividade de lectinas nos parâmetros hemostáticos

As lectinas de planta são pouco abordadas na área de hemostasia. As mais descrita são as lectinas tipo C de veneno de cobra (Snaclecs). De acordo com seus alvos de ligação, estas proteínas podem ser divididas em 3 grupos: lectina tipo C de cobra ligadora do FIX (FIX snaclec), snaclecs que se ligam ao FX e snaclecs FIX/X. A partir do veneno de *Agkistrodon acutus* foi purificado um fator anticoagulante (ACF I), uma

snalec que se liga ao FX (Xu *et al*., 2000b). ACF I apresenta uma forte atividade anticoagulante *in vivo* e inibe ambas as vias intrínseca e extrínseca da coagulação. Este fator também se liga com FIX na presença de Ca^{2+} 0.25 mM (Zhang *et al*., 2012). CrataBL, uma lectina de *Crataeva tapia*, foi capaz de prolongar o tempo de tromboplastina parcial ativada (TTPa), e inibir os fatores da coagulação da via intrínseca, sendo a primeira lectina descrita na literatura com atividade inibitória e anticoagulante (Araújo *et al*., 2011). Silva *et al*. (2012) reportaram que a lectina das sementes de *Bauhinia forficata*, prolonga apenas o TTPa, e este efeito não está relacionado com a inibição da atividade da calicreína plasmática humana nem com o fator Xa.

1.3. Morte celular

A morte celular, segundo Kroemer *et al*. (2009), pode ser classificada de acordo com vários aspectos: aparência morfológica (que pode ser apoptótica, necrótica, autofágica ou associada com mitose), critérios enzimáticos (com e/ou sem envolvimento de nucleases ou de distintas classes de proteases, como caspases, calpainas, catepsinas e transglutaminases), aspectos funcionais (programada ou acidental, fisiológica ou patológica) ou características imunológicas (imunogênica ou não imunogênica).

Para que uma célula seja considerada morta, um dos seguintes critérios morfológicos ou moleculares deve ser encontrado: perda da integridade de membrana plasmática, pela incorporação de corantes vitais *in vitro* (como o iodeto de propídeo); ou quando a célula, incluindo seu núcleo, sofre completa fragmentação em corpos discretos (conhecidos como “corpos apoptóticos”) e/ou quando verifica-se *in vivo* o englobamento da célula morta (ou fragmentos celulares) por uma célula adjacente (Kroemer *et al*., 2009). Esses eventos de morte celular podem ou não ter participação da mitocôndria.

A morte celular por apoptose difere da necrose com base em diversos aspectos bioquímicos e morfológicos. Apoptose está relacionada com insultos celulares mais amenos que não resultam em inflamação e sua ativação depende da produção de energia, ATP, ativação de caspases e outros fatores pró apoptóticos. A morfologia da apoptose é caracterizada pela integridade das organelas celulares, incluindo a da mitocôndria, condensação da cromatina, fragmentação do DNA nuclear e formação de corpos apoptóticos. Por outro lado, a necrose está relacionada a intensas agressões nas células associadas com a inflamação, processo que resulta na queda da produção de ATP e ou lesão da membrana celular, morfologicamente caracterizada por: tumefação, rompimento celular e das organelas, particularmente das mitocôndrias, aparecimento de vacúolos, acidofilia citoplasmática, e em suas etapas finais, a necrose é responsável pela degradação total das células (McConkey, 1998, Elmore, 2007, Kroemer *et al*., 2009).

As células possuem diversos mecanismos que regulam seu crescimento e morte. A morte celular programada ou apoptose é essencial para o desenvolvimento normal de qualquer organismo multicelular, apresentando diversas funções, tais como: dar forma aos órgãos pela remoção de células e estruturas desnecessárias e eliminar células que não são mais necessárias, células mutadas ou com infecções virais (Westphal e Kalthoff 2003, Yoshida 2003). Falhas nesses mecanismos podem gerar células tumorais ou desencadear doenças auto-imunes (Takahashi *et al*., 2004).

Os mecanismos de apoptose são altamente complexos e sofisticados, envolvendo uma cascata de eventos moleculares dependentes de energia. Até agora, pesquisas indicam que os mecanismos de apoptose são divididos em duas vias principais, a *extrínseca* ou via dependente de receptores de morte e a *intrínseca* ou via mitocondrial (Figura 4). Estas duas vias ocorrem independentes, sendo que a interação de ambas também pode ocorrer (Igney e Krammer, 2002; Takahashi *et al*., 2004). A via *intrínseca*

ou mitocondrial pode ocorrer de duas maneiras, como descrito a seguir. Em resposta a estímulos pró-apoptóticos tais como 1- DNA danificado, 2- inibidores de quinase e 3- ativação de receptores da morte celular, a proteína Bad, da família Bcl-2, se liga ao complexo Bcl-2/Bcl-xl presente na membrana mitocondrial (Budihardjo *et al*., 1999; Polster e Fiskum, 2004). Esta união promove a permeabilização da membrana externa pela formação de poros entre os dímeros de Bax (Gross *et al*., 1999). Dessa forma há efluxo mitocondrial de citocromo *c* e da proteína Apaf-1 para o citosol (Gross *et al*., 1999; Alirol e Martinou, 2006). No citosol, citocromo *c* e Apaf-1 se ligam ao dímero Bcl-2/Bcl-xl, clivando a pró-caspase-9 e formando o apoptossomo, complexo de alto peso molecular responsável pela ativação de várias pró-caspases (Green, 2005; Garrido *et al*., 2006). Em seguida há uma seqüência de clivagens promovendo a ativação proteolítica de precursores inativos das caspases, culminando na morte celular programada (Hengartner, 2000; Scorrano e Korsmeyer, 2003). Outra via mitocondrial de apoptose ocorre quando o efluxo de citocromo *c* é decorrente da formação da Transição de Permeabilidade Mitocondrial (TPM), em condições em que há aumento da concentração intramitocondrial de Ca^{2+} (Scorrano e Korsmeyer, 2003; Polster e Fiskum, 2004; Kowaltowski *et al*., 2009). Se por um lado a via intrínseca é desencadeada na mitocôndria, a via *extrínseca* tem início com a ativação dos receptores de morte (*death domains*), tais como Fas e TNF (fator de necrose tumoral), segue com a ativação da pró-caspase-8 (Kadenbach *et al*., 2004; Galluzzi *et al*., 2012) e culmina com a ativação de caspases efetoras, como a caspase-3 (Takahashi *et al*., 2004; Polster e Fiskum, 2004; Liu *et al*., 2011). A interação entre ambas as vias pode ocorrer quando a proteína citosólica Bid, outra proteína da família Bcl-2, é clivada e translocada à mitocôndria, onde interage com a membrana e permite a liberação de citocromo c (Fulda e Kroemer, 2011; Galluzzi *et al*., 2012).

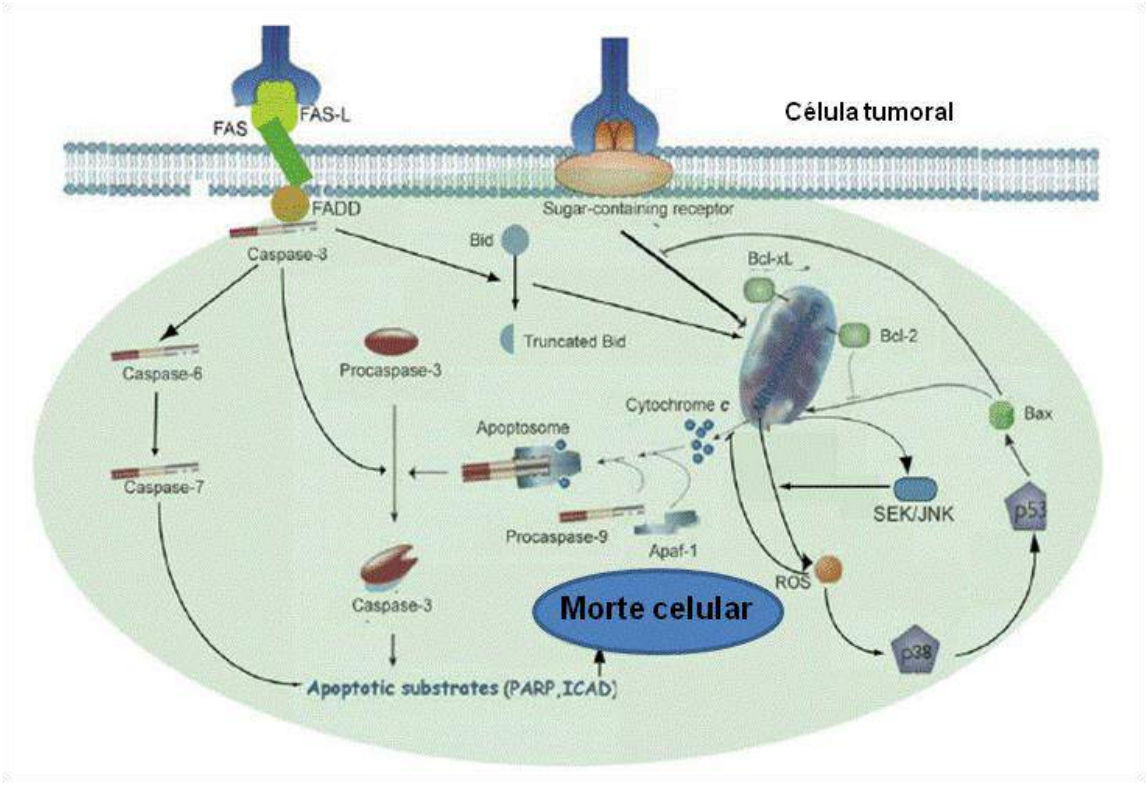

Figura 4. Sinalização intracelular das vias de apoptose. A via extrínseca pode ser iniciada após as interações de FAS com seus respectivos domínios de morte FADD (proteína associada a FAZ com um domínio de morte) ou TRADD (Receptor de TNF associado ao domínio de morte) (não representado). Esta via culmina na morte celular através da ativação de caspase-8 ou mediada por Bid e subseqüente apoptose dependente da via mitocondrial (intrínseca). A via intrínseca pode ser iniciada por uma infinidade de estresse intracelular como danos no DNA, estresse oxidativo, sobrecarga de Ca^{+2} citosólico que culminam em um mecanismo de ativação da apoptose dirigido pela mitocôndria. (Liu *et al*., 2010a).

1.3.1. Atividade antitumoral de lectinas

Atividade antitumoral de lectinas de planta sobre uma variedade de células malignas tem sido reportada ao longo dos anos. Lectinas como as MLs (*mistletoe lectin)* (Lyu *et al*., 2002), a ricina (Plattner *et al*., 2008) e a WGA (Narayanan *et al*., 2005) têm sido bem estudadas por apresentarem atividade antiproliferativa e indutora de apoptose em células tumorais. Além disso, outras lectinas como a concanavalina A (Con A) e a de *Polygonatum cyrtonema* (PCL) podem levar a morte celular por autofagia após internalização ou por ligação a receptores que contém carboidrato na superfície de células tumorais (Lei e Chang, 2007; Liu *et al*., 2009a). PCL, uma lectina ligadora de manose foi citotóxica para células HeLa (câncer cervical), MCF-7 (câncer de mama), A375 (melanoma humano) e L929 (fibrosarcoma murino) por interação complexa entre autofagia e apoptose, mas com baixa toxicidade para células normais (Wang *et al*., 2011a). Con A induziu apoptose em células balb/c 3T3 (3T3), fibroblastos gengivais humanos diplóides (HGF), melanoma humano A375 e carcinoma hepatocelular de fígado humano HepG2 (Liu *et al*., 2009d, 2010c). Recentemente, foi descrito o potencial antitumoral de uma lectina purificada de tubérculos de *Dioscorea opposita*. A lectina inibiu o crescimento de algumas linhagens celulares, incluindo câncer de mama (MCF-7), células de hepatoma (HepG2) e carcinoma nasofaríngeo (CNE2) com valores de IC_{50} de 3.71 μM, 7.12 μM e 19.79 μM, respectivamente, após 24 h de tratamento, no entanto o efeito anti-proliferativo não foi inibido na presença de galactose indicando que a atividade da lectina não está ligada ao sítio de reconhecimento a carboidrato (Chan *et al*., 2013).

Lectinas de planta têm sido utilizadas como candidatas a drogas antitumorais após avaliação com células de câncer humano *in vitro*, e mais importante ainda, algumas delas foram aplicadas em terapias pré-clínica e clínica no combate ao câncer

humano (Thies *et al*., 2005; Gupta *et al*., 2010). Em ensaios clínicos, ML-I tem sido amplamente utilizada como potencial droga antineoplásica ou como adjuvante de agentes terapêuticos. Esta lectina foi indicada para a redução dos efeitos colaterais associados ao tratamento quimioterápico ou radioterapia na Europa há várias décadas (Liu *et al*., 2010a).

1.4. A espécie Moringa oleifera

Moringa oleifera é uma planta tropical pertencente à família Moringaceae que possui 14 espécies conhecidas, sendo *M. oleifera* a mais divulgada e utilizada dentre elas (Abdulkarin *et al*., 2004). Tem sua origem no noroeste da Índia e se encontra amplamente distribuída nas regiões tropicais e subtropicais, ocorrendo na Ásia, África e América (Bhatia *et al*., 2007, Teixeira *et al*., 2012).

Segundo Joly (1979), *M. oleifera* é uma planta arbórea com longas vagens verdes (Figura 5A), flores brancas perfumadas (Figura 5B), sementes aladas (Figura 5C) e folhas grandes compostas (Figura 5D). As árvores de Moringa podem alcançar 4 m de altura, gerando flores e frutos em um ano; múltiplas colheitas de sementes são possíveis em muitas partes do mundo (Mcconnachie *et al*., 1999). A árvore é geralmente conhecida nos países em desenvolvimento como vegetal, planta medicinal e uma rica fonte de óleo (Katayon *et al.*, 2006). Nas Filipinas, as folhas jovens, flores e vagens verdes são comuns na dieta (Guevara *et al*., 1999). As folhas e as sementes jovens são ricas fontes de cálcio, ferro e vitamina C que servem como fontes de nutrientes para várias comunidades (Morton, 1991). Extrato etanólico e salino de diferentes tecidos de *M. oleifera* são potenciais fontes de antioxidantes (Santos *et al*., 2012)

Tradicionalmente, quase todas as partes da planta têm sido utilizadas para tratar doenças, tais como: tumores abdominais, histeria, escorbuto, ataques de paralisia, bexiga, problemas da próstata, feridas e infecções da pele (Sreelatha *et al*., 2011).

Extrato etanólico de folhas são usados como agente hipotensivo (Nikkon *et al*., 2003), anti-aterosclerose (Chumark *et al*., 2008) e como agente hipocolesterolêmico e anti-inflamatório (Cáceres *et al*., 1992; Ghasi *et al*., 2000). Extrato de sementes de moringa também foi eficiente na redução no número de ovos de helmintos em água com alta turbidez (Sengupta *et al*., 2012).

Nos diferentes tecidos de *M. oleifera* já foram identificados compostos com interessante atividade antitumoral e citotóxica. Guevara *et al*. (1999) isolaram de extratos etanólicos de sementes um composto capaz de inibir a progressão de câncer de pele em ratos. Extratos de raiz demonstraram atividade citotóxica contra células de leucemia (HL-60 e CEM) e melanoma (Costa-Lotufo *et al*., 2005). Sreelatha *et al*. (2011) demonstraram que extratos de folhas de moringa foram capazes de inibir a proliferação de células de tumor humano (KB) de uma maneira dose-dependente e também de induzir a apoptose das mesmas.

Em decorrência dos vários usos tradicionais que se tem relatado muitas pesquisas têm sido feitas para isolar compostos bioativos de várias partes da planta (Guevara *et al*., 1999).

Figura 5. A: Vagens de *M. oleifera*, B: Flores, C: Sementes, D: Folhas.

1.4.1. Sementes de *M. oleifera*

1.4.1.1 Propriedade coagulante

Sementes de *M. oleifera* apresentam óleos comestíveis e substâncias solúveis em água que indiscutivelmente constituem o coagulante natural mais estudado na comunidade científica (Yin, 2010). Coagulação e floculação constituem passos delicados no tratamento de água. Em uma estação de tratamento de água, convencionalmente, ela é agitada mecânica ou hidraulicamente, seguido da adição de coagulante, que atuam através da redução das forças de repulsão entre as partículas (impurezas) (Mcconhachie *et al*., 1999). A árvore de *M. oleifera* pode produzir cerca de 2000 sementes por ano. Este número de sementes seria capaz de tratar cerca de 6.000 L de água usando uma dose de 50 mg/L. As árvores no entanto, podem ser cultivadas para

produzir cerca de cinco a dez vezes esse rendimento (ou seja 10.000-20.000 sementes). Isso produziria até 60.000 L de água tratada por ano (Pritchard *et al*., 2010).

Estudos feitos por Fink (1984), Gassen (1990) e Gassenschmidt (1991) sugeriram que o componente coagulante ativo de *M. oleifera* deveria ser um peptídeo catiônico com peso molecular entre 6 e 16 kDa e ponto isoelétrico em pH 10. Em 1995, Gassenschmidt fez análises da composição de aminoácidos e seqüenciamento e mostrou que o componente apresentava grande quantidade de glutamina, arginina e prolina e um total de 60 resíduos ($MO_{2.1}$, $MO_{2.2}$). Muyibi e Okufu (1995) reportaram que *M. oleifera* não era um coagulante eficiente para água com baixa turbidez.

Ndabigengesere *et al*. (1995) estudou a eficiência e os mecanismos de coagulação de *M. oleifera* em água turva. Confirmou que o componente ativo era uma proteína dimérica com propriedades coagulantes mais eficientes do que o alumínio, pois o resíduo orgânico formado era inócuo para o meio ambiente e de 4 a 5 vezes menor que o encontrado em água tratada com alumínio. O coagulante natural também não alterava o pH, era solúvel em água, tinha peso molecular de 13 kDa e ponto isoelétrico (pI) entre 10-11. Também revelou que as sementes de *M. oleifera* podem ser usadas em sua forma íntegra ou descascadas, no entanto, sementes descascadas eram mais eficientes para coagulação. Em 1998, Ndabigengesere e Narasiah observaram que a dosagem ótima para coagulação era de 0,5 a 1 mg/L e que a proteína foi totalmente solúvel em água. *M. oleifera* como coagulante pode ser um substituto em potencial para o alumínio (Ndabigengesere *et al*., 1995).

Outro componente, com três frações ativas foi extraído de sementes de *M. oleifera* em tampão fosfato e cromatografia de troca iônica (Gassenschmidt *et al*., 1995). A fração floculante isolada de uma das proteínas apresentava peso molecular ao

redor de 6,5 kDa e pI acima de pH 10. A comparação da estrutura primária com seqüências de proteínas conhecidas não revelou significante homologia.

Okuda *et al*. (1999), estudaram que as sementes de *M. oleifera* possuem um coagulante extraído com NaCl 1 M com capacidade de coagulação 7,4 vezes maior do que o extraído em água. Em 2001, Okuda *et al*. isolaram outro componente com propriedades coagulantes em extratos salinos que não era proteína, polissacarídeo ou lipídio e sim, um polieletrólito com peso molecular em torno de 3,0 kDa e pH ótimo para coagulação acima de 8. Este coagulante também não aumentou a concentração de carbono orgânico residual.

Ghebremichael *et al.* em 2005, purificaram por um método simples uma proteína catiônica (MOCP) com pI maior do que 9,6 e massa molecular de 6,5 kDa. Estes estudos sugerem que componentes extraídos em soluções aquosas ou salinas são de naturezas diferentes.

O poder coagulante das sementes de *M. oleifera* tem sido aplicado na remoção de componentes diferentes em soluções aquosas e suspensões. Beltrán-Heredia *et al*. (2011) relataram que o extrato de sementes atua na remoção do surfactante aniônico lauril sulfato de sódio em soluções aquosas. Sharma *et al*. (2006) relataram a capacidade do pó das sementes em remover o cádmio [Cd (II)] por biossorção. Sementes de *M. oleifera* também foram testadas como adsorventes para a remoção de íons de prata (Ag^{I}) (Araújo *et al*., 2010). Sementes descascadas também demonstraram capacidade de descontaminar água que contém arsênico e pode ser usada como uma tecnologia doméstica segura e que não causa danos para o meio-ambiente (Kumari *et al*., 2006).

O interesse por coagulantes naturais tem ressurgido devido a serem biodegradáveis, seguros para a saúde humana e de baixo custo.

1.4.2. Lectinas de sementes de *M. oleifera*

O primeiro trabalho descrevendo a presença de lectina em sementes de *M. oleifera* foi reportado por Santos *et al.* (2005). Neste trabalho demonstrou-se a presença de uma lectina solúvel em água (WSMoL) em preparações obtidas após imersão de sementes intactas em água após 5, 15 e 37 h. As preparações foram principalmente ativas com células de coelho em pH 4,5 e reconhecem frutose e tiroglobulina de porco. WSMoL foi isolada por cromatografia em quitina e teve sua sequência N-terminal determinada (QAVQLTHQQQGQVGPQQVR). A sequência mostrou significante similaridade (70%) com $M0_{2.1}$ e $M0_{2.2}$, proteínas de *M. oleifera* (Coelho *et al*., 2009). Katre *et al.* (2008) reportaram a presença de MoL (Lectina de *M. oleifera*) uma proteína catiônica formada por subunidades de 7,1 kDa em presença de 2-mercaptoetanol, no entanto na ausência do mesmo duas bandas de 13.6 e 27.1 kDa aparecem. A proteína foi isolada por cromatografias em DEAE-Celulose e CM-Sephadex. Santos *et al*. em 2009 purificaram uma lectina de natureza catiônica, com atividade coagulante, formada por subunidades de 26,5 e 14,9 kDa. A lectina foi purificada por cromatografia de afinidade em gel de guar e chamada cMoL (Lectina coagulante de *M. oleifera*). WSMoL e cMoL apresentam atividade inseticida contra *Aedes aegypti* e *Anagasta kuehniella,* respectivamente (Coelho *et al*., 2009; Oliveira *et al*., 2011). WSMoL matou larvas L_4 de *A. aegypt*i promovendo alterações morfológicas em seu trato digestivo como hipertrofia dos segmentos, aumento do lúmen intestinal e rompimento da camada que delimita o epitélio (Coelho *et al*., 2009). WSMoL também apresentou efeito estimulante de oviposição para fêmeas grávidas de *A. aegypti* e reduziu a eclodibilidade dos ovos por matar os embriões (Santos *et al*., 2012a). cMoL causou distúrbios nutricionais e atrasou o desenvolvimento de larvas de *A. kuehniella* bem como reduziu o peso e a sobrevivência das pupas (Oliveira *et al*., 2011).

Adicionalmente, Ferreira *et al*. (2011) relataram que WSMoL também apresenta atividade coagulante e antibacteriana.

Rolim *et al*. (2011) investigaram os efeitos genotóxico e mutagênico do extrato aquoso e de WSMoL. A avaliação revelou que o extrato na concentração popularmente utilizada (0,2 μg/μL) e a lectina não foram mutagênicos nem genotóxicos. Por outro lado, o extrato em concentrações correspondentes a 4 e 7,5 vezes aquela usada pela população apresentou efeito mutagênico indicando que a concentração usual não deve ser aumentada.

Sementes de *M. oleifera* têm sido apontadas como rica fonte de proteínas bioativas, inclusive lectinas que, de acordo com a literatura desempenham diversas atividades biológicas. O presente trabalho descreve a investigação das características estruturais de cMoL e suas potenciais funções biológicas.

2. OBJETIVOS

2.1. Geral

Caracterizar estruturalmente a lectina coagulante de sementes de *M. oleifera* (cMoL), bem como investigar sua atividade na coagulação sanguínea *in vitro* e seu potencial citotóxico em células de melanoma murino B16-F10 e fibroblastos humanos normais.

2.2. Específicos

- Avaliar o grau de pureza da lectina utilizando cromatografia líquida de alta eficiência (HPLC);
- Obter a estrutura primária de cMoL;
- Estimar a estrutura secundária;
- Verificar possíveis modificações estruturais em função da variação do pH e temperatura utilizando dicroísmo circular;
- Caracterizar a atividade sob os parâmetros hemostáticos: avaliação dos Tempos de Protrombina (TP) e de Tromboplastina Parcial ativada (TTPa);
- Verificar a influência de cMoL na viabilidade e morte de células B16-F10;
- Avaliar a indução de apoptose em células B16-F10 através da determinação da ativação de caspases;
- Avaliar a participação mitocondrial na morte celular;
- Verificar produção de espécies reativas de oxigênio (EROs);
- Analisar a especificidade de ação da lectina determinando sua citotoxicidade sobre fibroblastos humanos normais (GN);

- Revisar os conceitos dos processos de coagulação, floculação, aglutinação e hemaglutinação; propor um mecanismo de ação para a lectina coagulante de *M. oleifera*.

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4. CAPÍTULO 1

Structural characterization of coagulant *Moringa oleifera* Lectin and its effect on hemostatic parameters

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ABSTRACT

Lectins are carbohydrate recognition proteins. cMoL, a coagulant *Moringa oleifera* Lectin, was isolated from seeds of the plant. Structural studies revealed a heat-stable and pH resistant protein with 101 amino acids, 11.67 theoretical pI and 81% similarity with a *M. oleifera* flocculent protein. Secondary structure content was estimated as 46% α-helix, 12% β-sheets, 17% β-turns and 25% unordered structures belonging to the α/β tertiary structure class. cMoL significantly prolonged the time required for blood coagulation, activated partial thromboplastin (aPTT) and prothrombin times (PT), but was not so effective in prolonging aPTT in asialofetuin presence. cMoL acted as an anticoagulant protein on *in vitro* blood coagulation parameters and at least on aPTT, the lectin interacted through the carbohydrate recognition domain.

1. Introduction

Lectins are a group of proteins which recognize and bind mono- and oligosaccharides [1,2]. They can bind to carbohydrate moieties on the surface of erythrocytes and agglutinate the cells, without altering the carbohydrate structure [3]. Lectins exist in viruses and all forms of life, however the best known are extracted from plants, especially seeds, organ of stock, which is a major source to obtain these molecules [4]. Hundreds of lectins have been purified and their sugar specificities identified, which has enabled their development into powerful tools for the purification, separation, and structural analysis of glicoproteins [5], as well as recognition molecules inside cells, on cell surfaces, and in physiological fluids [6]. They have shown inhibitory activity against fungi and bacteria [7,8], insects [9], viruses [10] and cytotoxic effects against tumor cells lines [11].

Physical-chemistry characterization of lectins is important to explain the function in different biological processes [12]. Structural biology of macromolecules searches products potentially useful for solving biochemical problems and eventually develops new therapeutical agents [13]. To study structures of macromolecule, spectroscopic techniques such as circular dichroism (CD) is used, where optically active substances will absorb differently left and right circularly polarized light, and the difference in absorption of components is measured. The method has been extensively used to unravel secondary structure of proteins giving information on the effect of added ligands [14]. Proteins with high α-helical content shows CD spectrum in the far UV region as two negative CD bands around 208–210 nm and 222–228 nm as well as one positive band near 190–195 nm [15].

Moringa oleifera, known as horseradish or drumstick tree, is widely found throughout India, Asia, some parts of Africa and America, belonging to the Moringaceae family [16,17]. Its constituents such as leaf, flower, fruit and bark have been anecdotally used

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as herbal medicines in treatments for inflammation, paralysis and hypertension [18]. The seeds contain edible oils and water soluble substances (coagulant proteins) that can be used in drinking water clarification [19–22] or for treatment of selected dyeing effluents [23]. Lectins with coagulant properties were purified from *M. oleifera* seeds. Santos et al. [24] purified and partially characterized coagulant *M. oleifera* lectin, cMoL, the first lectin with coagulant properties for contaminants in water. cMoL is a basic protein, active at pH range 4.0–9.0 and complex sugar specificity which recognized mainly the glycoproteins azocasein and asialofetuin. WSMoL, Water-Soluble *M. oleifera* Lectin, was detected by Santos et al. [25] as an acid glycoprotein, with higher hemagglutination activity at pH 4.5, recognizing mainly fructose and porcine thyroglobulin. Ferreira et al. [26] referred that this lectin has coagulant activity and is a natural coagulant for contaminants in water, reducing turbidity and bacterial proliferation. WSMoL and cMoL showed insecticidal activity against *Aedes aegypti* [27] and *Anagasta kuehniella* [28], respectively. Katre et al. [29] reported the presence of a *M. oleifera* lectin, MoL, a homodimer with molecular mass of 14 kDa and subunits (7.1 kDa) linked by disulfide bond(s). MoL is also a glycoprotein with high stability and agglutinates human as well as rabbit erythrocytes.

cMoL is a protein with important biological activities [24,28], however its structural characterization was not completely elucidated. In this article, we report the primary structure, CD characterization and for the first time the anticoagulant properties of a coagulant lectin from *M. oleifera* on *in vitro* hemostatic parameters of human blood coagulation.

2. Materials and methods

2.1. Isolation of coagulant M. oleifera lectin (cMoL)

M. oleifera seeds were collected on the *campus* of Universidade Federal de Pernambuco, in Recife city, Northeast of Brazil. The extract, fraction and lectin were prepared according to Santos et al. [24], with modifications. Seeds were ground to flour, which was extracted with 0.15 M NaCl at room temperature (25 °C) for 6 h, and a saline extract was obtained. Proteins were precipitated with ammonium sulphate (60%) at room temperature for 4 h; the fraction obtained after centrifugation (12,000 × *g* for 20 min at 4 °C) was dialyzed against water and 0.15 M NaCl. The fraction was applied (10 mg of protein) on a guar gel column (10 cm × 1.0 cm) previously equilibrated (20 mL/h flow rate) with 0.1 M NaCl. cMoL was eluted with a saline gradient of 0.15, 0.3, 0.5 and 1 M NaCl. UV absorbance was used to monitor samples. cMoL active fractions eluted with 0.3 M NaCl were pooled, analyzed by HPLC and used in the experiments. cMoL protein concentration was evaluated according to Lowry et al. [30] using bovine serum albumin as standard at a range of 0–500 μg/mL and absorbance at 720 nm.

2.2. Reversed-phase HPLC

cMoL was subjected to reverse-phase column C4 on HPLC system (Shimadzu) for purity analysis. The column was equilibrated with solvent A [0.1% (v/v) trifluoroacetic acid (TFA) in H_2O] and eluted using solvent B (90% acetonitrile in 0.1% TFA) in a linear gradient, where B = 5% when t = 0 min, B = 5% at t = 5 min, B = 100% at t = 60 min, B = 0% when t = 65 min. The elution profile was monitored at 215 and 280 nm.

2.3. Hemagglutination activity (HA)

Glutaraldehyde-treated rabbit erythrocytes were obtained as described by Bing, Weyand, and Stavinsky [31]. The lectin (50 μL) was serially two-fold diluted in microtiter V-plates containing 0.15 M NaCl before addition of 50 μL 2.5% (v/v) suspension of treated rabbit erythrocytes. The results were read after about 45 min when the control, containing only erythrocytes fully precipitated, appeared as a dot at the bottom of the well. HA (inverse of the titer) was defined as the highest sample dilution showing full hemagglutination [4,32].

2.4. Primary sequence determination

Edman degradation [33] was performed with an automatic gas-phase sequencer (492cLC; *Applied Biosystems*) using conditions recommended by the manufacturer. Samples (0.8 mg/mL) for sequencing were reduced in 200 μL of 0.25 M Tris–HCl buffer, pH 8.5 containing 6 M guanidine–HCl, 1 mM EDTA and 5 mg of DTT, and alkylated with iodacetamide [34]. Then, the protein was separated by reversed phase cromatography HPLC. The similarity of sequences was searched using the BLAST protein sequence database [35] and the sequences were aligned with the MULTALIN program [36]. Theoretical pI was calculated by ExPASy ProtParam tool through the primary sequence of the protein.

2.5. Spectroscopic measurements

CD data were recorded on a Jasco J-810 spectropolarimeter. Samples were placed in a 0.5 mm path length circular quartz cuvette. Lectin concentration was 0.2 mg/mL in phosphate–borate–acetate (PBA) buffer for measurements in the far UV region (250–190 nm), as an average of 8 scans. Data were expressed in terms of mean residue ellipticity [θ]. The secondary structure estimative was calculated by deconvoluting the CD spectrum using the CDPro software package, which contains three CD analysis programs, CONTINLL, SELCON3 and CDSSTR [37]. The three programs were used with a reference protein set consisting of 56 proteins, thus increasing the reliability of deconvolution. Results were expressed as a mean of the three programs. CDPro package also contains the Cluster program that was used to determine the tertiary structure class of cMoL [38]. To study the pH effect on cMoL, the protein (0.2 mg/mL) was incubated in phosphate–borate–acetate buffer (PBA), 10 mM, for 10 min, at pH values of 2.0, 4.0, 6.0, 7.0, 8.0, 10 and 12. The temperature effect on cMoL secondary structure was also analyzed. Protein samples were heated at 40, 60, 80 and 100 °C for 30 min and at 100 °C for 1 h. CD measurements were recorded as described above.

2.6. Determination of activated partial thromboplastin time – aPTT and prothrombin time – PT

aPTT and PT were determined in a semi-automated coagulometer BFT II (Dade Behring). Total plasma was obtained by centrifugation of human blood samples at 1726 × *g*, for 15 min (25 °C). PT assay control was made with 50 μL of saline and 50 μL of plasma, incubation for 60 s, with subsequent addition of 100 μL of reagent (Thromborel S-Dade Behring). cMoL in different concentrations (3.0, 15, 30, 37.5, 45 and 60 μg/mL) in 50 μL was incubated (60 s) with 50 μL of plasma, followed by subsequent addition of 100 μL of reagent. aPTT assay control was made with 50 μL of saline, 50 μL of plasma and 50 μL of aPTT reagent (Dade actin activated cephaloplastin reagent-Dade Behring), incubation for 120 s and subsequent addition (50 μL) of 0.025 M calcium chloride. cMoL (3.0, 15, 30, 37.5, 45 and 60 μg/mL) was incubated for 120 s with 50 μL of plasma and 50 μL of the aPTT reagent, followed by addition of 50 μL of 0.025 M calcium chloride. The blood coagulation assays were also performed with cMoL (50 μL) inhibited by glycoprotein. cMoL was previously incubated with asialofetuin (0.5 mg/mL) for 15 min e then the assay proceeded as described above. Each assay was made in duplicate and results were expressed as average of

3 independent protocols (±s.e.m). All experiments were approved by the Ethics Committee of the Universidade Federal de São Paulo, number CEP 1793/11, according to Brazilian federal law.

2.7. Statistical analysis

Differences between means values were analyzed using one-way ANOVA followed by Tukey's multi-comparison test in the coagulation assays. A p value < 0.05 was considered significant.

3. Results and discussion

3.1. Structural analysis of cMoL

cMoL is a coagulant *M. oleifera* lectin, purified by Santos et al. [24] after saline extraction and guar gel column chromatography. The coagulant property of this lectin is related to reduction of contaminants in water; cMoL promoted turbidity reduction of approximately 92% (in relation to negative control) similar to the positive control aluminum sulphate on water with high and low turbidity of kaolin clay [24]. By hemagglutination assays, the protein is resistant to change in pH, thermostable, agglutinates erythrocytes from rabbit and human blood types [24] and have insecticidal activity [28]. By a new and simple protocol, Santos et al. [24] purified and partly characterized a lectin different from those already reported, WSMoL [25] and MoL [29]. The knowledge of the structure and physicochemical properties of cMoL is needed to understand the mechanisms of action involved in the biological activities developed by the lectin and thus integrate the study of structure-function of the protein. Reverse phase chromatography was made to assess the purity of the fractions obtained in guar gel column. Fig. 1 shows the cMoL profile obtained by HPLC. The peak represents cMoL fractions from guar gel columm eluted with 0.3 M NaCl. The detection of a single peak shows homogeneity in the purification, so this step purified efficiently the lectin, biologically active.

Highly pure fractions of proteins are important for the performance of amino acid sequence and circular dichroism analysis. In this study, the complete sequence of cMoL was obtained and compared with other protein sequences of the NCBI-BLAST data bank; the search indicated similarity with the sequence of $M.O_{2.1}$, a flocculent active protein from *M. oleifera* [19]. These sequences were aligned using MULTALIN program (Fig. 2). The sequence revealed that cMoL is a protein with 101 amino acids, two chains, and has 81% of similarity with $M.O_{2.1}$, which presents 6.5 kDa and isoelectric point above pH 10 [19]. It is important to note that cMoL have eight cysteine residues, which may be involved in the disulfide bonds.

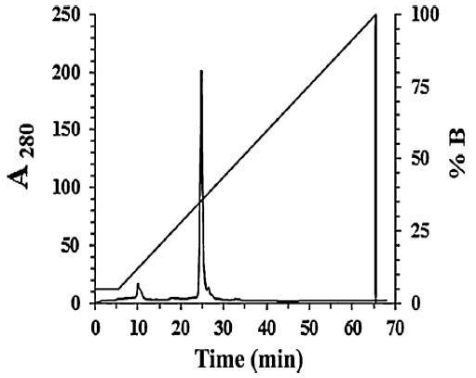

Fig. 1. cMoL profile by reverse phase chromatography using VYDAC C4 column in a HPLC system. Fractions eluted with 0.3 M NaCl on an affinity guar gel column were assessed for homogeneity evaluation. Absorbance was performed at 280 nm. The protein fraction was eluted using a linear gradient: solvent B (90% acetonitrile in 0.1% TFA), where B = 5% when t = 0 min, B = 5% at t = 5 min, B = 100% at t = 60 min, B = 0% when t = 65 min.

cMoL is a basic protein with a theoretical pI of 11.67 indicating a strong positive charge on the surface and confirming its cationic nature. High contents of glutamine (26.7%), alanine (6.9%), proline (6.9%) and 17 positively charged amino acids (16 arginines and 2 histidines) are also present in the lectin structure. Several protein families with flocculent/coagulant activity are present in *M. oleifera* seeds [19,39]. cMoL must develop its coagulant activity in water due to interactions among charges, as proposed to $MO_{2.1}$ [19,20] and not through binding to carbohydrate recognition site. Further studies on the genome and proteome of *M. oleifera* seeds could contribute to unravel the function of products from a unique gene(s) in the plant [24].

The native cMoL molecular weight obtained by Santos et al. [24] through Sephacryl S-300 size exclusion chromatography revealed a single peak corresponding to an apparent molecular mass of 30,000 Da; however, the molecular weight estimated to cMoL through the primary sequence revealed that the protein showed 11,928 Da. These results suggested that cMoL is a trimer consisting of three subunits of 11,928 Da. cMoL is not a glycosylated protein, which was confirmed through Schiff's reagent staining by Santos et al. [24].

Analyses of CD spectrum shows a typical shape and features of a mainly α-helical secondary structure, *i.e.*, two negative bands at 222 and 209 nm, and a positive band at 192 nm (Fig. 3). The secondary structure content of cMoL was estimated at 46% α-helix, 12% β-sheets, 17% β-turns and 25% unordered structures with root mean

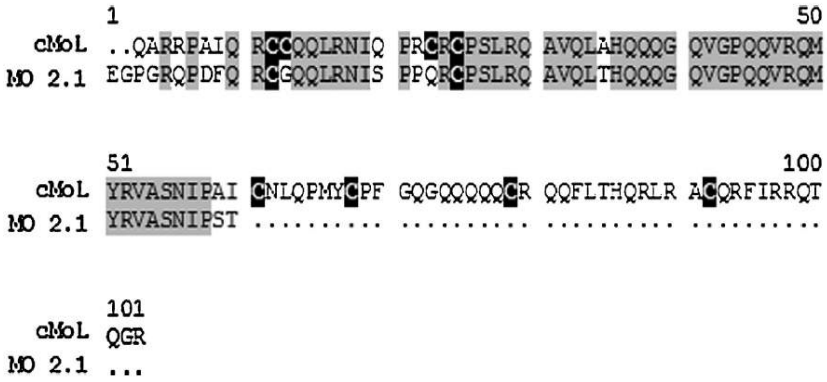

Fig. 2. Analysis of cMoL sequence and multiple sequence alignment of cMoL with flocculent *M. oleifera* protein $MO_{2.1}$ (2111235A). Identical residues among them are displayed in gray background. The cysteine residues of both proteins are indicated in black background.

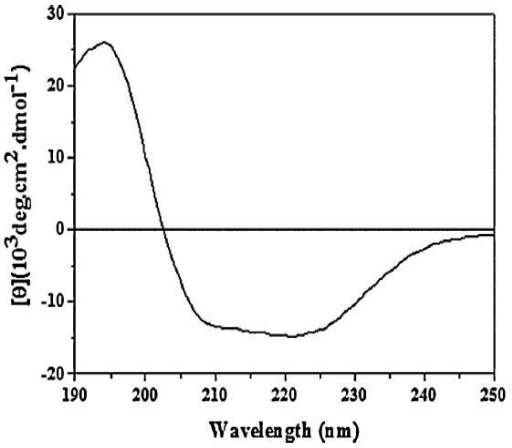

Fig. 3. CD spectrum of cMoL in 10 mM PBA, pH 7.0. Measurements were recorded as an average of 8 scans for protein solutions of 0.2 mg/mL, at 25 °C. CD spectrum deconvolution indicated 46% α-helix, 12% β-sheet, 17% β-turn, 25% unordered structure.

square deviation (RMS) lower than 2% for all structures. The Cluster analysis showed that cMoL belongs to the α/β tertiary structure class, corroborated by results of secondary structure estimative. For α/β proteins, the 222 nm band generally has a higher intensity than 208–210 nm band, whereas for the α+β proteins the reverse is observed [38]. The content of α-helices estimated for cMoL was similar to that determined for MO, the coagulant protein purified by Ndabigengesere and Narasiah [40]. Kwaambwa and Maikokera [41] reported that this protein presents secondary structural components of 58% α-helix, 10% β-sheet and 33% unordered structures, with RMS lower than 4% for all structures. But different from MoL, an alpha–beta lectin, isolated by Katre et al. [29] which containing 28% α-helix, 23% β-sheet, 20% turn and 28% of unordered structure. The secondary structure content of cMoL and MoL revealed that these lectins have different content of secondary structures but consist of a mixture of alpha and beta structures.

Generally, the functional stability is accompanied by structural stability. Fig. 4a shows CD spectra of cMoL in pH values of 2.0, 7.0 and 12. The secondary structure of the protein did not change in acidic and alkaline conditions as shown in the CD spectra, corroborating with the data on the stability of biological activity in function of pH. The content of each secondary structure of cMoL at different pH range is shown in Table 1. No significant change due to pH was observed in the estimated secondary structures from CD spectra. Sample spectra from other pH values were omitted since

Table 1
cMoL secondary structure estimated from CD spectra at different pH values.

pH	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Unordered (%)
2.0	43	13	18	26
7.0	46	12	17	25
12.0	39	15	19	27

Quantitative predictions were performed using CDPro software.

Table 2
cMoL secondary structure estimated from CD spectra at different temperatures.

Temperatures	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Unordered (%)
40 °C	51	9	16	25
60 °C	51	8	16	25
80 °C	42	10	21	27
100 °C (30 min)	18	25	23	33
100 °C (1 h)	7	26	27	40

Quantitative predictions were performed using CDPro software.

small changes were detected. cMoL and MoL have as a common feature activity in acidic and alkaline conditions [24,29]. Other lectin with similar pH stability is a chitin-binding lectin from rhizomes of *Setcreasea purpurea* (SPL). SPL is a homotetrameric protein with hemagglutinating activity stable in pH range of 2.0–9.0 [42].

Treating cMoL at 100 °C and subsequent evaluation by HA revealed the lectin as a thermostable protein [24], however by accurate CD analysis, the protein conformation is maintained only until 80 °C for 30 min (Fig. 4b). When cMoL was subjected to heating at 80 °C, change in α-helix content was verified, followed by a gentle increase of β-structures while unordered structures did not alter. The estimated unordered structure content increased when cMoL was heated at 100 °C for 30 min and 1 h (Table 2). High temperature is a powerful denaturing agent leading to protein unfolding through breaking of hydrogen bonds that maintain protein structure [43]. Thermostability is a common feature of proteins from *M. oleifera* seeds. MOCP, a coagulant protein isolated by Ghebremichael et al. [21] remained active after 5 h heat treatment at 95 °C. Katre et al. [29] also reported that MoL is a heat-stable protein, which retains the activity even after incubating at 85 °C up to 30 min at pH 7.2.

3.2. cMoL effect on hemostatic parameters

The influence of cMoL on blood coagulation was determined by activated partial thromboplastin time (aPTT) and prothrombin time (PT) (Fig. 5). The positive control used in the assays was plasma of healthy volunteer donors with normal values for clotting times. A variety of new anticoagulants are being developed and tested

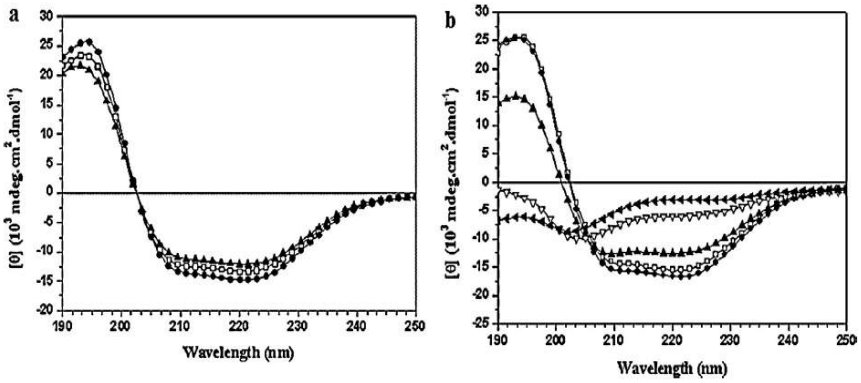

Fig. 4. (a) CD spectra of cMoL (0.2 mg/mL), in 10 mM PBA buffer, at pH 2.0 (□), 7.0 (●) and 12 (▲) and (b) cMoL spectra after heating at 40 (□), 60 (●), 80 (▲) and 100 °C for 30 min (▽). The spectra after heating at 100 °C for 1 h is also represented (◄).

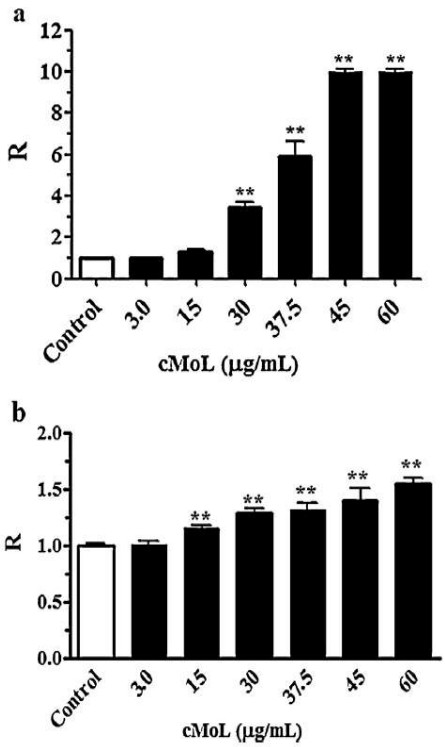

Fig. 5. *In vitro* effect of cMoL in hemostatic parameters. Activated partial thromboplastin time (aPTT, a) and prothrombin time (PT, b) were determined. The statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. A p-value < 0.05 was considered to indicate significance. p-Value < 0.05 (*) and p-Value < 0.001 (**). R: ratio of sample coagulation time with control coagulation time. Data represent means ± s.e.m., $n = 3$.

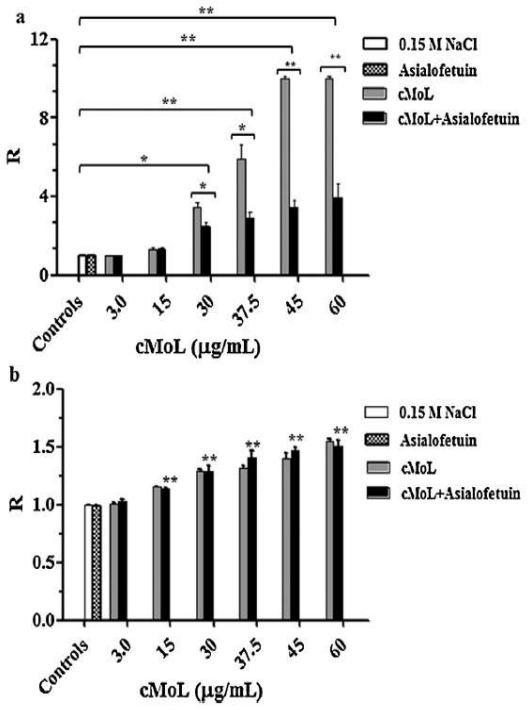

Fig. 6. Blood coagulation assays in the presence of cMoL inhibited by asialofetuin (0.5 mg/mL). Lectin was previously incubated (15 min) with asialofetuin. (a) Activated partial thromboplastin time (aPTT); (b) prothrombin time (PT). The statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. A p-value < 0.05 was considered to indicate significance. $p < 0.05$ (*) and $p < 0.001$ (**) represent comparative analysis between cMoL inhibited by asialofetuin and controls. Statistical significance of cMoL in the presence and absence of glycoprotein was also showed. R: ratio of sample coagulation time with control coagulation time. Data represent means ± s.e.m., $n = 3$.

to inhibit the various steps in the coagulation cascade [44]. The determination of aPTT is particularly useful in monitoring the effect of heparin and determining deficiencies of factors VIII, IX, XI and XII. TP reflects the activity of factor II (prothrombin), V, VII and X, whose deficiency is accompanied by a prolongation of time required for clot formation [45].

cMoL significantly prolonged aPTT (to more than 300 s) and PT (Fig. 5b) coagulation time, at the assayed concentrations (3.0, 15, 30, 37.5, 45 and 60 µg/mL) but the most affected parameter was aPTT (Fig. 5a). Prolongation of aPTT suggests inhibition of the intrinsic pathway and/or common blood coagulation, while prolongation of PT suggests inhibition of the extrinsic pathway [46]. The intrinsic and extrinsic pathways converge in the formation of factor Xa [47]. Other lectins also significantly prolongs both coagulation times. *Cratylia mollis* seed lectin (Cramoll 1,4), a mannose/glucose binding lectin, promotes almost two-fold increase of coagulation times [48]. *Bauhinia forficata* lectin, BfL, increased only aPTT coagulation time but this effect was not related to human plasma kallikrein or human factor Xa inhibition [49]. Thus cMoL showed an anticoagulant activity, since in determining the R, the ratio between sample coagulation time and control coagulation time [50] was higher than 1.0.

Significant correlation between recognition and binding of lectins to carbohydrates or glycoconjugates on cell surface or free in biological fluids are involved in many lectin effects, such as activity against insects, viruses, bacteria and cytotoxicity [27,42,51,52]. Then, to investigate cMoL interaction with coagulation factors, blood coagulation assays were performed in the presence of asialofetuin. This glycoprotein blocks the site of carbohydrate recognition of the lectin. aPTT assays showed a reduction in the time required for blood coagulation in the presence of the inhibited lectin by glycoprotein in the same assayed concentrations (Fig. 6a), while PT was not affected (Fig. 6b). These results suggest that cMoL interacts with the carbohydrate portion of serine proteases of the coagulation cascade from intrinsic pathway. No decrease in PT in the presence of inhibited lectin suggests that the lectin should act on the factors of the extrinsic pathway and/or under the factor Xa by a different domain from the carbohydrate recognition.

4. Conclusions

In this article structural characteristics of cMoL were described; also, for the first time, the anticoagulant activity was detected on human blood coagulation process by a coagulant *M. oleifera* seed lectin. Structural analysis revealed cMoL common features with another coagulant protein from *M. oleifera*. cMoL interactions in blood coagulation must occur at least partially, by the carbohydrate recognition domain. cMoL is the only currently described protein from *M. oleifera* seeds that exhibits anticoagulant activity.

Author contributions

Conceived and designed the experiments: LAL, MCCS, RSF, MLVO, LCBBC. Performed the experiments: LAL, MCCS, RSF, LAS, RM. Contributed to reagents and analysis tools: RM, MLVO, PMGP, LCBBC. Analyzed and interpreted the data: LAL, MCCS, RSF, RASL, MLVO, PMGP, LCBBC. Wrote the paper: LAL, MCCS, RASL, PMGP and LCBBC. All authors read and approved the final manuscript.

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5. CAPÍTULO 2

Cytotoxicity by coagulant *Moringa oleifera* lectin (cMoL) in B16-F10 melanoma cells

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Cytotoxicity by coagulant *Moringa oleifera* lectin (cMoL) in B16-F10 melanoma cells

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ABSTRACT

Ethnopharmacological relevance: Different parts of *Moringa oleifera* tree are widely used as edible vegetal; seeds are especially used in folk medicine to treat many diseases and for water treatment by populations of tropics and subtropics regions around the world.

Aim of the study: To examine the cytotoxic and apoptosis inducing effects of coagulant *M. oleifera* lectin (cMoL) on B16-F10 murine melanoma cells.

Materials and methods: cMoL cytotoxic effect was studied by testing the cell viability using trypan blue assay and cell death by flow cytometry. To investigate the mitochondrial involvement it was performed the estimative of mitochondrial membrane potential ($\Delta\Psi m$). Production of Reactive Oxygen Species (ROS) and caspases activation were also measured. Cell images after treatment were captured for morphological analysis. Citotocity to normal human fibroblasts was also evaluated.

Results: cMoL reduces cell viability and causes cell death (47.6%) in melanoma cells when compared with control cells in the concentration of 250 µg/mL after 48 h treatment. At this same concentration, the lectin shows higher specificity for tumor cells, since normal human fibroblasts (GN) presented approximately 12.6% cell death. cMoL increased ROS production and promoted caspases 3, 8 and 9 activation which characterizes apoptotic cell death and involvement of both extrinsic and intrinsic pathways. The images corroborate with these results showing cells in apoptotic process. Furthermore, the cell death was independent of mitochondrial permeability transition (MPT).

Conclusion: This study demonstrate for the first time the cytotoxic effects of cMoL especially against tumor cell line by induction of apoptosis.

1. INTRODUCTION

Moringa oleifera (Lam) or "drumstick tree" is a medium-sized tree autochthonous from north-west India and now widely distributed throughout the tropics and subtropics regions, occurring in Asia, Africa and America (Bhatia *et al*., 2007, Teixeira *et al*., 2012). Moringa is one of the 14 known species of the monogeneric family Moringaceae and it is generally known in the developing world as a vegetable, a medicinal plant, and a source of vegetable oil (Katayon *et al*., 2006). It is one of the 11 species of plants more used in Bangladeshi folk medicine (Costa-Lotufo *et al*., 2005); seeds and different parts of the plant have long been used to treat diseases such as abdominal tumors, hysteria, scurvy, paralytic attacks, helmintic bladder, prostate problems, sores and skin infections (Anwar *et al*., 2007; Fuglie, 1999). Seed extract already demonstrated reduction in liver fibrosis in rats (Hamza, 2011) and from a seed ethanolic extract a compound was isolated with potent antitumor effect in chemical carcinogenesis (Guevara *et al*., 1999). Furthermore, ethanolic and saline extracts from distinct *M. oleifera* tissues are potential sources of antioxidants (Santos *et al*., 2012). *M. oleifera* seeds are well-known to have coagulant proteins used in water and wastewater treatment (Gassenschmidt *et al*., 1995; Ndabigengesere *et al*., 1995; Ghebremichael *et al*., 2005; Vieira *et al*., 2010). A coagulant *M. oleifera* lectin (cMoL) was isolated from seeds. The lectin is basic, pH resistant and a trimer consisting of three subunits of 11,928 Da; also, belongs to the α/β tertiary structure class (Luz *et al*., 2013). cMoL already demonstrated coagulant properties for contaminants in water (Santos *et al*., 2009), insecticidal action against *Anagasta kuehniella* (Oliveira *et al*., 2011) and anticoagulant properties on hemostatic parameters of human blood coagulation (Luz *et al*., 2013).

Lectins cytotoxic for tumor cells have been reported over the years, and some of them have already being used for alternative cancer therapy or as adjuvant agents, during the chemotherapy and radiotherapy in Europe (Schumacher *et al*., 2003).

Chemicals as well as biological agents that induce apoptosis have been reported to be promising interventions in the management of malignant cancer (Sreelatha *et al*., 2011). Mitochondria play an important role in the cell death process since they regulate energy production and execution of cell death (Fulda and Kroemer, 2011). Several conditions that lead to mitochondrial injury with functional impairment, such as lipid peroxidation of the inner membrane or mitochondrial permeability transition (MPT) cause cell death either by necrosis or apoptosis (Green and Reed, 1998; Kroemer *et al*., 1998; Fiers *et al*., 1999). The MPT is a non-selective permeabilization of the inner mitochondrial membrane which results in loss of components of the mitochondrial matrix, impaired mitochondrial function with consequent disruption of the outer mitochondrial membrane and release of proteins from intermembrane space (Zoratti e Szabo, 1995; Lemasters *et al*., 1998; Kowaltowski *et al*., 2001).

In the present work we reported the *in vitro* cytotoxic effects and apoptosis-inducing activities of cMoL using B16-F10 melanoma tumor cells. In addition it was evaluated the citotoxicity of the lectin to normal human fibroblasts.

2. MATERIAL AND METHODS

2.1 Chemicals

Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (CCCP), adenosine 5-diphosphate (ADP) and carboxyatractiloside (CAT) were purchased from Calbiochem of EMD Chemicals Inc; digitonin was obtained from Sigma (ST. Louis, MO, USA). All other chemicals were standard commercial products of reagent-grade quality.

2.2 Lectin purification

cMoL was obtained in accordance with Santos *et al*. (2009) with modifications. Seed flour was extracted with 0.15 M NaCl at room temperature (25°C) for 6 h. The proteins present in the extract were precipitated with ammonium sulphate at 25°C for 4 h and a protein fraction was obtained. The fraction was applied (10 mg of protein) on a guar gel column (10 cm × 1.0 cm) previously equilibrated (20 mL/h flow rate) with 0.1 M NaCl. cMoL was eluted with a saline gradient of 0.15, 0.3, 0.5 and 1 M NaCl. UV absorbance was used to monitor samples; fractions eluted with 0.3 M NaCl were pooled and used in the experiments. cMoL protein concentration was evaluated according to Lowry *et al*. (1951) using bovine serum albumin as standard at a range of 0–500 µg/mL and absorbance at 720 nm and hemagglutinating activity (HA).

2.3 Cell culture

B16-F10 cells were obtained from American Type Culture Collection, USA and cultured in plastic bottles 75 cm^2 (TPP, Trasadingen, Switzerland) in RPMI 1640 (Vitrocell, Brazil) supplemented with 10% fetal bovine serum (FBS, Vitrocell), 100 µg/mL gentamycin (Vitrocell), 100 IU/mL penicillin (Vitrocell), and 100 µg/mL streptomycin (Vitrocell) at 37°C in a humidified atmosphere with 5% CO_2. The cells used in the experiments were subcultured when reached a confluency of approximately 70-80% and subcultured up to ten passages.

The primary fibroblasts all line GN were obtained from normal gingival biopsy and kindly provided by Dr. Ricardo Della Coletta (University of Campinas, Piracicaba, UNICAMP). GN cell line was grown in the DMEM containing high glucose supplemented with 10% FBS (Vitrocell), 100 µg/mL gentamycin (Vitrocell), 100 IU/mL penicillin (Vitrocell), and 100 µg/mL streptomycin (Vitrocell) at 37°C in a humidified atmosphere with 5% CO_2.

2.4 Cell Viability

To determine the anti-proliferative and cytotoxic effects of cMoL, cells were stained with 0.1% trypan blue and counted in a Neubauer chamber. Viability was determined by exclusion of stained cells. Briefly, B16-F10 cells were plated in 6-well culture plates (3.5×10^4 cells per well) and after 24 h, the medium was replaced and cells were treated with lectin in different concentrations (50, 100, 250 and 500 µg/mL). After further 48 h, cells were washed in PBS, trypsinyzed, centrifuged and resuspended in 300 µL of medium containing FBS. The total number of cells, the number of viable cells (non-stained) and inviable cells (stained blue) were counted. Cell viability was expressed as the percentage of viable cells relative to control.

2.5 Analysis of Cell Death

Samples were analyzed in a FACSCalibur flow cytometer (BD Biosciences, USA) equipped with an argon laser and Cell-Quest software (version 4.1). Ten thousand events were acquired for each sample. B16-F10 populations were analyzed for the intensity of the fluorescent probe signals, in FL1 and FL2 channels. Cells (10^6) were washed with PBS and resuspended in a binding buffer (10mM HEPES pH 7.4, 150mM NaCl, 5mM KCl, 1mM $MgCl_2$, and 1.8mM $CaCl_2$) for the analysis of cell death. Then cells were incubated for 20 min, at 25°C in the dark, with annexin V-FITC (0.2 µg/mL, 1:500, Invitrogen, USA) and propidium iodide (0.2 µg/mL, 1:500, Sigma). Apoptosis

was quantified by FACS analysis as the number of annexin V-FITC-positive and PI-negative cells divided by the total cell number, while necrosis was quantified as the number of PI-positive cells and annexin V-FITC negative cells divided by the total cell number.

2.6 Determination of Reactive Oxygen Species (ROS)

Cells were treated with cMoL (250 µg/mL) for 6 h, then harvested and staining with 5 µM MitoSox (Molecular Probes) at 37°C, 10 min, to quantify mitochondrial superoxide generation. The samples were analyzed in the FL-2 channel of the flow cytometer (Payne *et al*., 2007).

2.7 Detection of Caspase 3, 8 and 9 activation

To determine caspase 3, 8 and 9 activation cells were treated with 250 µg/mL of cMoL; after 48 h, 10^6 cells were stained with fluorescent markers FITC-DEVD-FMK, FITC-IETD-FMK or FITC-LEHD-FMK (1:300, Calbiochem, USA) in serum-free medium for 40 min at 37°C in a humidified atmosphere with 5% CO_2. Then, cells were washed, resuspended in the same medium and analyzed by flow cytometry, according to the manufacturer instructions. B16-F10 cells treated with 1.25 µg/mL cycloheximide (Sigma) and 10 nM tumor necrosis factor α (TNFα, Peprotech, USA) for 24 h were used as a positive control.

2.8 Determination of mitochondrial membrane potential (ΔΨm) in digitonin-permeabilized B16-F10 cells

Mitochondrial membrane potential (ΔΨm) in digitonin permeabilized cells was estimated as change in fluorescence of safranine O (Figueira *et al*., 2012), recorded using a spectrofluorometer (Hitachi, model F4500, Tokyo, Japan), operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit width of 5 nm. After 48 h of treatment with cMoL (250 µg/mL), B16-F10 viable cells ($2x10^6$

cells/mL) were permeabilized with 15 mM digitonin (Campos *et al*., 2004) in 2 mL of standard reaction medium (125 mM sucrose, 65 mM KCl, 10 mM de HEPES, 10 mM Tris–HCl (pH 7.2), 2 mM Na_2HPO_4, 1 mM de magnesium, 0.33 mM EGTA, complex I substrates (2 mM malate, α-ketoglutarate, 1 mM pyruvate and 1 mM glutamate), 0.01 % BSA and 5 µM safranine O) under constant stirring at 37ºC.

2.9 Observation of cellular morphologic changes

B16-F10 melanoma cells (3.5 x 10^4 cells/mL) were incubated with increasing concentrations of cMoL (50, 100, 250 and 500 µg/mL) for 48 h and photographed under a microscope Leica DFC360 FX, using LAS AF software (Leica Microsystems).

2.10 Data analysis

All results were expressed as the mean ± the standard error of the mean (SEM) for at least five independent experiments. Differences between means values were analyzed using one-way ANOVA followed by Tukey's multi-comparison test. A p value < 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1 cMoL reduces cell viability and causes cell death on B16-F10 melanoma cells

M. oleifera is a plant broadly used in folk medicine which lack experimental studies about cytotoxicity of seed compounds. In the present study, the effect of cMoL on the viability and cell death of B16-F10 cells was followed using trypan blue exclusion assay and flow cytometry after 48 h of exposure in culture. The treatment with increasing concentrations of cMoL (50, 100, 250 and 500 µg/mL) significantly reduced cell viability (44% ± S.E.M at 250 µg/mL) as shown by the reduction of viable cells **(Fig. 1A and B)**.

To better characterize the cell death, the rates of apoptosis and necrosis were analyzed by annexin V and propidium iodide staining. Cells treated with increasing concentrations of cMoL showed higher percentage of double-positive cells (Anx V^{pos}/ PI^{pos}) indicating apoptotic cell death **(Fig. 2)**. Since cMoL induced 47.6% ± S.E.M. cell death in B16-F10 cells at 250 µg/mL, this treatment was established to the following experiments. Furthermore, when cells were cultured with the concentrations mentioned above for 48 h, marked apoptotic morphologic alterations were gradually observed by optical microscopy. **Figure 3** shows decrease in cell number and morphological changes as rounded cells, reduction of cellular volume and formation of apoptotic bodies (indicated by arrows). Apoptosis is accompanied by cell rounding-up, retraction of pseudopodes, reduction of cellular volume (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), classically little or no ultrastructural modifications of cytoplasmic organelles and plasma membrane blebbing (Kroemer *et al*., 2009). Altogether, these results suggest that cMoL induces apoptotic cell death in B16-F10 melanoma cells.

3.2 cMoL revealed lower cytotoxicity for normal cell line

The effect of cMoL on normal fibroblast cell line (GN) was also analyzed. In contrast with the results showed to B16-F10 melanoma cells, GN cell line showed approximately 12.6% ± S.E.M of cell death when treated with 250 µg/mL of cMoL for 48 h **(Fig. 4).** These results suggest that the lectin bears a greater susceptibility for malignant cells. Lectin binding to cell surface receptors is known to trigger cell signaling and to play a key role in displaying the cytotoxic activity of lectin molecules against tumor or normal cells. In other words structurally different lectins can interact with identical carbohydrate ligands and exhibit different biological effects (Wang *et al*., 2000). Ohba *et al*. (2003) reported the different expression of biological activity by

wheat germ agglutinin isolectins (WGA-1, WGA-2 and WGA-3) against normal lymphocytes and cultured leukemic cell lines (Jurkat, MOLT-4, Raji, Daudi and K-562). The isolectins interacted in different degrees with leukemic cells and manifested significantly different cytoagglutinating and cytotoxic activities against assayed cells.

3.3 cMoL induces apoptosis in a caspase-dependent manner

Caspases are a specific class of cysteine proteases involved in the signaling cascade of cell death leading to the discrimination between caspase-dependent and caspase-independent cell death (Galluzzi *et al*., 2011). cMoL induces apoptotic cell death in a caspase-dependent manner. The results show activation of caspases 3, 8 and 9 **(Fig. 5)**. The activation of caspase 3 confirms apoptotic cell death; activation of caspase 8 and 9 reflects the involvement of both extrinsic and intrinsic signaling pathways, respectively. *Polygonatum odoratum* lectin (POL) is a mannose-binding specific GNA-related lectin which possesses antiproliferative activity toward murine fibrosarcoma L929 cells. This lectin was also able to promote cell death with participation of both caspase 8 and 9 (Liu *et al*., 2009f). The cascade involved in extrinsic and intrinsic pathways are different, however both can be merged into caspase activation. To initiate extrinsic or death receptor pathway of apoptotic cell death it is necessary that extracellular stimuli such as UV, X-ray and cytotoxin occur which promotes combination between Fas-L (Fas ligand) and Fas (membrane receptor), thus forming a death complex, resulting in the intracellular activation of initiator caspase 8 and executioner caspase 3 and 6 (Lavrik *et al*., 2005; Wajant, 2002). cMoL must be or induce the formation of substances that act as potent stimulus for activation of extrinsic pathway of cell death.

3.3 ROS production by B16-F10 cell line

To better understand the mechanisms involved in the apoptotic cell death promoted by cMoL, the rates of reactive oxygen production by mitochondria were estimated by the MitoSox method in the initial periods of treatment. After 6 h, cMoL promoted a two-fold increase of mitochondrial ROS **(Fig. 6)**. *Polygonatum cyrtonema* lectin (PCL) also induces cell death by increasing ROS levels. PCL induces apoptosis and autophagy via a mitochondrial-mediated ROS–p38–p53 pathway (Liu *et al*., 2009b). Apoptosis is a major pathway of cell death which can be activated in response to various intracellular and extracellular signals, where mitochondria may participate direct or indirectly in activation of this death. Numerous pro-apoptotic signals are stimulated by several factors, among them, increased ROS production, that in turn characterizes the condition of oxidative stress which can precede cell death (Kowaltowski and Vercesi, 1999). The increased levels of ROS may result in oxidations of amino acids in proteins, polydesaturated fatty acids in lipids, as well as DNA damage and apoptosis (Mazure and Pouysségur, 2010).

3.4 Mitochondrial membrane potential (ΔΨm)

It is already well established in the literature that intrinsic apoptosis pathway responds to a wide array of intracellular stress conditions such as DNA damage and oxidative damage controlled by mitochondria, whose permeabilization constitutes a point-of-no-return in the signaling pathway (Kroemer *et al.*, 2007). For this purpose, we investigated the mitochondrial involvement in B16-F10 cell death. As depicted in **Figure 7**, the mitochondrial membrane potential of the cells after treatment was maintained, and the organelle could phosphorylate ADP to ATP. These findings demonstrated that B16-F10 cell death was independent of MPT phenomenon and

suggest that caspase 9 activation may have been coordinated by Bcl-2 family members (Garrido *et al*., 2006).

4. CONCLUSIONS

In summary, it was reported for the first time that a lectin from *M. oleifera* seeds (cMoL) showed cytotoxic effects on a tumor cell line (B16-F10) and that this activity was less specific for normal cell lines such as human fibroblasts (GN). cMoL promoted reduction of the number of viable cells and induced apoptosis in a caspase-dependent manner. The cell death caused by cMoL on B16-F10 melanoma cells had no involvement of MPT.

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Figure captions

Figure 1. cMoL reduces B16-F10 melanoma cell viability. B16-F10 cells were treated with increasing concentrations of the lectin for 48 h. (A) Cell viability was expressed as the percentage of viable cells related to control. (B) After treatment the number of viable cells were determined using 0.1% trypan blue. The statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. Values are mean ± s.e.m. of at least five independent experiments. A p-value < 0.05 was considered to indicate significance. p-value < 0.05 (*****) and p-value < 0.001 (******).

Figure 2. cMoL induces cell death in B16-F10 melanoma cells. B16-F10 cells were treated with increasing concentrations of cMoL for 48 h and staning with annexin V and propidium iodide. The percentages of necrotic (PI+) or apoptotic (AnxV+) cells were determinate by flow cytometry. The statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. Values are mean ± s.e.m. of at least five independent experiments. A p-value < 0.05 (*) was considered to indicate significance.

Figure 3. Morphological alterations of B16-F10 melanoma cells after treatment with cMoL. (A) Control, (B) 50 µg/mL, (C) 100 µg/mL, (D) 250 µg/mL and (E) 500 µg/mL. Images were provided by a Leica DFC360 FX, using the LAS AF software (Leica Microsystems). Bars represent 100 µm.

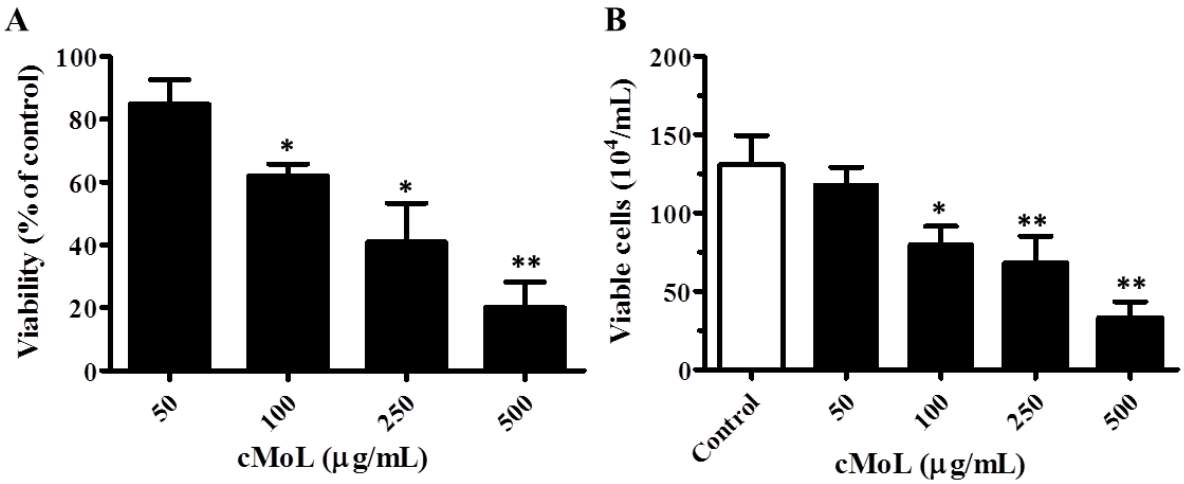
Figure 4. GN cell lines (human fibroblast) viability after cMoL treatment. After trypsinization, GN (3.5×10^4 cells/mL) were incubated in RPMI-1640 medium with 10% fetal bovine serum in the presence of increasing cMoL concentrations for 48 h. Statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. Data are the average ± s.e.m of five independent experiments.

Figure 5. cMoL treatment induces caspase 3 (A), 8 (B) and 9 (C) activation. Caspase activation was determined by flow cytometry using FITC-DEVD-FMK after 48 h of treatment with the lectin. Statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. Values are mean ± s.e.m of at least 5 independent experiments. p-value < 0.05 (*****) and p-value < 0.001 (******).

Figure 6. ROS levels in B16-F10 melanoma cells. Cells were treated with cMoL for 6h. Cells were washed and then probed with 5 µM MitoSox. ROS production was analyzed using flow cytometry. Statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. Values are mean ± s.e.m. of at least 5 independent experiments. *Significantly different from control at p < 0.05 (*) and p-value < 0.001 (******) level.

Figure 7. B16-F10 mitochondrial membrane potential ($\Delta\Psi m$) after exposure to cMoL for 48 h. Cells (2×10^6/mL) were added to a reaction medium containing PBS (pH 7.2), 1 mM $MgCl_2$, 5 µM safranine O and 5 mM succinate in a total volume of 2 mL. Black line represents control cells (untreated). Yellow line represents cells treated with cMoL. The arrows indicate additions of 15 mM digitonin, 200 µM ADP, 5 µM CAT and 1 mM CCCP. The figure is representative of four independent experiments.

Figure 1


A
Viability (% of control)
100
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*
*
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50
100
250
500
cMoL (µg/mL)
B
Viable cells (10^4/mL)
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50
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cMoL (µg/mL)

Figure 2

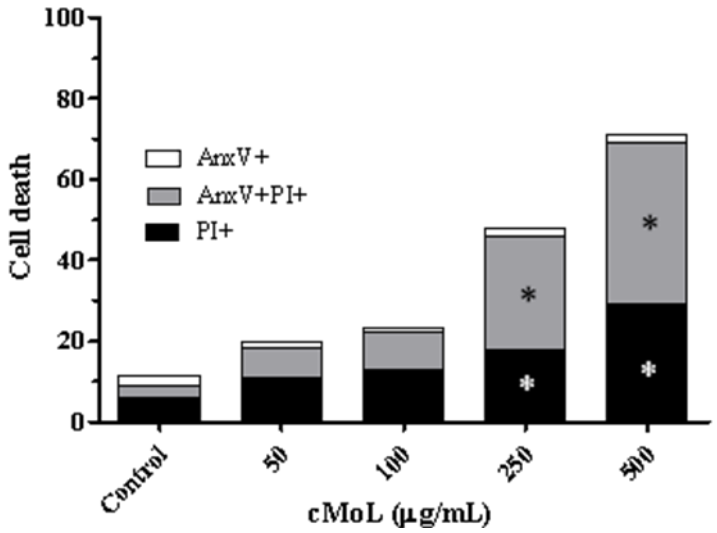

100
80
60
40
20
0
Cell death
AnxV+
AnxV+PI+
PI+
*
*
*
*
Control
50
100
250
500
cMoL (µg/mL)

Figure 3

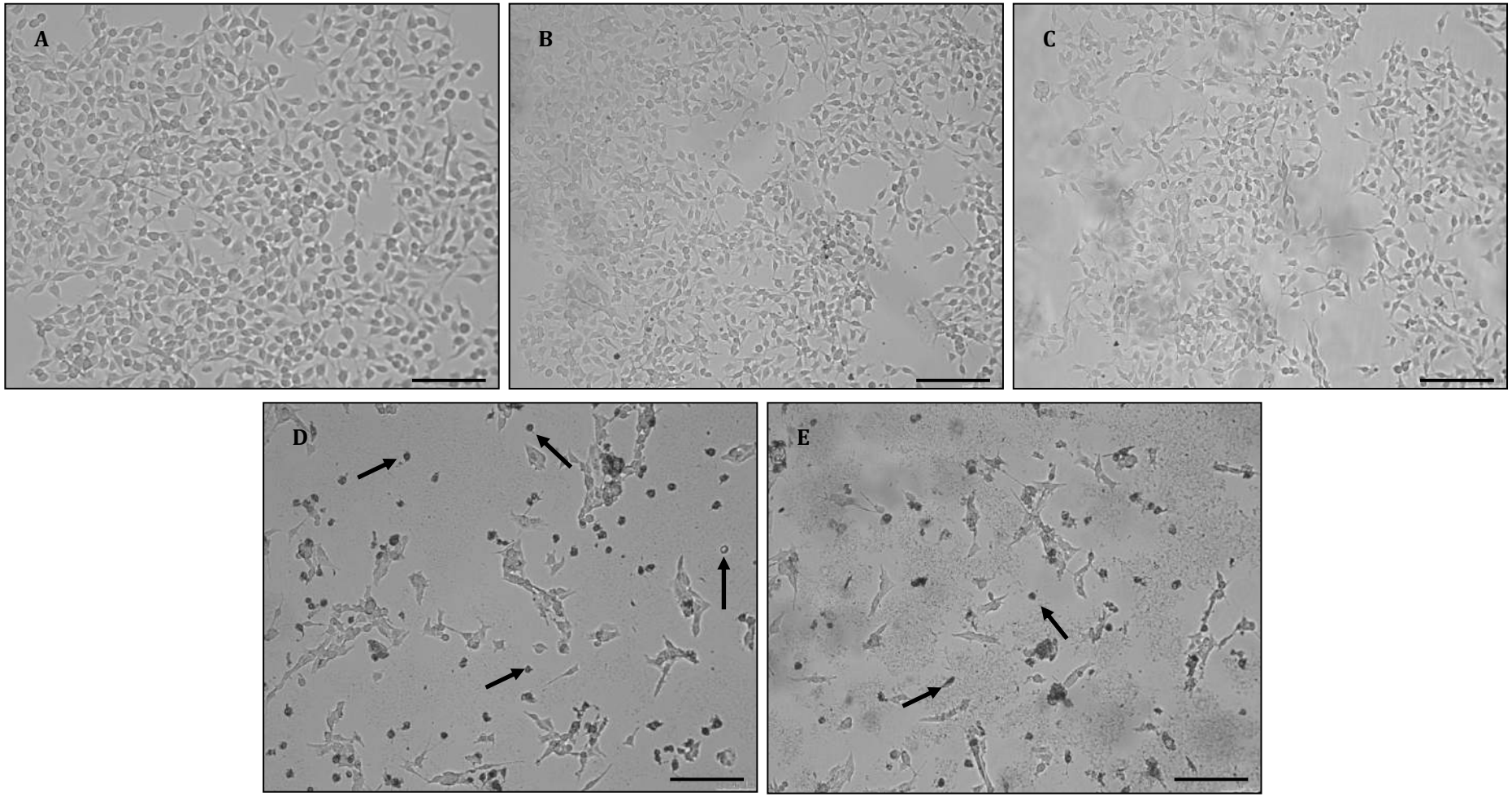
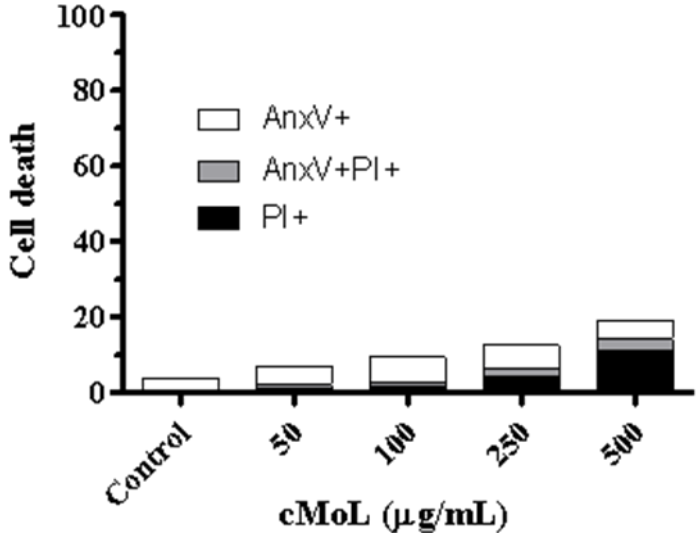


Figure 4


100
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Cell death
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50
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cMoL (µg/mL)

Figure 5

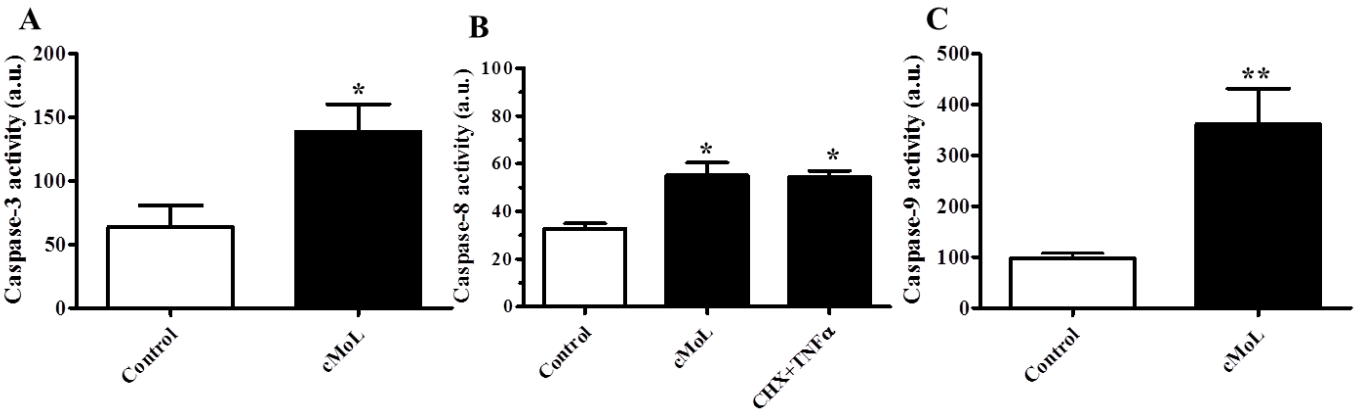

A
Caspase-3 activity (a.u.)
200
150
100
50
0
*
Control
cMoL
B
Caspase-8 activity (a.u.)
100
80
60
40
20
0
*
*
Control
cMoL
CHX+TNFα
C
Caspase-9 activity (a.u.)
500
400
300
200
100
0
**
Control
cMoL

Figure 6

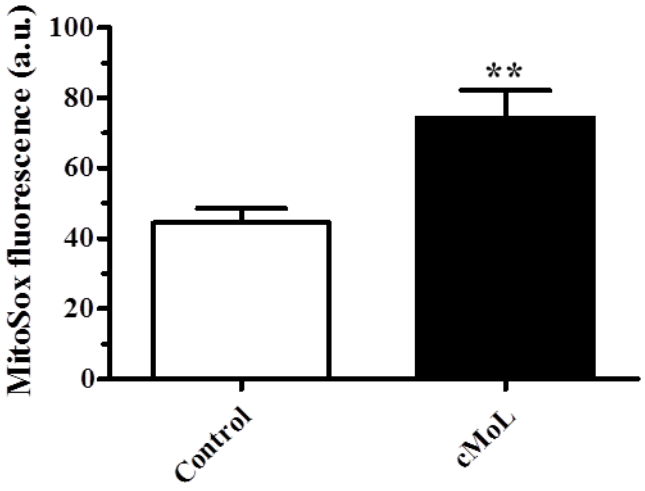
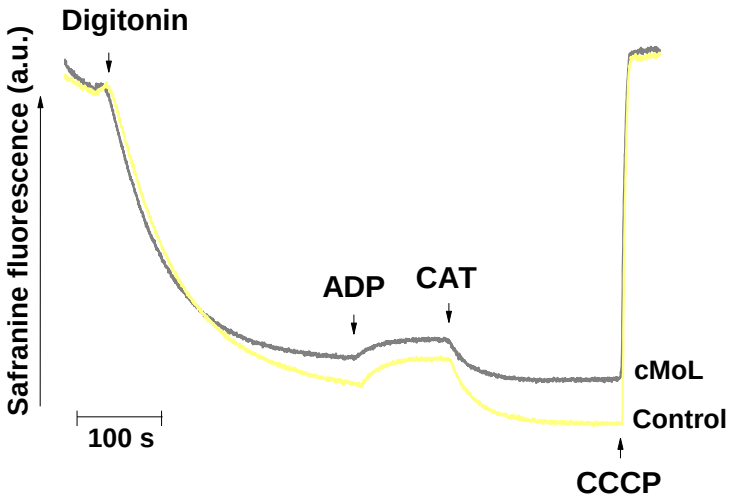


Figure 7


Digitonin
Safranine fluorescence (a.u.)
ADP
CAT
cMoL
Control
100 s
CCCP

6. CAPÍTULO 3

Coagulation, flocculation, agglutination and hemagglutination are similar properties?

CAPÍTULO DE LIVRO ACEITO PARA PUBLICAÇÃO NA

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Chapter

COAGULATION, FLOCCULATION, AGGLUTINATION AND HEMAGGLUTINATION ARE SIMILAR PROPERTIES?

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ABSTRACT

Coagulation, flocculation and agglutination are terms that usually cause confusion. Coagulation is a process of making colloidal matter dispersed/suspended in a liquid to join in a coherent mass. Flocculation is a physical process of contact and adhesions wherein the aggregates form larger-size clusters called flocs beeing excluded from suspension. These processes have several remarkable applications such as water treatment. The agglutination phenomena can be defined as the linkage of particles or cells in a liquid resulting in formation of clumps. In detail, aggregation/agglutination is the outcome of connections established by agglutinating agents among different components of the particulate materials. Antibodies and lectins, proteins which bind to specific targets (epitopes or carbohydrates, respectively) can be highlighted as agglutinating agents. The interaction can be used to identify microorganisms, proteins and carbohydrates; when agglutination involves erythrocytes, the technique is called hemagglutination. The aim of this chapter is to clarify differences about the important phenomena coagulation, flocculation, agglutination, and hemagglutination; characteristics and applications of molecules and substances able to exert these processes are also discussed.

Keywords: Coagulation; flocculation; agglutination; hemagglutination; lectins

1. INTRODUCTION

The processes of coagulation, flocculation and agglutination involve the union of substances, particles or cells dispersed or suspended in a liquid forming aggregates which remain or not in suspension. These processes have several applications such as water treatment in which synthetic coagulants are used; the latter compounds are often toxic to animals and harmful to human health. As an alternative environmentally friendly, synthetic coagulants may be substituted for natural coagulants from plants.

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Aggregation/agglutination is the outcome of connections established by agglutinating agents among different components of particulate materials. Agglutination is a term broadly used in medical-clinical area to designate the formation of visible aggregates as a result of interaction among specific antibodies and insoluble particles containing antigenic determinants; agglutination interactions are employed for laboratory diagnosis of diseases.

Lectins, carbohydrate recognition and surface interactive proteins have a plethora of applications and can contribute as useful coagulants and agglutinants in distinct research areas.

2. COAGULATION AND FLOCCULATION

Coagulation and flocculation are related phenomena which can usually occur together and involve the clumping of particles with consequent destabilization and coming out of the aggregates from suspension. In a chemical perspective, coagulation is defined as the process of making the colloidal matter dispersed/suspended in a liquid to join in a coherent mass. The flocculation is an essentially physical process of contact and adhesions wherein the aggregates form larger-size clusters called flocs which are excluded of the suspension (IUPAC). Flocculation is widely used as synonymous with agglomeration, aggregation, and coagulation.

Coagulants and flocculants are useful in food and beverage industries to remove microscopic particles that affect water taste, appearance and texture (Wong et al., 2007). Nevertheless, these processes are mainly known due to their great importance in the water treatment, which is discussed below.

2.1. Coagulation and Flocculation in Water Treatment

Water is a natural element essential to life; freshwater comprises only 3% of the total water on Earth and only a small percentage (0.01%) of this water is available for human use (Hinrichsen and Tacio, 2002). It is worryingly that this small fraction of freshwater is under stress due to the exponential population growth, increasing and disordered urbanization, and unsustainable consumption by industry and agriculture (Azizullah et al., 2011).

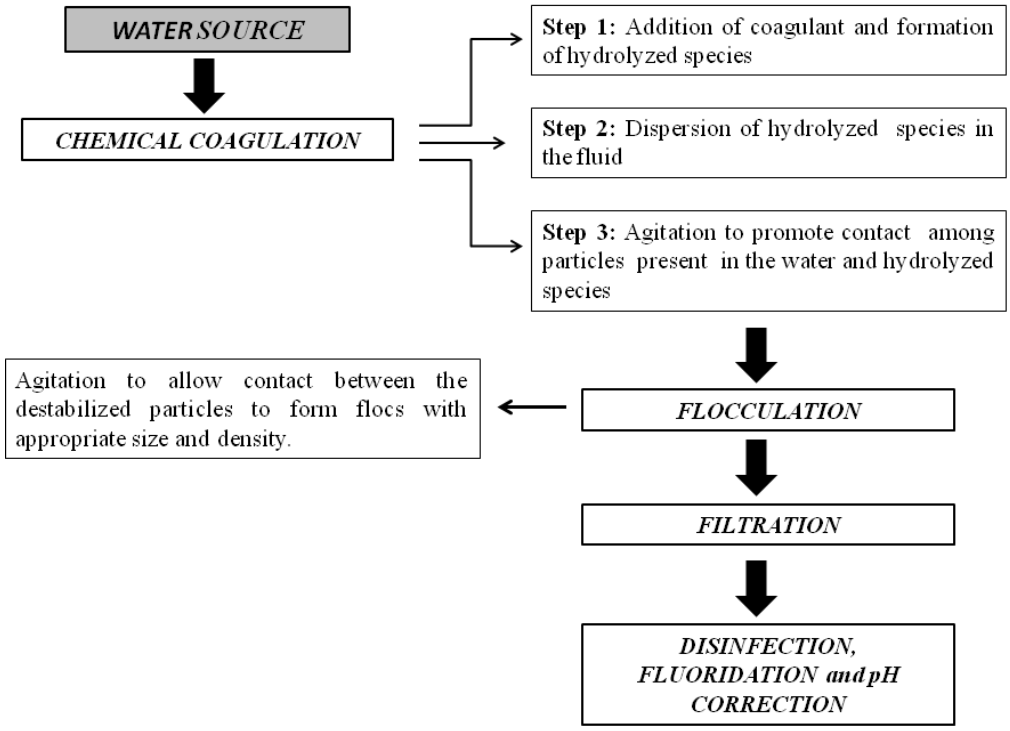

Figure 1. Steps of water treatment.

The water, due to its physical-chemical properties, is not found in pure state in nature but accompanied by foreign dissolved substances as well as particles and microorganisms in suspension. Microbial pathogens (bacteria, virus and protozoa), inorganic pollutants (acids, salts and toxic metals), ions (nitrates, phosphate, sulphates, Ca^{+2}, Mg^{+2} and F^{-}) and toxic products (detergents, disinfectants, pharmaceuticals, chemical reagents, radioactive elements) are often present contaminating water leading to problems at public health, economics and environmental levels (Zhicong et al., 2011, Jean et al., 2012). In addition, pesticides and organic compounds like oils are also threats to water quality (Azizullah et al., 2011). Then, there are many sources of water contamination, primarily substances derived from agricultural and industrial activities, as well as those present in soil (Gopal et al., 2007).

The conventional water treatment includes the steps coagulation, flocculation, sedimentation, filtration, and disinfection. Coagulation and filtration are the most critical processes determining the success or failure of water treatment system (Ghebremichael, 2004). Coagulation has been employed to decrease turbidity, color and to remove pathogens (Matilainen et al., 2010). When properly operated, the other processes such as flocculation and sedimentation, may not be required (Conley, 1961) and the role of disinfection can be significantly reduced (Ghebremichael, 2004). **Figure 1** shows the stages of water treatment, detailing the steps of coagulation and floculation.

Coagulation and flocculation constitute delicate steps of water treatment. Conventionally, the water is mechanical or hydraulically stirred, followed by the addition of coagulants, which act by reducing the repulsive forces between particles (impurities) increasing collisions and floc formation (Mcconhachie et al., 1999). The efficiency of the coagulation-flocculation method depends on the water characteristics, pH and temperature of solution, the type and quantity of coagulants as well as intensity and duration of mixing (Radoiu et al., 2004). These processes are interconnected and must be lucrative and easy to operate (Bromley et al., 2002).

Coagulation, in an electrostatic approach, starts with the reduction of zeta potential, which is a measure of particle stability and represents the potential required to breaking the protective layer of ions surrounding a particle and depends upon the electrostatic forces between charges carried by the colloidal particles (Ndabigengesere et al., 1995). The coagulation process can be achieved by adding cationic electrolytes that promote a compression of the electrical double layer surrounding

suspended particles destabilizing them by decreasing the magnitude of the repulsive interactions and allowing their attraction through van der Waals forces (Prabu et al., 2011).

Adsorption-charge neutralization occurs, after diffuse layer compression, when the addition of coagulant releases metal cations which trigger a hydrolysis reaction with production of soluble hydrolysable species (positively charged) promoting aggregation of negatively charged suspended particles (Gassenschmidt et al., 1995). Adsorption and bridge formations occur by addition of synthetic or natural organic materials that have ionizable sites along their chains.

Flocculation as a physical phenomenon follows the rapid mixing and coagulation. In this process the size of particles increases as a result of collisions among them. The large particles formed can be easily removed by inexpensive procedures, such as gravity sedimentation and filtration (Metcalf, 2003). The chemical coagulation process can occur in a few seconds, while the aggregation of destabilized particles forming flocs can take hours and is usually held in mechanical or hydraulic units. The speed of flocculation depends on temperature, time and intensity of agitation (Wong et al., 2007).

The flocculation of negatively charged particles occurs due to Coulomb forces among their surfaces and positively charged macromolecules, resulting in a neutral charge. It is possible to connect simultaneously several particles, since only a little moiety of the macromolecule binds to the surface of a single negatively charged particle leading to formation of flocs (Gassenschmidt et al., 1995).

Metal salts such as polyaluminium chloride and aluminium sulphate, and synthetic polymers such as polyacrylamide are frequently used as coagulant agents for water treatment (Duan et al., 2002, Tzoupanos and Zouboulis, 2011). These compounds can promote deleterious effects on the environment and aquatic organisms such as fishes (Thomas and Jurgen, 2002). Particularly, the polyacrylamide residues (acrylamide) are toxic for humans and other animals by affecting the peripheral nervous system (Smith et al., 1996).

Several studies have been performed in order to optimize the coagulant action, such as determination of optimal pH and addition of flocculants, to reduce the environmental impact and health damage (Tatsi et al., 2003). Natural compounds, in this sense, have been considered as substitutes for chemical coagulants due to their abundance, low price, innocuity and biodegradability. More details on natural coagulants are presented in section 1.3.

3. SYNTHETIC COAGULANT SUBSTANCES

Chemical coagulation is a complex phenomenon involving several inter-related parameters. Hence, it is very critical to define if a coagulant will operate efficiently under given conditions. The chemical coagulants can be classified as acidic (aluminum sulfate, ferrous sulfate, ferric chloride, ferric sulfate) and basic (sodium aluminate). Coagulant dosages vary in a wide range aiming maximum removal efficiency of pollutants using minimum doses at optimum pH (Szpak et al., 1996).

The main inorganic coagulants used are salts of aluminum and ferric ions. The latter compounds are often chosen to destabilize colloidal and suspended solids (Tak-Hyun et al., 2004); the most common coagulants used in water and wastewater treatment are the $Al_2(SO_4)_3.4H_2O$ and the polyaluminum chloride (PAC) due to their effectiveness in treating a wide range of water types at relatively low cost (Hassani et al., 2008).

The aluminum sulfate is probably the most widely used chemical for coagulation of public water supplies, due to excellent floc formation, low cost and relative economy; it is ease to handling, transport, and management. Also, aluminum sulfate is very effective for reduction of color, turbidity, chemical oxygen demand (COD) and biochemical oxygen demand (BOD). However, depending on the dosage, the ingestion of drinking water containing residues of this coagulant can cause renal failure and, when carried to the brain, can lead to dementia, loss of motor coordination, cognitive decline, and Alzheimer's disease (Flaten, 2001, Rondeau et al., 2009).

Synthetic polymeric forms of Al, such as polyaluminium chloride (PAC) and polyaluminium sulphate, have become the most common alternative coagulants (Hassani et al., 2008). The use of PAC has some advantages over aluminum sulphate including reduced acidity, positively charged monomers and polymers, rapid formation of denser flocs, and reduced sludge (Tang et al., 1998). The higher charge density of PAC species often results in a decrease in the coagulant dose and the associated solid production.

Other coagulants such as ferrous sulfate ($FeSO_4.7\ H_2O$) are very useful to treat waters with pH in the range of 8.5 to 11.0. The ferric sulfate ($Fe_2(SO_4)_3$) is convenient for treatment of acidic or highly colored water and is effective in the pH range 5.0 to 11.0. The ferric chloride ($FeCl_3$) produces good flocs also in the pH range 5.0 to 11.0. In most water treatment systems, synthesized polymers have been used such as polyacrylamide.

The textile industry is one of the most chemically intensive industries and the major polluter of water. Its effluents are discharged as a wastewater which has high color, BOD, COD, pH, temperature, turbidity and toxic chemicals. Pre-hydrolyzed coagulants such as PAC, polyaluminium ferric chloride (PAFCl), polyferrous sulphate (PFS) and polyferric chloride (PFCl) are effective in color removal even at low temperature and produce lower volume of sludge (Verma et al., 2012). It has been reported that pre-hydrolyzed metallic salts are often found to be more effective than hydrolyzing metallic salts which are readily soluble in water (Jiang and Graham, 1998).

4. NATURAL COAGULANT SUBSTANCES

Although the use of natural coagulants of plant and mineral origin for water treatment was very common in the past, the lack of scientific knowledge about their action mechanisms and the modernization of techniques using chemical coagulants led to a decrease in their use (Ndabigengesere and Narasiah, 1998). Recently, the interest in natural coagulants has risen due to their biodegradability, safety for human health and low cost. Natural coagulants can also be extracted from microorganisms and animal tissues (Šciban et al., 2009).

Seeds of 14 species from *Moringa* genus have been reported to possess coagulating properties in different degrees (Jahn, 1988) and the species *Moringa oleifera* is the most studied. Aqueous extracts from dry *M. oleifera* seeds have been extensively used for removal of water turbidity due to their natural coagulant ability; Gassenschmidt (1991) suggested that a cationic peptide with molecular weight between 6 and 16 kDa with an isoelectric point at pH 10.0 was the active principle. Ndabigengesere et al. (1995) confirmed that the active component was a dimeric protein with coagulant properties better than those of aluminum salts. Also, the authors reported that the use of *M. oleifera* coagulant generated less residues than when aluminum salts were employed and that residual material was innocuous to the environment. Gassenschmidt et al. (1995) isolated two flocculating and basic proteins (pI>10) from *M. oleifera* seeds called $MO_{2.1}$ and $MO_{2.2}$ with molecular masses of 6.5 and 7.0 kDa, respectively. Amino acid sequencing of $MO_{2.1}$ revealed 60 residues in the full sequence (ZGPGRQPDFQRCGQQLRNISPPQRCPSLRQAVQLTHQQQGQVGPQQVRQMYRVASNIPST) and high contents of glutamine, arginine and proline. The authors also demonstrated that $MO_{2.1}$ showed a flocculant capacity higher than a cationic polymer on polyacrylamide basis.

Okuda et al. (2001) isolated a non-proteinaceous coagulant from saline extracts of *M. oleifera* seeds; this compound corresponded to a polyelectrolyte with molecular mass around 3.0 kDa. Other coagulant proteins from *M. oleifera* seeds were subsequently isolated. Ghebremichael et al. (2005) purified a cationic protein on a cation exchanger column which showed pI greater than 9.6, molecular mass lower than 6.5 kDa and flocculant and antimicrobial properties. Santos et al. (2009) purified a cationic lectin (carbohydrate-binding protein) which showed coagulant property and was named coagulant *M. oleifera* lectin (cMoL). Further, Ferreira et al. (2011) reported that another lectin isolated from Moringa seeds called WSMoL (water-soluble *M. oleifera* lectin) was able to reduce turbidity in water.

M. oleifera seeds, in addition to coagulant power, have been applied to remove different components in aqueous solutions and suspensions. Beltrán-Heredia (2011) reported that *M. oleifera* seed extract acted as an agent for removal of the anionic surfactant sodium lauryl sulphate in aqueous solutions (removal of 65% was reached). Sharma et al. (2006) and Meneghel et al. (2013) reported the seed powder ability to remove cadmium (Cd) by biosorption and results suggested that the interaction between amino acids of seed proteins and Cd was mainly responsible for the removal of Cd(II) ion. *M. oleifera* seeds were also tested as a sorbent for removing Ag(I) in aqueous solutions and the best results were obtained using 2 g of adsorbent with particle size of 75-500 μm, at pH 6.5 (Araújo et al., 2010). *M. oleifera* seed lectin was effective in promote sedimentation of bacteria present in water (Ferreira et al., 2011).

Other natural coagulants from plants have been searched and studied. In Venezuela, the coagulant potentials of *Cactus latifaria* and seeds of *Prosopis juliflora* were tested using synthetic water formulated to resemble drinking water. When starting from high (100-200 NTU) and low (30-40 NTU) initial turbidities, both materials promoted reduction in turbidity and final results were close to the required standard of 5 NTU. Their optimum dose was 20-40 mg/L which is comparable with that achieved using extracts from *M. oleifera* (50 mg/L) and was about 75% lower than aluminum sulphate (Diaz et al., 1999).

Crude extract from common bean (*Phaseolus vulgaris*) seeds showed the ability to act as a natural coagulant for water treatment with a few advantages over *M. oleifera* seeds, such as, no oil presence. In this study the authors reached partial purification of the coagulant components through anion exchange chromatography. The fraction having the highest coagulation activity (72.3%) was eluted with 0.875 mol/L NaCl and the optimal coagulation dosage was 0.081 mg/L. Coagulation activity of partially purified common bean coagulant was almost 22 times higher than that of crude extract (Antov et al., 2010).

Seed extracts from Horse chestnut (*Aesculus hyppocastanum*), and Common oak (*Quercus robur*), Turkey oak (*Quercus cerris*), Northern red oak (*Quercus rubra*) and European chestnut (*Castanea sativa*) were also investigated for potential use in water treatment. The natural coagulants were extracted with water or NaCl solutions and all these plant materials showed coagulant properties, although extracts from seeds of *C. sativa* and *Q. robur* were the most efficient expressing coagulant activities of 80% and 70%, respectively (Šciban et al., 2009).

Chitosan is a polysaccharide prepared by de-N-acetylation of chitin, which is the main constituent of crustacean shells (Chen et al., 2007). A number of studies have assessed its use as a coagulant or flocculant for the removal of mineral colloids (Huang et al., 2000, Roussy et al., 2004) and it has been used to treat inorganic solid suspensions in wastewater treatment systems (Roussy et al., 2005). Chitosan is a polymer with a moderate to high molecular weight and cationic charge; the coagulation process using chitosan seems to be charge neutralization (Huang et al., 2000). Chen and Chung (2011) compared the coagulation performance of acid-soluble chitosan, water-soluble chitosan, a coagulant mixture of chitosan with alum, and a coagulant mixture of chitosan with PAC. The results showed that when chitosan was mixed with alum or PAC in a mass ratio of 1:1, the coagulation efficiency of the mixtures was better than those of chitosan, alum, or PAC alone in terms of a wider dosage range and high settling velocity. These findings suggest that alum or PAC can be partially replaced by chitosan as a simple and cost-effective alternative.

Tannins are mostly water-soluble plant polyphenolic compounds with molecular weight ranged between 500 and some thousand daltons. These compounds contain enough hydroxyl groups for effective cross linking of other compounds and are actually a natural and feasible source for coagulant synthesis. The production process of these kinds of coagulant is well-known and possible even to optimize it in terms of efficiency (Beltrán-Heredia et al., 2010). The tree *Acacia mearnsii* (Black wattle) is a well-known tannin source and was revealed as an efficient product in anionic surfactant removal (Sánchez-Martín et al., 2009).

The studies regarding the application of plant-based coagulant can be considered a promising alternative to remove contaminants from water destined for public supply. The use of natural coagulants can avoid solid residues that are generated in conventional water treatment processes.

5. AGGLUTINATION AND HEMMAGLUTINATION

The agglutination phenomena can be defined as the linkage of particles or cells suspended in a liquid, resulting in formation of clumps. In detail, this aggregation is the outcome of connections established by agglutinating agents among different components of the particulate material.

Agglutination is a term broadly used in medical-clinical area to designate the formation of visible aggregates as a result of interaction among specific antibodies and insoluble particles containing antigenic determinants. The agglutination can occur with particles having natural antigenic determinants on their surface (erythrocytes, bacteria, protozoa, etc.) and with inert particles (latex, polystyrene, bentonite, etc.), or even with antigenically unrelated cells (blood cells) which adsorb or attach to soluble antigens. When agglutination interactions use erythrocytes can be called hemmaglutination.

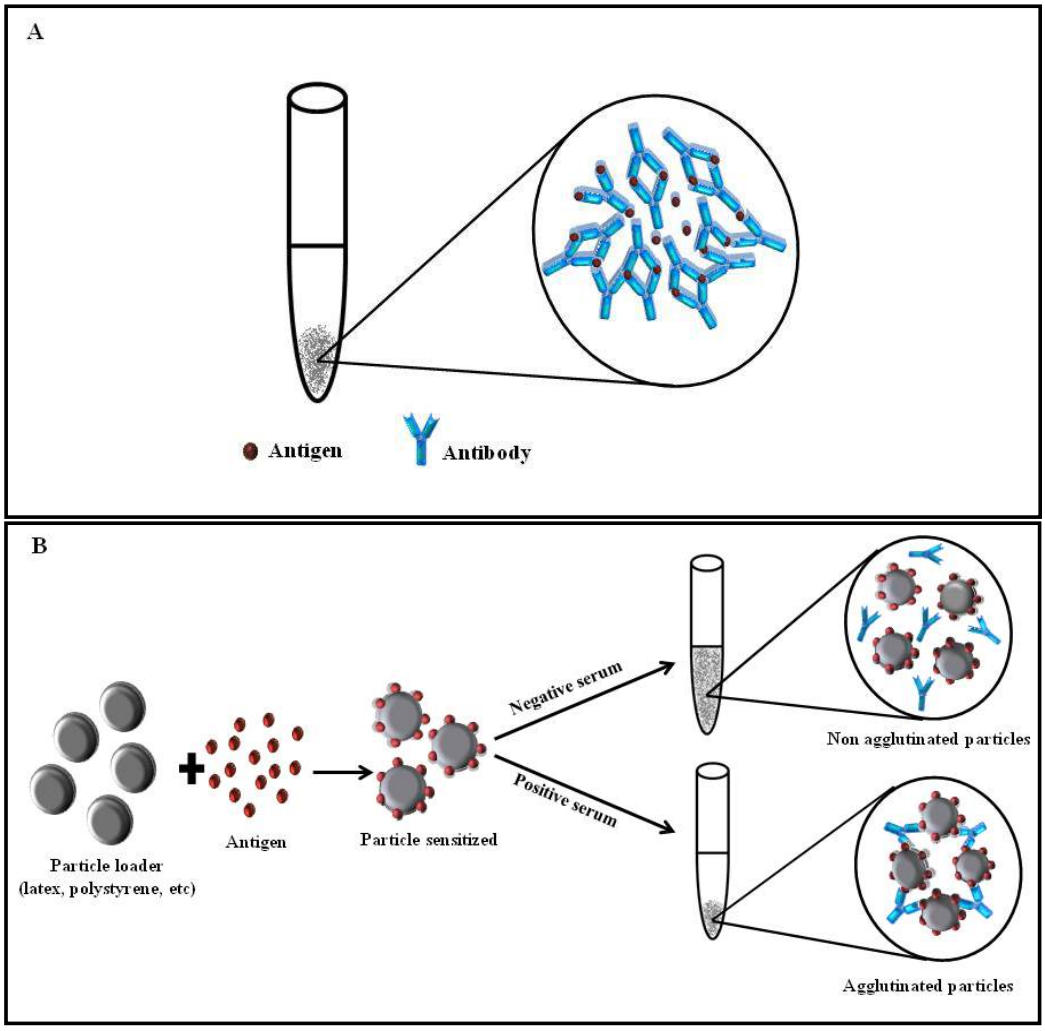

Figure 2. Schematic representation of agglutination interactions. A: Direct agglutination reaction where antibodies recognize and establish links with antigens forming aggregates. B: Indirect agglutination using particles. These reactions occur when antigens or parts of antigens are first bound (adsorbed) to a carrier particle to become resistant and more easily recognized by specific antibodies. Sensitized carrier particles amplify the recognition by antibodies and clump.

Agglutination interactions may occur through a direct or indirect form **(Figure 2)**. In both agglutination reactions, the biological fluid is tested for the presence of antibodies that will bind the

antigens (Stanley, 2002). Insoluble antigenic particles in direct agglutination are used at their entire or fragmented forms. Bacteria, fungi, protozoa and erythrocytes can be directly agglutinated by antibody. On the other hand, in the indirect agglutination the erythrocytes and inert particles (latex, bentonite, yeast, etc) can be sensitized by passive adsorption via chemical agents such as tannic acid and chromium chloride, and by conjugation of the antigen by means of covalent chemical bonds by providing stable reagents. The agglutination assays can be performed in tubes or plates.

6. DIAGNOSTIC APPLICATIONS

A diagnostic assay for an infectious agent can be used to demonstrate the presence or absence of infection, or to detect evidences of a previous infection (for example, the presence of antibodies). Agglutination reactions are much employed for the laboratory diagnosis of diseases caused by viruses, bacteria, protozoa, fungi, and autoimmune diseases (Stanley, 2002). In Microbiology, agglutination is an important technique for diagnosis commonly used as a method of identifying bacteria and its specific antigens (Gaidamashvili et al., 2002).

Direct or indirect agglutination and hemagglutination assays have been increasingly applied in various research fields and clinical diagnosis of several diseases such as visceral leishmaniasis (Srivastava et al., 2011) and typhoid fever (Abdoel et al., 2007). This technique has been mainly performed as a qualitative analysis but demonstrate versatility, reliability and speed of execution.

Leptospirosis, a zoonosis with worldwide distribution, is an acute febrile illness caused by spirochaetes of the pathogenic *Leptospira interrogans* group. Latex beads sensitized with recombinant LipL32 (a highly conserved leptospiral antigen) were used to detect specific antileptospiral antibodies from human and dog sera. Recombinant antigen-based latex agglutination assay is a suitable technique for the examination of a large number of sera which involves the LipL32 immunodominant antigen. The test was found to be sensitive, specific and accurate as compared to the standard microscopic agglutination assay, mainly in the acute phase of the illness (Dey et al., 2007). Hemagglutination assays are also widely used in clinical laboratories for the diagnosis of leptospirosis (Levett and Whittington, 1998).

Brucellosis is a zoonotic disease that, despite being long recognized, continues to afflict humans and domestic animals in many countries around the world (Araj, 2010). A rapid latex agglutination test was developed and evaluated for the serodiagnosis of human brucellosis (Abdoel and Smits, 2007). Latex particle agglutination test (LPA test) is also used to detect bacterial antigen in cerebrospinal fluid to diagnose bacterial meningitis. The LPA test was positive in 30 of the 36 cases studied, with a sensitivity and specificity of 83% and 100%, respectively (Das et al., 2003).

Chagas' disease is a complex zoonosis caused by the parasite *Trypanosoma cruzi*. Serological assays are widely used for the diagnosis, particularly regarding the diagnostic of disease chronic stage. The indirect hemagglutination is included among these techniques as the most commonly used approaches and is also used in epidemiological surveys, in medical care tasks and in scientific research (Souza and Neto, 2012).

The determination of C-reactive protein (CRP) is an assay that measures general levels of inflammation in human body. In recent years it has been given particular interest to the measurement of serum CRP as a marker of inflammation associated with cardiovascular diseases. In clinical procedures/protocols, lipid agglutination and latex agglutination are among the most used techniques to analyse CRP. The basic biochemical reaction that constitutes blood CRP detection is the selective association of the protein with a specific analyte adsorbed onto particles of cholesterol or latex (Algarra et al., 2012).

Ye et al. (2011) developed a latex agglutination inhibition reaction test (LAIRT) to detect aflatoxin B_1 (AFB_1) in agricultural commodities, foods and feeds. Aflatoxin B_1 is a toxic metabolite produced mainly by *Aspergillus flavus* and *A. parasiticus* and humans would be exposed to AFB_1 directly by eating contaminated products (Tan et al., 2009). The method developed was simple, easy

to perform and interpret, and the process could be completed within 10 min using minimal equipment.

7. LECTINS AS COAGULANT AND AGGLUTINATING AGENTS

Lectins are proteins or glycoproteins able to bind reversibly to carbohydrates without altering the covalent structure of any of the recognized ligands (Sharon, 2007). These proteins are broadly distributed in nature (Santos et al., 2013). Lectins can precipitate soluble glycoconjugates and bind to carbohydrate of membrane glycoproteins and glycolipids thus inducing agglutination of various cell types (Vazquez et al., 1996). These proteins play an important role in immunological defense systems since they can sequester viruses, bacteria and other cellular-micro invaders, as well as substances that they secrete. Also, these proteins are involved in other cellular events besides agglutination process; they act in proliferation, opsonization, signal transduction, metastasis and apoptosis (Dutta et al., 2005, Nunes et al., 2012, Coriolano et al., 2012a, Coriolano et al., 2012b).

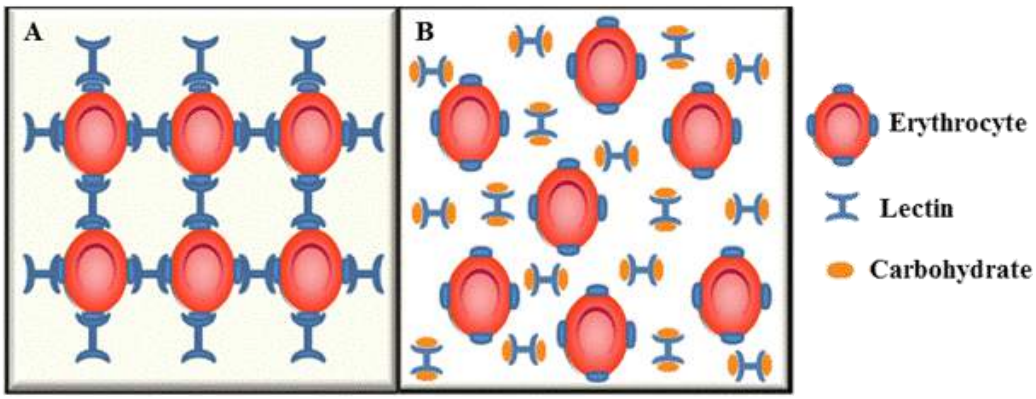

Figure 3. Schematic representation of lectin hemagglutination (A) and carbohydrate inhibition assays (B). Carbohydrates present on the surface of erythrocytes are recognized by the binding sites of the protein forming the network (A). Lectins with more than one binding site are capable of promoting the agglutination phenomenon. The lectin binding sites also recognize carbohydrates free in solution (B) and interaction is inhibited; free erythrocytes precipitate.

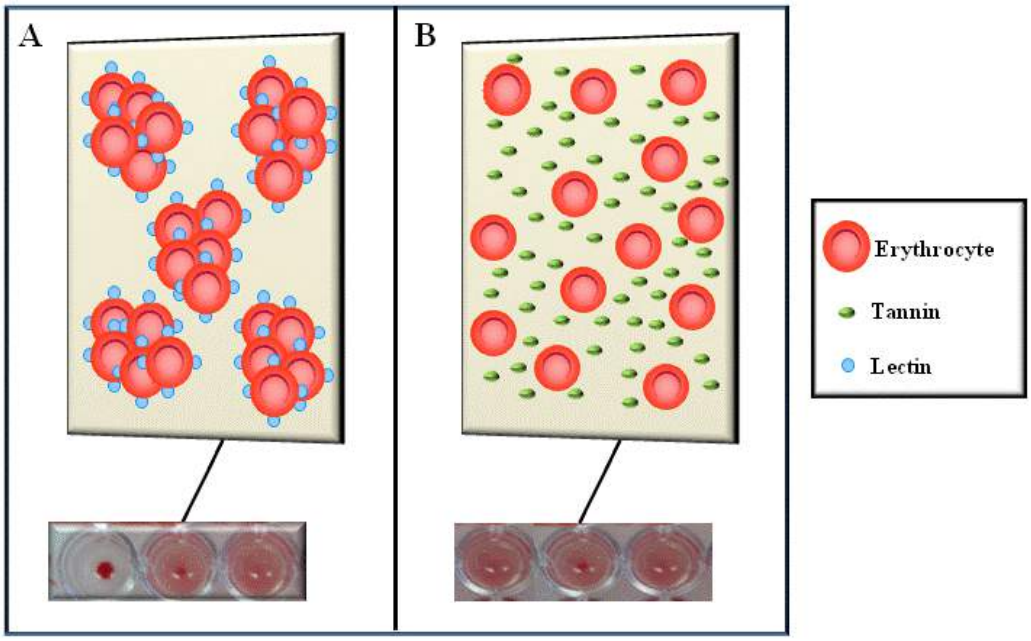

Figure 4. Schematic representation of hemagglutination and pseudo-hemagglutination assays as revealed by optical microscopy. A: Hemagglutination with formation of red blood cell aggregates due to the presence of lectin. B: Pseudo-hemagglutination with dispersion of red blood cells induced by substances such as tannin.

The presence of a lectin – carbohydrate recognizing protein – is detected using a hemagglutination assay. These molecules have the ability to induce cell agglutination phenomenon (Kennedy et al., 1995). The hemmaglutination assay is performed by serial dilution of a sample containing lectin and incubation with human or animal red blood cells; in this process, the carbohydrate-binding sites interact with carbohydrate or glycoprotein present in the erythrocyte surfaces forming a network of agglutination among the cells (Correia et al., 2008). This process is shown in **Figure 3A**. The assurance that the agglutinating agent is a lectin is provided by inhibition assay of hemagglutinating activity using a solution of a specific carbohydrate or glycoproteins (Correia et al., 2008). If the lectin binding sites are occupied by free carbohydrate, the lectin binding to erythrocyte surface is blocked and the network agglutination will not be formed (Figure 3B).

Lectin hemagglutination is distinct from tannin induced pseudo-hemagglutination; lectins bind cell polysaccharides differently from tannins (**Figure 4, A and B**). Thus the connection between tannins and polysaccharides present in the membrane of erythrocytes promote a wrap that induces repulsion between cells; this phenomenon is seen macroscopically as an apparent agglutination, but at the microscopic level it is, in fact, a pseudo-hemagglutination. Beside tannins compounds also able to cause pseudo-hemagglutination are lipids or bivalent cations at high concentrations (Rüdiger, 1998).

7.1. Bacterial Agglutination by Lectins

The ability of lectins to interact with bacteria has already been reported for different purposes. Commercial lectins of *Canavalia ensiformis, Ulex europaeus, Phaseolus vulgaris, Triticum vulgaris,* and *Swartzia pickellii* of undefined specificity interacted with *Yersinia pestis* strains isolated from rodent fleas and human biological fluids. Most of the *Y. pestis* strains did not agglutinate with *U. europaeus* or *C. ensiformis* lectin; *P. vulgaris* lectin agglutinated suspensions of all the bacillus strains used. Fifteen of the 19 strains tested positive for assays using *S. pickellii* lectin. A similar agglutination pattern was obtained for lectins with specificity for oligosaccharides containing *N*-acetylglucosamine and *S. pickellii* lectin, which did bind to the affinity matrix chitin, a polysaccharide of *N*-acetylglucosamine. The use of bacterial strains and commercial lectins of

defined specificity may be an approach to provide evidence about lectin binding sites of undefined monosaccharide specificity (Cavalcanti et al., 1990).

7.2. Lectins with Antibacterial Activity

Some lectins have antibacterial activity through cell agglutination and variable effects against different microorganisms (Oliveira et al., 2008; Nunes et al., 2011). Glycoconjugates such as peptidoglycans, lipopolysaccharides and teichoic acids are present on bacterial cell surfaces and constitute potential lectin targets (Nunes et al., 2011).

A lectin from *Bothrops leucurus* snake venom (BlL) exhibited antibacterial effects against human pathogenic Gram positive bacteria and was not effective against Gram negative bacteria. A possible reason for the difference in susceptibility is the difficulty that BlL encounters in crossing the outer cell wall of Gram-negative bacteria to reach the periplasmic space. This lectin may interact with the peptidoglycan present in the Gram-positive bacteria cell wall while may not be able to bind peptidoglycans of Gram-negative bacteria, whether it does not enter in the periplasmic space. In the presence of 200 mM galactose this lectin loses its antibacterial effects and agglutination properties; so, the carbohydrate-binding property of BlL is linked with its antibacterial activity (Nunes et al., 2011).

A lectin from *Phthirusa pyrifolia* leaf exhibited antibacterial activity and was more effective for Gram-positive than for Gram-negative species. This greater interaction observed with Gram-positive bacteria may be explained by the high levels of peptidoglycan on the wrapper. Probably, this protein was able to agglutinate the bacteria, promoted their immobilization, and inhibited their growth or even destroyed the bacteria (Costa et al., 2010). This kind of interaction (lectin-bacteria cells) may exist by covalent/or noncovalent aggregation, depending on the molecular weight of the oligomers and its subunits (Rittidach et al., 2007). A lectin from *Eugenia uniflora* seeds demonstrated a remarkable nonselective antibacterial activity. This lectin strongly inhibited the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella*; it moderately inhibited the growth of *Bacillus subtilis*, *Streptococcus* sp. and *Escherichia coli* (Oliveira et al., 2008).

7.3. Lectins with Coagulant Properties

Some proteins have coagulant properties and can be used in water treatment. *M. oleifera* is a plant whose seeds contain natural coagulant proteins (Okuda et al., 2001; Ghebremichael et al., 2005; Bhuptawat et al., 2007, Santos et al., 2009, Ferreira et al., 2011). It is widely known that the plant have numerous uses (Santos et al., 2011). Antibacterial activity has been attributed to different parts of the plant, such as leaves, roots, seeds, flowers, fruit peel and unripe pods (Anwar et al., 2007).

Coelho et al. (2009) purified by chitin chromatography a lectin from *M. oleifera* seeds called WSMoL (water-soluble *M. oleifera* lectin). Ferreira et al. (2011) demonstrated that this lectin has coagulant activity, reducing turbidity and bacteria contamination. The study showed that WSMoL aggregated and coagulated these microorganisms from environmental water as well as interfered in the growth of *S. aureus* and *Escherichia coli*.

The coagulant *M. oleifera* lectin (cMoL) is a native basic protein that was isolated after saline extraction and guar gel column chromatography. This lectin was active at pH range 4.0-9.0 and its hemagglutinating activity was inhibited by carbohydrate and abolished by azocasein as well as asialofetuin. Polyacrylamide gel electrophoresis under reduced conditions revealed a main polypeptide band of 26.5 kDa; cMoL showed coagulant activity in turbid water, similar to aluminium sulphate, the coagulant most widely used in water treatment (Santos et al., 2009). Santos et al. (2012) showed that a saline extract from *M. oleifera* seeds with lectin activity removed humic

acid from water; this preparation acted better than aluminum sulphate and can be an interesting natural alternative to remove humic acid.

Figure 5. Aspect of coagulation assay using kaolin clay 10 g/L (a model of turbid water) and cMoL (1mg/mL) as coagulant. Left tube represents the control and right tube water treated with cMoL evidencing clarification.

cMoL coagulant property is showed in a simple assay. First, a tap water sample was treated with kaolin clay, stirred for 30 min and allowed to settle for 24 h to complete hydration (Figure 5, left). cMoL (200 µL, 1 mg/mL) was then incubated with kaolin suspension (1 mL), pH 6.0, for 30 min (**Figure 5**, right). Kaolin was used to give the desired turbidity to water sample which was clarified with cMoL. Water remained turbid in the control tube.

7.4. Coagulation Mechanism Proposal of Coagulant *M. oleifera* Lectin (cMoL)

The understanding about the mechanisms involved in the coagulation process by proteins from *M. oleifera* seeds has always been a challenge for researchers, usually because this activity is reported only for crude extracts. cMoL is thermostable, pH resistant and have a molecular weight of 26.5 kDa (Santos et al., 2009). Okuda et al. (2001) proposed a model where coagulation by purifed coagulant solution (MOC-SC-pc) from *M. oleifera* seeds occurs due to interaction of MOC-SC-pc with bivalent cations, forming *net-like* structures. This model can not be applied to cMoL since the presence of bivalent ions (Ca^{+2} and Mg^{+2}) did not improve the efficiency of coagulation (Santos et al., 2009).

Molecules vary in their charge properties; all molecules with ionizable groups can be titrated and their net surface charge is highly pH dependent. Proteins are built up with many different amino acids containing weak acidic and basic groups; their net surface charge will change gradually as the pH of the environment changes, so the proteins are amphoteric components.

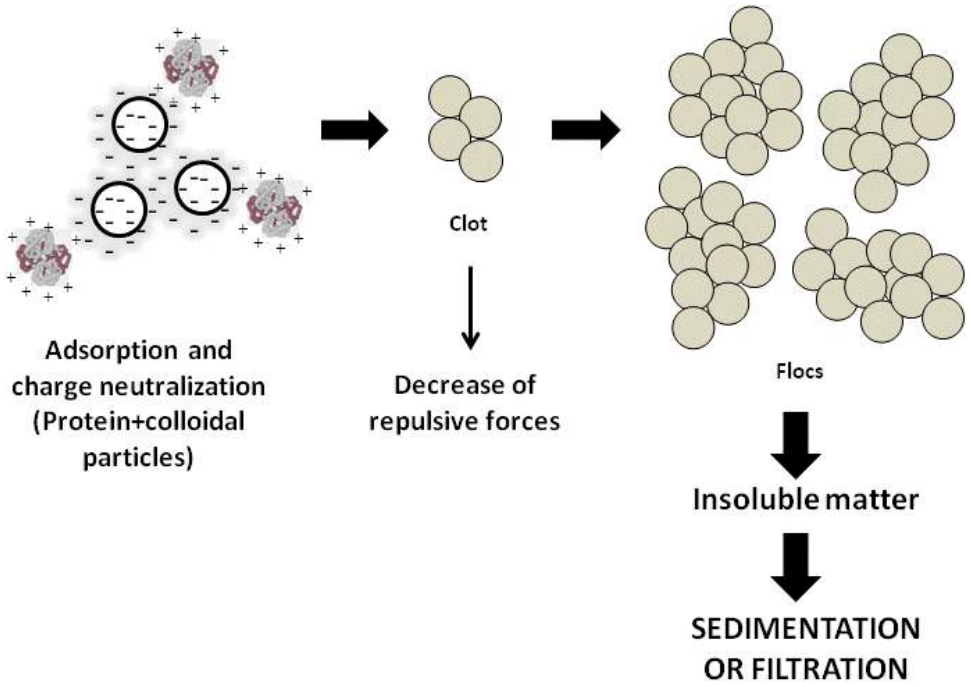

Figure 6. Coagulation mechanism proposed to coagulant *Moringa oleifera* lectin (cMoL).

Table 1. Zeta potential of kaolin clay in different pH values.

Kaolin clay 0.5 g/L	Zeta potential (mV)
pH 5	-4.66 +/- 0.32
pH 6	-7.75 +/- 0.04
pH 7	-2.51 +/- 0.15
pH 8	-4.66 +/- 0.25

Molecules vary in their charge properties; all molecules with ionizable groups can be titrated and their net surface charge is highly pH dependent. Proteins are built up with many different amino acids containing weak acidic and basic groups; their net surface charge will change gradually as the pH of the environment changes, so the proteins are amphoteric components.

Figure 6 shows a proposal for the interaction mechanism involved in cMoL coagulation process. cMoL, a basic positively charged protein (Santos et al., 2009), interacts with colloidal particles like kaolin, which zeta potential is negative (**Table 1**). The decrease of repulsive forces leads to formation of aggregates, which become increasingly larger and denser forming an insoluble material subsequently removed by sedimentation or filtration. Zeta potentials of kaolin (0.5 g/L) in different pH values were determined using a Malvern Zetasizer instrument equipped with the zeta potential cell DTS1060 at 20 ◦C. Values were derived from the electrophoretic mobility using the Smoluchowski approximation (Hunter, 1981).

Coagulant activity of high-molecular cationic polyacrylamide derivatives has been coagulant activity of high-molecular cationic polyacrylamide derivatives has been explained by the bridge formation model. Coagulation of negatively charged particles is a result of binding by Coulomb forces of positively charged particles and neutralization of part of the surface charge. Reduced electrostatic repulsion leads to the agglomeration and formation of flocs by interaction between negatively charged particles (Gassenschmidt et al., 1995); cMoL may acts in a similar way.

CONCLUSION

The purpose of this chapter was to review the differences among coagulation, flocculation, and agglutination processes and the applications of molecules and substances able to exert these effects. Coagulation, flocculation, and agglutination differ in the manner how linkage occurs among aggregate components and whether aggregates remain in suspension or not. The agglutination reaction by antibodies or lectins can be employed for different purposes such as identification of bacterial isolates or diagnosis of infection diseases. Coagulant and agglutinating properties of lectins from *M. oleifera* seeds confer to these proteins a potential as water treatment agents by removing particulate materials and microorganisms.

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7. CONCLUSÕES

- A sequência primária de cMoL revela que a proteína apresenta 101 aminoácidos, duas cadeias e 81% de similaridade com uma proteína floculante de sementes de *M. oleifera* ($MO_{2.1}$). O alto grau de similaridade entre essas proteínas sugere que várias famílias de proteínas com atividade floculante/coagulante estão presentes em sementes de *M. oleífera.*
- Deconvolução do espectro de DC indicou a presença de 46% de α-hélice, 12% folhas-β, 17% voltas-β e 25% de estruturas desordenadas. O conteúdo de α-hélices estimados para cMoL é similar a outras proteínas coagulantes de *M. oleifera.*
- A conformação da proteína é mantida até 80 °C e em toda a faixa de pH 2.0-12.
- cMoL prolonga os tempos de coagulação sanguínea (TTPa e TP). As interações de cMoL com os fatores da coagulação ocorrem, pelo menos parcialmente, pelo domínio de reconhecimento a carboidrato da proteína.
- cMoL atua como uma proteína anticoagulante sob parâmetros hemostáticos *in vitro* e é atualmente, a única proteína de sementes de moringa com esta propriedade;
- cMoL reduz a viabilidade e causa morte nas células de melanoma B16-F10 após 48h de tratamento na concentração de 250µg/mL.
- A lectina demonstra elevada especificidade para células tumorais;
- Os mecanismos envolvidos na morte celular incluem: aumento da produção de espécies reativas de oxigênio (EROs) e ativação de caspases 3, 8 e 9;
- cMoL é uma lectina citotóxica pois induz morte celular por apoptose em células de melanoma B16-F10;
- A morte celular causada por cMoL não teve envolvimento da Transição de Permeabilidade Mitocondrial;
- Os conceitos dos processos de coagulação, floculação, aglutinação e hemaglutinação foram revisados e foi proposto um mecanismo de ação em água para a lectina coagulante de *M. oleifera.*

8. ANEXOS

Anexo I - Regras da revista

JOURNAL OF ETHNOPHARMACOLOGY

An Interdisciplinary Journal Devoted to Indigenous Drugs

AUTHOR INFORMATION PACK

GUIDE FOR AUTHORS

INTRODUCTION

The *Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their biological and pharmacological effects based on the principles established through international conventions. Early people, confronted with illness and disease, discovered a wealth of useful therapeutic agents in the plant and animal kingdoms. The empirical knowledge of these medicinal substances and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on *materia medica*. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of indigenous remedies. Chemists continue to use plant-derived drugs (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.

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Evaluation of seed coagulant *Moringa oleifera* lectin (cMoL) as a bioinsecticidal tool with potential for the control of insects

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ABSTRACT

Lectins have demonstrated significant levels of protection against different pests when expressed in transgenic plants. The effects of the coagulant *Moringa oleifera* lectin (cMoL) on moth flour (*Anagasta kuehniella*) were tested by incorporating the protein in an artificial diet at levels of 0.5%, 1% and 2% (w/w), respectively. cMoL showed a dose-dependent effect on average larval weight and a series of nutritional disturbances. A significant increase in total development time of 15 days was observed in the group fed with cMoL at 1%, increasing the rate of pupal mortality by 27.6%. The *A. kuehniella* midgut proteases were unable to digest cMoL for up to 12 h of incubation. The lectin presented a tight binding to a chitin column, suggesting that the insecticidal activity of cMoL involves carbohydrate–lectin interactions on the surface of the digestive tract, with glycoproteins and others glycolsylated structures in the midgut and resistance to enzymatic digestion.

1. Introduction

The steady increase in worldwide agricultural production and the creation of large crop areas has facilitated the spreading of insect pests that increase production costs, in addition to reducing farming productivity and causing losses in stored products. Problems associated with widespread insecticide usage, together with the development of insect resistance to *Bacillus thuringiensis* (Bt) toxins in genetically engineered crops, have resulted in a greater interest of scientists in exploiting the potential of using plant defensive proteins, such as lectins, to help in combating crop damage [1].

Plant seeds contain several biologically active proteins that play various specialized functions. The most representative molecules are hydrolytic enzymes, inhibitors, lectins and the ribosome inactivating proteins [2,3]. Plant lectins are defined as proteins possessing at least one non-catalytic domain, which binds reversibly to specific mono or oligosaccharides [4]. Indeed, many highly-abundant plant lectins have been found to combine a role in storage with a role in plant defense whenever the plant is under attack by predators [5]. Several lectins have shown effects during different life stages of many insect orders such as Coleoptera [6–8], Diptera [9], Hemiptera [10], Homoptera [11], Hymenoptera [12], Isoptera [13] and Lepidoptera [14–16]. This feature demonstrates the potential of using plant lectins as naturally occurring insecticide agents against the pests that diminish crop production [16]. Generally, *in vitro* bioassays are undertaken to judge this biological characteristic, which consists of inclusion of the studied lectin into artificial diets offered to the target insect during a given period of time [8].

Lectins are naturally occurring proteins/glycoproteins with substantial structural diversity [17]. Transgenic crops expressing genes that confer resistance against insect herbivory have been produced for most of the world's economically important crops [18] and several studies have shown the efficiency of lectin expression in transformed crops, such as the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA), which is one of the most studied lectins. Genes that encode GNA have been incorporated into a range of crops, including potato [19,20], rice [21–23], maize [24], tobacco [25], wheat [26], tomato [27] and sugarcane [28–30]. In addition to GNA (mannose specifies), ConA (mannose/glucose specifies), PNA (galactose specifies), morniga-G (galactose/N-acetylgalactosamine specifies) and WGA (N-acetylgalactosamine specifies) are examples of other lectins that have also proven insecticidal activity and are widely studied. These observations support the search for new

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lectins with insecticidal activity of different carbohydrate specificities, to act against the most distinct pest species.

cMoL (coagulant lectin from *Moringa oleifera*), belongs to a group of lectins isolated from *Moringa oleifera* seeds (Moringaceae family) [31,32]. This lectin is constituted of a monomeric protein with an approximate weight of 26.5 kDa, as revealed by SDS-PAGE and showed coagulant activity, similar to aluminium sulphate, the coagulant most widely used in water treatment [31]. Another lectin from *M. oleifera*, WSMoL (water-soluble *M. oleifera* lectin), presented larvicidal activity to *Aedes aegypti*, the vector of dengue [33]. The aim of this study was to evaluate the insecticidal activity of cMoL, as judged by its effect on the survival and growth of the Mediterranean flour moth, *Anagasta kuehniella* (Zeller) (Lepidoptera: Pyralidae). This is a polyphagous pest that feeds on a wide variety of stored products, particularly in stored grains, such as dried cocoa beans, dried grains, nuts, tobacco, coconut and dried fruits [34]. *A. kuehniella* is of major economic importance as a flour and grain feeder; it is often a severe pest in flour mills [16,35].

2. Materials and methods

2.1. cMoL purification

Seeds of *M. oleifera* were collected in Recife city, northeastern Brazil and purified according to Santos et al. [31]. Seed flours of *M. oleifera* were extracted with 0.15 M NaCl for 6 h at room temperature. The proteins in this extract were precipitated using 0–60% ammonium sulphate fractionation for 4 h at room temperature. The 0-60F was dialyzed with distilled water (two changes) and 0.15 M NaCl, overnight. The 0-60F was chromatographed (10 mg of protein) on a guar gel column (10.0 cm × 1.0 cm), previously equilibrated with 0.15 M NaCl (20 ml/h flow rate). cMoL was eluted with 1.0 M NaCl.

2.2. Insects and effects of cMoL feeding on insect growth, survival and development

The eggs of flour moths (*A. kuehniella* (Zeller); Phycitinae, Pyralidae, Lepidoptera) were supplied by the Laboratório de Purificação de Proteínas e suas Funções Biológicas (LPPFB), Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brazil. The colony was housed in standard conditions of 28 ± 1 °C, 65–75% relative humidity and a 16:8 (light:dark) photoperiod and routinely maintained on an artificial diet prepared by mixing whole wheat flour, whole wheat husks, whole wheat, and yeast (8:2:1.9:0.1, w/w), according to Macedo et al. [16].

To examine the effects of cMoL on *A. kuehniella* development, neonate first instar larvae were selected and fed an artificial diet containing 0.5%, 1% and 2% of cMoL (w/w). Controls were fed with untreated diet. Each treatment was set up in 250-mg clear plastic, airtight containers and five larvae were transferred to each plastic container ($n = 60$). After the larvae reached the 4th instar at standard conditions, the relationship between protein content and the weight and number of larvae were determined. Larval consumption and faecal production were analyzed on a dry mass basis. The protein content and tryptic activity of the faecal and midgut samples were also determined.

Linear regression analysis was used to evaluate the response of *A. kuehniella* to the concentrations of cMoL. The treatment that provided the effective dose for a 50% response (ED_{50}), defined as the concentration of cMoL that reduced the larval mass by 50% compared to the control larvae, was utilized for further assays. The fresh weight of the resulting pupae and adults was determined on the first day following pupation and upon adult eclosion, respectively. The number of adults that emerged was counted in order to determine the percentage survival to adult emergence (S). The time at which the adults emerged was also recorded to allow estimation of the mean time of development (T). Howe's index [36] was calculated by dividing the log10 of the percentage survival to adult emergence (%S) by the mean time of development (T).

2.3. Measurement of nutritional parameters

Several nutritional parameters were used to compare fourth instars fed on the control diet with those fed on a diet containing 1% cMoL. The larvae, faeces and remaining uneaten food were separated, dried and weighed. The indices of consumption, digestion and food utilization were calculated as described by Scriber and Slansky [37]:

The efficiency of the conversion of ingested food (ECI) estimates the percentage of ingested food that is converted to biomass, and was calculated as: [biomass gained (mg fresh mass)/food ingested (mg dry mass)] × 100.

The efficiency of the conversion of digested food (ECD) estimates the efficiency with which digested food is converted to biomass, and was calculated as: biomass gained (mg fresh mass)/[food ingested (mg dry mass) − feces (mg dry mass)] × 100. Approximate digestibility (AD) estimates the amount of ingested food that is digested, and was calculated as: [food ingested (mg dry mass) − feces (mg dry mass)]/food ingested (mg dry mass) × 100.

Metabolic cost (MC) was calculated as: 100 − ECD.

2.4. Midgut preparation

Homogenates of the larval guts were prepared according to Macedo et al. [38]. Fourth instar larvae were cold-immobilized and dissected in cold 150 mM NaCl. The midguts were surgically removed from the larvae using tweezers. The gut portion taken was posterior to the proventriculus and anterior to the malpighian tubules. After removing all extraneous tissue and freeing the lumen of its contents by rinsing in 150 mM NaCl, the midgut tissues were homogenized in cold 150 mM NaCl in a hand-held Potter–Elvehjem homogenizer immersed in ice. Midgut homogenates were centrifuged at 14,000 × g for 20 min at 4 °C and the supernatants were collected in a known volume of phosphate buffer and used immediately as enzymes sources for enzymatic assays or stored at −20 °C.

2.5. Faecal pellet preparations

Faeces were prepared according to Ramos et al. [34]. Faeces were collected and homogenized in cold 150 mM NaCl in a hand-held Potter–Elvehjem homogenizer. The homogenates were centrifuged at 14,000 × g for 20 min at 4 °C and the supernatants were collected in a known volume of phosphate buffer and used as a source of enzymes for enzymatic assays or stored at −20 °C.

2.6. Protein quantification

Protein concentrations were determined by the dye-binding method of Bradford [39], with bovine serum albumin (1 mg/mL) as the standard.

2.7. Enzymatic assays

Trypsin-like enzymes from gut extracts and faecal samples from *A. kuehniella* larvae were determined using the chromogenic substrate, N-benzoyl-DL-arginine-p-nitroanilide (BAPNA), prepared in 1% (v/v) DMSO (dimethyl sulfoxide) and 100 mM Tris–HCl, pH 8.0 buffer. Samples of 10 μL of trypsin-like enzymes were added to 60 μL of assay buffer, and then 200 μL of BAPNA was added a final concentration of 1 mM. Six replicates were made for each assay. The reaction rate was determined by monitoring the absorbance change at 410 nm for 30 min in a VersaMax Microplate Reader (Molecular Devices, US).

2.8. Digestion of cMoL

The digestion of lectin was carried out according to Macedo et al. [16]. cMoL (2 mg/mL) was incubated with midgut homogenate in Tris–HCl 100 mM pH 8.0 buffer. The cMoL/midgut protein ratio was 1:10 (w/w). Digestion was performed for 1, 3, 6, 12, 24 and 48 h at 37 °C and was stopped by immersing the tubes in boiling water for 2 min. The degradation of bovine serum albumin was used as a positive control for protease activity. The proteins were subsequently separated by SDS-PAGE on 12.5% as described by Laemmli [40]. The proteins were detected by silver staining.

2.9. Protease activity of midgut and faecal extracts in polyacrylamide gels containing 0.1% gelatin

Proteins extracted from the midguts and faecal extracts of *A. kuehniella* larvae fed on control diet and diet containing 1% cMoL were run on 12.5% SDS-PAGE containing 0.1% gelatin [41]. The samples were incubated without prior boiling for 30 min at 37 °C. The samples had also been incubated with TLCK, a synthetic trypsin inhibitor (N-p-tosyl-lysine chloroketone, 1 mM) for 30 min at 37 °C to inhibit proteolytic activity prior to SDS-PAGE. Following electrophoresis at 4 °C, the gels were washed with 2.5% Triton X-100 solution for 2 h with shaking to remove the SDS, after which the gels were incubated with 100 mM Tris–HCl buffer, pH 8.0, for 2 h. The gels were subsequently stained with Coomassie brilliant blue R-250. Bands of proteolytic activity appeared as clear (white) zones against a blue background.

2.10. Chitin-column chromatography

To examine the possible interaction of cMoL with chitin, cMoL was chromatographed on a chitin column (2 ml) and equilibrated with 150 mM NaCl. After adsorption of the protein (2 mg), the column was washed with 150 mM and 1 M NaCl, respectively. The bound cMoL was then eluted with 1 M acetic acid. The bound cMoL was then eluted with 0.1 M acetic acid. Fractions (2 ml) were collected, dialyzed against water and used in assays of hemagglutinating activity and protein quantification.

2.11. Hemagglutinating activity assays

HA was performed in microtiter plates, according to Correia and Coelho [42]. The peaks from chromatography (50 μL) were serially two-fold diluted in 150 mM NaCl before addition of 50 μL 2.5% (v/v) suspension of rabbit glutaraldehyde treated erythrocytes. The titer was expressed as the highest dilution exhibiting hemagglutination.

For hemagglutinating activity inhibition, carbohydrate or glycoprotein in solution (50 μL) were added to microtiter plates. Samples of peaks (50 μL) were serially two-fold diluted in monosaccharide solution and incubation (45 min, 27 °C) was performed before the addition of a 2.5% (v/v) suspension of rabbit erythrocytes (50 μL) and HA was observed after 45 min. Inhibition of HA was performed using a D-fructose solution (200, 100, 50 and 25 mM) and azocasein (0.5 mg/mL).

2.12. Statistical analysis

For each treatment and control, data regarding the reduction in weight gain of the larvae, pupae and adults are expressed as means ± SEM. To detect significant differences between treatments, data were analyzed by one-way analysis of variance (ANOVA) and then means ± SEM were separated using a post-hoc Tukey test. A p value ≤ 0.05 was considered to be significant.

3. Results and discussion

The effect of cMoL incorporated in artificial diet on growth, nutritional physiology and survival of larvae was accompanied until fourth instar, pupae and adults (Fig. 1). All nutritional parameters, as well as the total development time (TDT) and growth index (GI), were evaluated together with survival and weight gain to better understand the action of cMoL on this insect. Fig. 1(A and B) shows the effect of cMoL on weight and survival of the larvae of the fourth instar, respectively. According to the graph, it may be clearly seen that the reduction in larval weight gain is dose-dependent. At 1%, cMoL caused a reduction in the average weight of ca. 45%, approaching the ED_{50}. Regression analysis demonstrated that the addition of cMoL to the artificial diet, at 0.1%, resulted in a 0.25 mg decrease in mass; however, no concentration of cMoL tested caused any significant reduction in larval survival. Similar results have been reported for other lectins. Coelho et al. [43] incorporated ACLEC (*Anona coriacea* lectin) in an artificial diet at a level of 1% for *A. kuehniella* larvae and observed a reduction in larval mass of ca. 50%. A lectin from *Koelreuteria paniculata* seeds (Kplec), added to the artificial diet at a concentration of 1%, was toxic to *A. kuehniella*, reducing the larval weight by 84% [44]. Recently, Macedo et al. [16] tested a *Bauhinia monandra* leaf lectin (BmoLL) for anti-insect activity against *A. kuehniella*, *Callosobruchus maculatus* and *Zabrotes subfasciatus*. BmoLL in the artificial diet at 1% produced a decrease in larval weight of 40% for *A. kuehniella* while at 0.5% and 0.4% it produced 20% and 50% decreases in the mass of *Z. subfasciatus* and *C. maculatus*, respectively.

With regard to mortality rate, the same lectin may present different lethality for different insects species. For example, transgenic rice plants expressing GNA show resistance to both *Nephotettix virescens* and *Nilaparvata lugens* [45,46]. In both studies, the survival of nymphs on GNA-expressing plants was significantly reduced (up to 50%) [47]. Nagadhara et al. [48] tested resistance against *Sogatella furcifera* by expressing GNA specifically in the phloem of transgenic rice plants. Survival of nymphs and fecundity of adults both dropped by 90%, feeding decreased, and surviving nymphs suffered delayed development [47]. However, Gatehouse et al. [49] reported the expression of GNA in transgenic potatoes for the control of the tomato moth, *Lacanobia oleracea*, with a significant effect on larval size and adverse affect on the developmental rate, whilst effects on survival were not so marked. This difference in larvicidal activity of GNA towards different insect species illustrates the peculiar specificity of lectins as insect pest management molecules. Therefore, we can infer that other factors intrinsic to each species of insect study can result in different effects on larval mortality; this does not rule out an insecticide role for cMoL, but indicates that significant rates

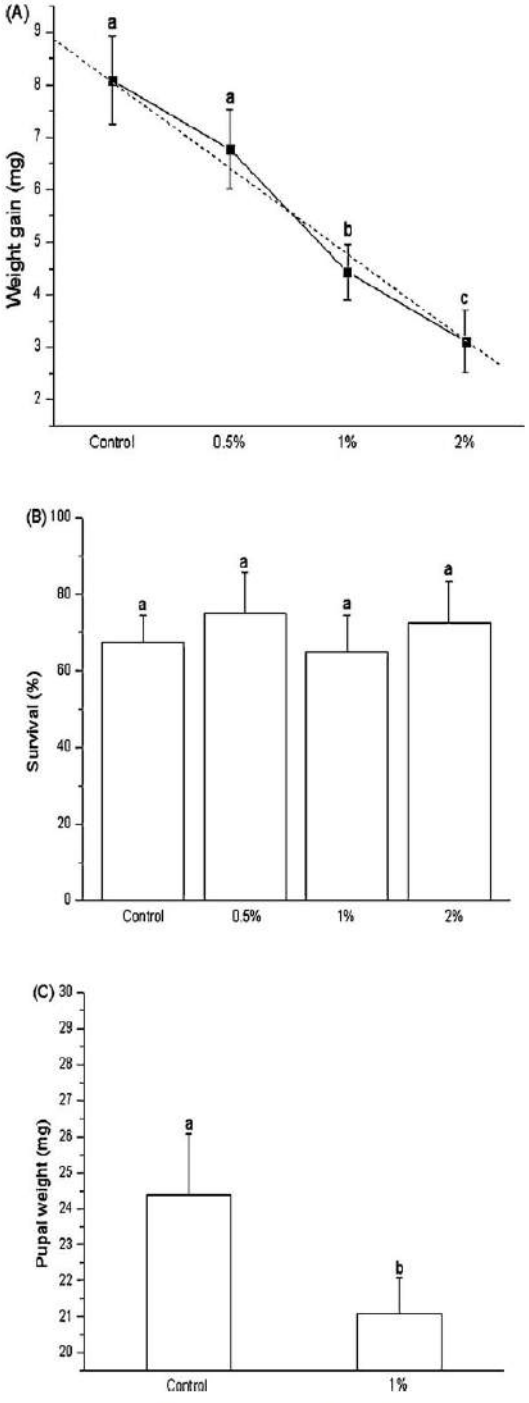

Fig. 1. Effect of dietary cMoL on the (A) mass and (B) survival of *A. kuehniella* larvae. Inset: variation in the size of fourth-instar larvae fed on the control diet (1) or a diet containing 0.5% (2), 1.0% (3) or 2.0% (4) cMoL. (c) Effect of cMoL against pupa of the *A. kuehniella*, resulting from larvae fed on diet containing 1% cMoL. Inset: variation in the size of control pupa (1) or that of larvae fed on 1% cMoL (2). Each point is the mean ± S.E. of ≥60 larvae. The different letters indicate significant differences ($p < 0.05$; Tukey test). Bar = 1 cm.

of larval mortality were not observed for *A. Kuehniella* larvae. The cMoL concentration in the artificial diet ranged from 0.5% to 2%, as these values constitute the range of lectin concentrations found in transgenic crops and were similar to concentrations used in other studies with purified plant lectins [16,20,50,51].

Table 1
Nutritional parameters of *A. kuehniella* fourth-instar larvae fed on 1% cMoL-treated and control diets.

Treatment (%)	ECI (%)	ECD (%)	AD (%)	CM (%)
Nutritional parameters (mean ± SE)				
Control	26.58 ± 1.1a	31.13 ± 1.5a	80.67 ± 4a	68.87 ± 1.5a
1	18.28 ± 1.9b	19.42 ± 1.8b	94.06 ± 2.3b	80.23 ± 1.6b

ECI: efficiency of the conversion of ingested food; ECD: efficiency of the conversion of digested food; AD: approximate digestibility; CM: 100 – ECD.
Means within a column followed by a different letters are significantly different, $p < 0.05$; based on Tukey's test.

Table 2
Effect of cMoL (1% in the diet) on the development of the *A. kuehniella* larvae.

Treatment (%)	%S[a]	TDT[b]	GI[c]
Control	90.1 ± 1	46	0.0424
1	62.5 ± 1.9	61	0.0294

Values are the mean ± S.E. $p < 0.05$ compared to corresponding control (Student's *t*-test).
[a] Survival during the adult stage.
[b] TDT = total developmental time (mean developmental time per insect in days).
[c] GI = growth index [(in % adult emergence)/TDT].

Food intake and faecal production of larvae of *A. kuehniella* treated on a diet containing 1% cMoL decreased by 18.7% and 66.5%, respectively. These results were followed by profound nutritional changes (Table 1). The diet containing 1% cMoL decreased (by approximately 14%) the efficiency by which ingested food was assimilated (AD) by the larvae of *A. kuehniella*, when compared to the control group. The efficiency by which *A. kuehniella* larvae converted food into body mass as shown by the amount of food consumed (ECI), or the amount of food absorbed in the intestine (ECD) decreased by 31.2% and 37.6%, respectively, while the metabolic cost (MC) increased by 16.5%, compared to respective controls. The decrease in dietary utilization suggested that the reduced growth and decrease in faecal production resulted from behavioral and physiological (post-ingestive) effects [43,52,53], possibly caused by the longer retention of food in the gut to maximize AD. A greater AD would help to meet the increased demand for nutrients [54,55] and compensate for the deficiency in foodstuff conversion (reduction in ECI and ECD), perhaps by diverting energy from biomass production into detoxification [43,56]. ECD also decreases as the proportion of digested food metabolised for energy increases [57]. The low values of ECI and ECD observed suggest that chronic toxicity and the reduction in ECD likely resulted from an increase in the proportion of assimilated energy diverted from growth to cover MC associated with detoxification and excretion of cMoL. Boleti et al. [15] described an increase in MC in *A. kuehniella* larvae that was similar to that seen in our study. Coelho et al. [43] observed similar results in their study, where *A. kuehniella* and *C. cephalonica* larvae feed on ACLEC at 2% presented lower food consumption (33% and 28%) and faecal production with decreases of 25% and 40%, respectively, accompanying similar nutritional changes.

The vast majority of individuals fed on cMoL at 1% developed to the pupal stage. Interestingly, a significant delay in larval development time of 15 days was found, compared to the control group. In addition to the significant increase in TDT, treatment with cMoL at 1% caused an increase in pupal mortality of 27.6%, where the surviving pupa were adversely affected and presented a smaller size and a reduction in the average weight of 13.5%, compared to the control group (Fig. 1C). Neither type of deformation was observed in the pupae in both groups. Li and Romeis [30] reported that the presence of GNA in food of green lacewing *Chrysoperla carnea* significantly prolonged the larval development time by 3.7 days on average. Machuka et al. [50] incorporated different lectins from African yam beans (*Sphenostylis stenocarpa*) for *C. maculatus* larvae. The Enugu 95-3 lectin caused an average delay in developmental time of 7.5, 7.8 and 11.3 days at 0.2%, 2.0% and 5.0% dietary levels, respectively, compared to susceptible control seeds. Murdock et al. [55] showed that WGA, lectin, which has N-acetylglucosamine carbohydrate binding specificity, delays *C. maculatus* development by 6.5 and 22.8 days at 0.2% and 1.0%, respectively. The growth index (GI) measures the effects of the food substrate, such as dietary lectin, on both survival and developmental time [36]. The principle behind this parameter is that adverse conditions, such as an unsuitable food substrate, may prolong the developmental period, while fewer individuals survive, resulting in a low GI value. Conversely, a suitable food substrate should result in a high GI value [50,58,59]. At 1%, cMoL caused a significant drop in GI value of more than 30% (Table 2). Similar effects of plant lectins on the development of insects are well documented [60]. The low mortality observed for *A. Kuehniella* larvae fed with cMoL was offset by low rates of adult emergence. Another important result was the delayed development. The extension of the life cycle of a pest is very desirable, since any delay in its development is advantageous because it reduces the emergence ratio of new generations and, thus, reduces the damage caused by the species in the target crop.

A prerequisite for toxicity is that the lectin should be able to survive the hostile proteolytic environment of the insect midgut [43]. The susceptibility of cMoL degradation by proteases of *A. kuehniella* was tested by incubating the lectin with these enzymes in a 1:10 ratio, followed by SDS-PAGE (Fig. 2A). In an attempt to degrade the lectin under study, the larvae of *A. kuehniella* did not show any changes in its enzymatic profile, in the total amount of enzymes or the production of different classes of proteases when analyzed by gel electrophoresis and enzymatic activity *in vitro* (Fig. 2A). cMoL was resistant to digestion during the first 12 h of incubation (Fig. 2B), in contrast to the control that was prepared with BSA, where digestion of protein occurred during the first 3 h (data not shown). Macedo et al. [60] observed that the incubation of TEL (*Talisia esculenta* lectin) with purified insect cysteine-like proteases from *C. maculatus* was unable to digest the lectin at up to 15 h. Another lectin, BmoLL was resistant to gut proteolysis by *C. maculatus* and *Z. subfasciatus* for up 48 h [16]. These results vary according to insect species. The incubation of ACLEC with gut proteases from *C. cephalonica* larvae resulted in the degradation of the lectin that started within first minutes but required several hours for completion. In contrast, ACLEC was more resistant to *A. kuehniella* gut enzymes [43]. This result confirms the effects on larval physiology, reaffirming that the lectin appears to have maintained its structure to exert its insecticidal activity.

Many proteins that bind to chitin are related to plant defense mechanisms against organisms that contain this polysaccharide as a constituent of their membranes. This group includes wheat germ agglutinin (WGA) and other lectins and proteins that are toxic to insects and affect the development of fungi [61]. The mode of action of these proteins is unknown but may interfere with the uptake of nutrients by binding to chitin in larval midgut membranes. This membrane exists in most phytophagous insects and is composed primarily of chitin (containing N-acetylglucosamine residues) and proteins [61,62]. The chromatography of cMoL on the chitin column resulted in two peaks, one of which eluted in the equilibrium buffer and the other with 1 M acetic acid. According to the results of HA assays, we can affirm that the peak eluted from the column with acetic acid is cMoL, as evidenced by the high HA and its complete inhibition by azocasein, characteristic of cMoL (data not shown).

We suggest that the binding of cMoL to chitin demonstrates the interactions of lectin with chitinous structures present in the midgut of larvae. As such, cMoL binds strongly to chitin and presents insecticidal activity, similarly to other insecticidal proteins such as the vicilins [63,64], Talisin [65] and zeatoxin [66], in addition to other lectins [16,43,44]. Because the occurrence of

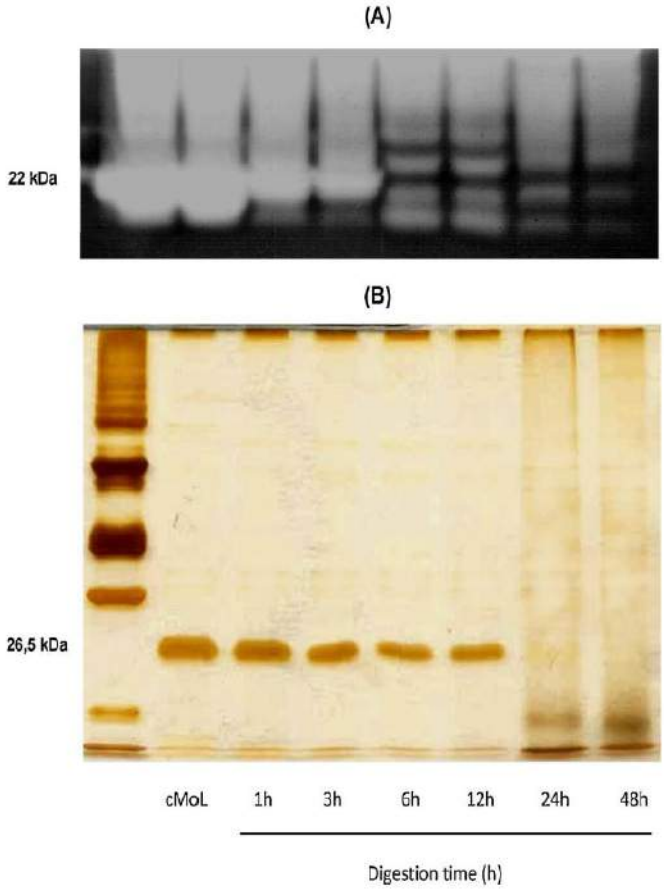

Fig. 2. (A) Proteolytic activity of *A. kuehniella* larvae fed on cMoL. Protease assay SDS-PAGE containing 0.1% gelatin: (1) midgut extract from larvae fed on the control diet, (2) midgut extract from larvae fed on 1% cMoL, (3) faecal extract from larvae fed on the control diet, (4) faecal extract from larvae fed on 1% cMoL, (5) midgut extract from larvae fed on the control diet with TLCK, (6) midgut extract from larvae fed on 1% cMoL with TLCK, (7) faecal extract from larvae fed on the control diet with TLCK, (8) faecal extract from larvae fed on 1% cMoL with TLCK. Proteolytic activity appeared as a clear zone against a dark blue background. (B) SDS-PAGE of cMoL digested by midgut extracts of *A. kuehniella*. cMoL and cMoL digested by 1 h, 3 h, 6 h, 12 h, 24 h and 48 h.

this carbohydrate as a structural component is limited to a few organs, such as the integuments of arthropods, nematodes and the gut linings of insects, chitin metabolism is an excellent target for selective pest control strategies [67]. Eisemann et al. [68] reported that ConA (a mannose/glucose ligand) bound to and increased the permeability of peritrophic membranes of the blowfly *Lucilla cuprina*. These authors concluded that the ingested lectin may restrict the bi-directional movement of nutrients and digestive enzymes across pores in these membranes, thus leading to nutritional deprivation [8,68]. Macedo et al. [16,60] suggest that the alteration in intestinal protein content may result from an interference in the digestive environment, which could increase the difficulty of the transit of molecules through the perotrophic membrane by interfering with recycling and absorption, culminating in a decrease in the protein concentration of gut contents and a greater removal of proteins in the faeces. SDS-PAGE, containing 0.1% gelatin (Fig. 2A), demonstrated that cMoL did not cause any change in the enzymatic content of the larvae, as shown by activity levels and by the similarity of the enzyme profiles obtained from the control group and that fed on cMoL. Another important observation is that, in both groups, the serine proteases are largely digestive enzymes, since the addition of a synthetic inhibitor (TLCK) upgraded its inhibition.

The insecticidal activity of lectins makes them good candidates for the control of insect pests. However, the specific interaction of lectins with the gut epithelium of insects may have a wider potential for biotechnology than the insecticidal property of lectins alone [69]. By developing chimeric proteins that use lectins as a binding domain or subunit, they might be used, (*i*) to assist in concentrating bioactive polypeptides such as Bt toxins and insect hormones [70] at or near vulnerable target sites in the insect alimentary tract, (*ii*) to facilitate the transfer of cytotoxins across the epithelial cell membrane or (*iii*) to help prevent proteolytic digestion of other bioactive proteins to which they have been bound [69]. Other possible biotechnological applications of lectin may include the site direct mutagenesis of cMoL in order to improve its insecticide activity, similar to that performed by Zhu-Salzman et al. [69] for *Griffonia simplicifolia* lectin II (GSII). Through mutagenesis studies, the authors showed that recombinant GSII with an increase in sugar binding also increased the insecticidal activity for *Callosobruchus maculatus*.

In conclusion, this study showed that cMoL is a lectin with a significant negative impact against *A. kuehniella* and suggests that the use of this molecule may constitute an alternative method for the combat this pest. The lectin demonstrated a tight interaction with chitin and proved to be refractory to digestion by *A. kuehniella* midgut proteases, interfering in the digestive environment together with the glycosylated enzymes, other glycoproteins or even in the peritrophic membrane structure, which may hinder the transport of enzymes and their hydrolysis products, reducing the availability of amino acids for larval growth, leading to

the poor development observed. cMoL possesses a great potential as a biotechnological tool, promoting an environmentally friendly agriculture and being less harmful.

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Effect of lectins from *Opuntia ficus indica* cladodes and *Moringa oleifera* seeds on survival of *Nasutitermes corniger*

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ABSTRACT

Biodegradation by termites is a serious problem for wood and crop industries worldwide, and new environmentally friendly alternatives for termite control have been developed. This work investigated the effects of crude and purified preparations containing lectins from *Opuntia ficus indica* cladodes (OfiL) and *Moringa oleifera* seeds (WSMoL and cMoL) on *Nasutitermes corniger* workers and soldiers. Purified OfiL was more active than cladode extracts, showing a stronger termiticidal activity against workers (LC_{50} of 0.116 mg ml^{-1}) than against soldiers. OfiL was active against soldiers only at 1.5 mg ml^{-1}. All preparations containing WSMoL and cMoL were active only at concentrations of 1.0 and 1.5 mg ml^{-1}. The tested preparations did not exert repellent activity against *N. corniger*. OfiL was able to kill workers and therefore is potentially a new tool for *N. corniger* control; as a consequence, this lectin could disturb organization, structure, and maintenance of termite colonies.

1. Introduction

The use of chemical insecticides is still the main strategy used to control insect pests in agriculture. However, when applied on a large scale, these products have been associated with the emergence of resistant pest populations as well as damage to the environment and human health (Barros et al., 2006; Leite et al., 2007; Liu et al., 2011). Phytoinsecticides have been intensively studied in programs of integrated pest management, and today they are considered an effective and environmentally friendly alternative (Bernardi et al., 2010). When compared to ordinary chemical insecticides, these products generally present lower persistence and residual action, being more rapidly degraded. Although bioinsecticides may not be as effective as synthetic chemicals, they minimize the risk of elimination of beneficial species, decrease the emergence of resistant populations and phytotoxicity, and reduce human health hazards (Menezes, 2005).

Insecticidal activity of lectins – hemagglutinating proteins with carbohydrate-binding ability – against agricultural insect pests has been reported for *Bauhinia monandra* leaf lectin on *Callosobruchus maculatus*, *Zabrotes subfasciatus*, and *Anagasta kuehniella* (Macedo et al., 2007). The lectins from *Arisaema intermedium* and *Arisaema wallichianum* have been reported to delay the development of the melon fruit fly, *Bactrocera cucurbitae*, inhibiting pupation and emergence (Kaur et al., 2009). Induction of mortality on *Acyrthosiphon pisum* (pea aphid) and *Myzus persicae* (green peach aphid) were observed after exposure to lectins from *Allium sativum* and *Xerocomus chrysenteron*, respectively (Fitches et al., 2008; Jaber et al., 2008). Chitin-binding lectins are able to interact with chitinous structures in insect guts, promoting deleterious effects (Coelho et al., 2009).

Termites belong to a group of decomposer insects called Isoptera, closely related to cockroaches. They are long-lived eusocial insects that form large colonies consisting of reproductive forms, sterile workers, soldiers, and immature individuals. These insects produce their nests in trees, roots, or soil, since they are able to feed on woody tissue of plants, intact or partially decayed, as well as leaf litter, soil, and animal dung (Scheffrahn et al., 2002; Verma et al., 2009). Termites are abundant in tropical and subtropical regions, where their tunneling efforts help aerate the soil; also, these organisms help in breaking down and recycling plant materials (Thompson, 2000; Costa-Leonardo, 2002; Verma et al., 2009).

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Termites also improve soil quality by ingesting and transporting organic and mineral particles; they are nitrogen fixers and can function as bio-indicators of environmental quality and soil fertility in agriculture ecosystems (Brown, 1997; Kaschuk et al., 2006; Yamada et al., 2006).

However, their specialization to feed on cellulosic materials, along with the ability to damage harvested kernels, have given termites the reputation of being among the most important pests, due to the economic losses they cause. These insects destroy wood, building materials, and other commercial products (Meyer, 2005; Verma et al., 2009). Biodegradation by termites is considered the most serious problem in the wood industry, and severe economic losses caused by termite action have been reported worldwide (Meyer, 2005). In China, the economic losses exceed 200 million dollars a year, while in Japan values reach 800 million/year and in the U.S. these losses are above 1 billion/year; in Brazil termites were responsible for 42.7% of the overall damage to buildings (Zhong and Liug, 2002; Verma et al., 2009). Miranda et al. (2004) showed that *Cryptotermes nordenskioeldi* damages sugarcane crops and *Amitermes nordestinus* is a potential pest in this culture. Joshi et al. (2005) and Constantino (2007) have reported on termites damaging maize, eucalyptus, and rice.

Controlling termites is a complex task, since these insects live in highly organized colonies. Due to this difficulty, different termite-control methods have historically been devised (Myles, 2005). Physical barriers to termites include the use of heat, freezing, electricity, and even microwaves. However, chemical treatment stands as the most widely adopted approach to reduce infestation by termites and includes soil termiticidal injection, baits, and chemical fumigation. Plant-derived biopesticides, nematodes, bacteria, and fungi have several desirable properties such as insecticidal, repellent, and deterrent action that inhibit feeding activities, apart from acting as insect growth regulators (Bogús et al., 2005; Chai et al., 2007; Zhou et al., 2007; Bedmutha et al., 2011).

Nasutitermes corniger Motsch. (Isoptera, Termitidae) is broadly distributed in America, being found from southern Mexico to northern Argentina. In the urban environments of the Brazilian semi-arid region, *N. corniger* has been responsible for the destruction of furniture as well as of building structures (Paes et al., 2002). Termiticidal activity on *N. corniger* has been reported for lectins isolated from *Cladonia verticillaris* lichen, *B. monandra* secondary roots, and *Myracrodruon urundeuva* bark, heartwood, and leaf, as well as secondary metabolites from *Bowdichia virgilioides*, *Anadenanthera colubrina*, and *Hymenaea stigonocarpa* (Sá et al., 2008; Silva et al., 2009; Santana et al., 2010; Napoleão et al., 2011; Souza et al., 2011). The termiticidal activities of lectins were reported recently, and commercial formulations are currently being developed. In this work, the insecticidal activity of lectins aroused our interest in shedding new light on how plants could be used as novel sources of these proteins as bioactive agents against *N. corniger*.

Cladodes of *Opuntia ficus indica* Mill. are used in folk medicine for their diuretic, antiulcer, and wound-healing properties (Galati et al., 2001, 2002; Trombetta et al., 2006). Chitin-binding lectin isolated from *O. ficus indica* cladodes (OfiL) was thermo-stable and ion dependent and showed antifungal activity against *Candida albicans*, *Colletotrichum gloeosporioides*, *Fusarium decemcellulare*, *Fusarium lateritium*, *Fusarium oxysporum*, and *Fusarium solani* (Santana et al., 2009).

Moringa oleifera Lam. is a tree native to India; it is also found in several African, Asian, and South American countries. This plant is of significant economic, industrial, and medicinal importance (Makkar and Becker, 1996). Two chitin-binding lectins isolated from *M. oleifera* seeds have shown insecticidal activity. The water-soluble *M. oleifera* lectin (WSMoL) promoted mortality of *Aedes aegypti* fourth-stage larvae (Coelho et al., 2009) and the coagulant *M. oleifera* lectin (cMoL) was able to reduce growth of larvae and kill pupae of *A. kuehniella* (Oliveira et al., 2011).

This work reports on the effect of OfiL, WSMoL, and cMoL on *N. corniger* survival, as well as on the response of termites to lectin in a repellence assay. In addition, some properties of OfiL, cMoL, and WSMoL are comparatively analyzed.

2. Materials and methods

2.1. Plant material

O. ficus indica Mill. (Family Cactaceae) has the vernacular names of "palma-forrageira" in Portuguese, "nopal de castilla" in Spanish, and Indian fig opuntia in English. The cladodes from different specimens were collected in Limoeiro City, State of Pernambuco, northeastern Brazil. The cladodes were air-dried, powdered, and stored at 4 °C.

M. oleifera Lam. has the vernacular names "moringa" in Portuguese, "árbol del ben" in Spanish, and horseradish tree or drumstick in English. Seeds from 10 to 15-year-old trees were collected in Recife City, State of Pernambuco, northeastern Brazil. The seeds were air-dried, powdered, and stored at 4 °C.

2.2. Insects

Colonies of *N. corniger* were kept in a greenhouse (28 ± 2 °C; 75 ± 5% relative humidity) at the Departament of Agronomy of the Federal Rural University of Pernambuco. Termite colonies were selected according to overall integrity criteria. One colony was used in experiments with *O. ficus indica* preparations and another in those with samples from *M. oleifera* seeds.

2.3. Isolation of *O. ficus indica* lectin (OfiL)

The OfiL was isolated according to the protocol described by Santana et al. (2009). Cladode powder (10 g) was homogenized (16 h, 4 °C) in 0.15 M NaCl (50 ml). After filtration through gauze followed by centrifugation (4000 g, 15 min), the crude extract (E_{OfiL}) was loaded (22 mg of protein) onto a chitin column (6.5 × 1.5 cm) equilibrated (flow rate of 20 ml h^{-1}) with 0.15 M NaCl. The column was washed with the equilibrium solution. Afterwards, OfiL was eluted from the column with 1.0 M acetic acid and dialyzed (3.5 kDa cut-off membrane) against 0.15 M NaCl (6 h at 4 °C) for eluent elimination.

2.4. Isolation of water-soluble (WSMoL) and coagulant (cMoL) *M. oleifera* seed lectins

The WSMoL was isolated according to the procedure described by Coelho et al. (2009). Powdered seeds (10 g) were homogenized with distilled water (100 ml) in a magnetic stirrer (16 h, 4 °C). Next, the mixture was filtered through cotton gauze and centrifuged at 3000 g for 15 min. The crude extract (E_{WSMoL}) was treated with a 60% saturated ammonium sulphate solution (Green and Hughes, 1955) and the precipitated fraction containing WSMoL (F_{WSMoL}) was collected after centrifugation (3000 g, 15 min), dissolved in 0.15 M NaCl, and submitted to dialysis (3.5 kDa cut-off membrane) against 0.15 M NaCl (6 h at 4 °C). The dialyzed F_{WSMoL} (80 mg of proteins) was then applied to a chitin column (18 × 1.5 cm) equilibrated (flow rate of 20 ml h^{-1}) with 0.15 M NaCl. After extensive washing with the equilibrating solution, WSMoL was eluted with 1.0 M acetic acid and dialyzed (3.5 kDa cut-off membrane) against distilled water (6 h at 4 °C).

Isolation of cMoL was done according to Santos et al. (2009). Powdered seeds (10 g) were homogenized with 0.15 M NaCl

(100 ml) in a magnetic stirrer (6 h, 28 °C). Afterwards, the mixture was filtered through cotton gauze and centrifuged at 3000 g for 15 min. The proteins in saline extract (E_{cMoL}) were precipitated using ammonium sulphate (60%) and the precipitated protein fraction (F_{cMoL}) was dialyzed against distilled water (4 h) and 0.15 M NaCl (16 h). The F_{cMoL} was loaded (10 mg of protein) onto a guar gel column (10 × 1 cm) equilibrated (flow rate of 20 ml h^{-1}) with 0.15 M NaCl. The cMoL was eluted with 1.0 M NaCl and dialyzed (3.5 kDa cut-off membrane) against 0.15 M NaCl (6 h at 4 °C).

2.5. Protein content

The protein concentration was estimated according to Lowry et al. (1951) using bovine serum albumin (31–500 μg ml^{-1}) as standard.

2.6. Hemagglutinating activity

The assay was conducted in microtiter plates (Kartell S.P.A., Italy) according to Paiva and Coelho (1992) using a suspension (2.5% v/v) of rabbit erythrocytes treated with glutaraldehyde (Bing et al., 1967). Hemagglutinating activity (HA) was determined by mixing a twofold serial dilution of each sample (50 μl) with 0.15 M NaCl in microtiter plates. A 2.5% (v/v) suspension of erythrocytes (50 μl) was added to each well. Incubation at 27 °C (45 min) ensued. One hemagglutination unit was defined as the reciprocal value of the highest dilution of sample that promotes full agglutination of erythrocytes. Specific hemagglutinating activity was defined as the ratio between titer and protein concentration (unit mg^{-1}). HA inhibitory assays were performed by incubation (45 min) of lectin sample with 200 mM monosaccharide or 500 μg ml^{-1} glycoprotein solutions before erythrocyte suspension addition.

2.7. Polyacrylamide gel electrophoresis

Evaluation of OfiL and WSMoL was done by PAGE for native (10% w/v, gel) basic or acidic proteins performed according to Reisfeld et al. (1962) and Davis (1964), respectively. Basic polypeptides were stained with 1% (w/v) Amido Black in 10% (v/v) acetic acid. Acidic polypeptides were stained with 0.02% (v/v) Coomassie Blue in 10% (v/v) acetic acid.

2.8. Termiticidal assay

Termiticidal activity was evaluated by a no-choice bioassay based on the method described by Kang et al. (1990). Each experimental unit consisted of a petri plate (90 × 15 mm) with the plate base covered with filter paper. Termiticidal activity was evaluated for E_{OfiL} (0.25, 0.5, 1.0, and 1.5 mg ml^{-1} of protein) and OfiL (0.25, 0.5, 1.0, and 1.5 mg ml^{-1}). *M. oleifera* seed preparations containing WSMoL (E_{WSMoL}, F_{WSMoL}, and WSMoL) and cMoL (E_{cMoL}, F_{cMoL}, and cMoL) were evaluated at concentrations of 0.125, 0.25, 0.5, 1.0, or 1.5 mg ml^{-1} of protein. In each replicate, a filter paper disk (4 cm in diameter) impregnated with 200 μl of sample in 0.15 M NaCl was placed in the plate. In negative controls, paper disks were impregnated with 0.15 M NaCl solution containing neither extract nor lectin. A total of 20 active termites (workers and soldiers, in the proportion of 4:1, respectively) were transferred to each plate; these were maintained at 28 °C in darkness. Evaluation of insect survival was made daily until the death of all insects. Bioassay was achieved in quintuplicate for each concentration and survival rates (as percentages) were obtained for each treatment.

2.9. Repellence assay

The repellence assay was based on Su et al. (1982). Petri plates (100 × 15 mm) were filled up with 2% agar solution to the plate covers. After solidification, wells are made in agar by removing a central cylindrical core 25 mm in diameter and 10 peripherical cylinders (6 mm in diameter). In each peripherical well a piece of filter paper was soaked in 15 μl of 0.15 M NaCl containing neither extract nor lectin (negative control). Petri plates contained the OfiL, WSMoL, and cMoL preparations at the same concentrations used in the termticidal assay. Termites (16 workers and four soldiers) were then transferred to the central well and plates were maintained at 28 °C in darkness. Assays were made in triplicate. The parameters observed for 15 days were the absence or presence of termites in peripherical wells, the construction standards of tunnels in agar, and the closing by insects of constructed galleries.

2.10. Statistical analysis

Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) and data were expressed as a mean of replicates ± SD. Significant differences between treatment groups used in the termiticidal assay were analyzed by Student's *t*-test (significance at $p < 0.05$) using the Origin 6.0 program. The lethal concentrations required to kill 50% (LC_{50}) of termites after 4 days were calculated by probit analysis with a reliability interval of 95% using the computer software StatPlus® 2006 (AnalystSoft, Canada).

3. Results

A protein level of 11 mg ml^{-1} and specific hemagglutinating activity of 3.0 were observed in E_{OfiL}. In turn, a specific hemagglutinating activity of 40 and a single polypeptide band in PAGE for acidic proteins were detected in isolated OfiL (Fig. 1A). No band was detected in PAGE for basic proteins.

Termiticidal assays using E_{OfiL} showed that this preparation did not interfere with survival of soldiers (Fig. 2C), though it exerted termiticidal action against workers ($p < 0.05$) when used at 1.5 mg ml^{-1} (Fig. 2D). Significantly different soldier mortality rates ($p < 0.05$) were observed for OfiL 1.5 mg ml^{-1} only, as compared to the control (Fig. 2A), while on workers (Fig. 2B) lectin promoted a 100% mortality rate after 4 (1.5 mg ml^{-1}), 5 (1.0 mg ml^{-1}), and 6 (0.25 and 0.5 mg ml^{-1}) days. Student's *t*-test revealed that effects of concentrations 0.5, 1.0, and 1.5 mg ml^{-1} were significantly ($p < 0.05$) different in comparison with the control, and that the LC_{50} determined after 4 days was 0.116 mg ml^{-1}.

The E_{OfiL} and OfiL did not repel workers or soldiers, since the insects built galleries randomly, with no differences observed between samples and the negative control (0.15 M NaCl). Also, it is important to note that these groups of insects maintained these galleries open and active throughout the experiment.

For E_{WSMoL}, the protein level was 5.42 mg ml^{-1} and specific hemagglutinating activity was 0.73, while F_{WSMoL} was richer in protein (13.64 mg ml^{-1}) and lectin (specific hemagglutinating activity of 1.17). The WSMoL, isolated by chitin chromatography, showed high specific hemagglutinating activity (6826) and a single polypeptide band in PAGE for acidic proteins (Fig. 1B). Polypeptide bands were not detected on PAGE for basic proteins.

The E_{WSMoL} was termiticidal ($p < 0.05$) on soldiers only when used at 1.5 mg ml^{-1} (Fig. 3A), but it was active against workers when used as 1.0 and 1.5 mg ml^{-1} preparations (Fig. 3B). The F_{WSMoL} and WSMoL were able to kill soldiers and workers only when used as 1.5 mg ml^{-1} preparations ($p < 0.05$) (Fig. 3C, D, E, and

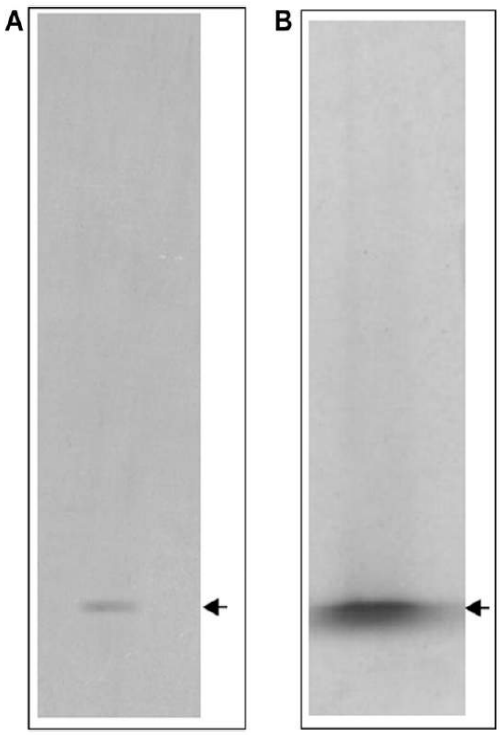

Fig. 1. Electrophoresis profiles of OfiL (A) and WSMoL (B) on PAGE for native acidic proteins stained with Coomassie Brilliant Blue.

F). No WSMoL preparation induced a rejection response by termites in the repellence assay.

The E_{cMoL} (8.04 mg ml^{-1} of protein and specific hemagglutinating activity of 7.96) was treated with ammonium sulphate and the resultant F_{cMoL} showed the highest protein concentration (15 mg ml^{-1}) and specific hemagglutinating activity (17.06). cMoL isolated after chromatography on guar gel column showed specific hemagglutinating activity of 200 that was not inhibited by *N*-acetylglucosamine (Table 1).

The E_{cMoL} was active ($p < 0.05$) against soldiers only as a 1.5 mg ml^{-1} preparation (Fig. 4A), although it promoted worker mortality ($p < 0.05$) at protein concentrations of 1.0 and 1.5 mg ml^{-1} (Fig. 4B). No F_{cMoL} concentration tested exerted a termiticidal effect against soldiers or workers (Fig. 4C and D). Purified cMoL was not active against soldiers (Fig. 4E), and was termiticidal ($p < 0.05$) against workers when used as 1.5 mg ml^{-1} preparations (Fig. 4F). There were no repellent properties shown for E_{cMoL}, F_{cMoL}, or cMoL.

4. Discussion

Termite control has been performed using chemical insecticides; however, the adverse effects of these chemicals on human health and the environment at large, such as neurotoxicity and contamination of food, as well the emergence of resistant insect strains and long-term ecological imbalance, have become issues of increasing concern. For these reasons, natural products have been screened for termiticidal and repellent activities with a view to preventing termite attack (Verma et al., 2009).

Here, the availability of simple, efficient, and reproducible procedures that yield milligram quantities of OfiL, WSMoL, and cMoL, as well as previous reports of insecticidal activity of chitin-binding lectins, facilitated the evaluation of lectin preparations (E_{OfiL}, E_{WSMoL}, E_{cMoL}, F_{WSMoL}, F_{cMoL}, OfiL, WSMoL, and cMoL) for

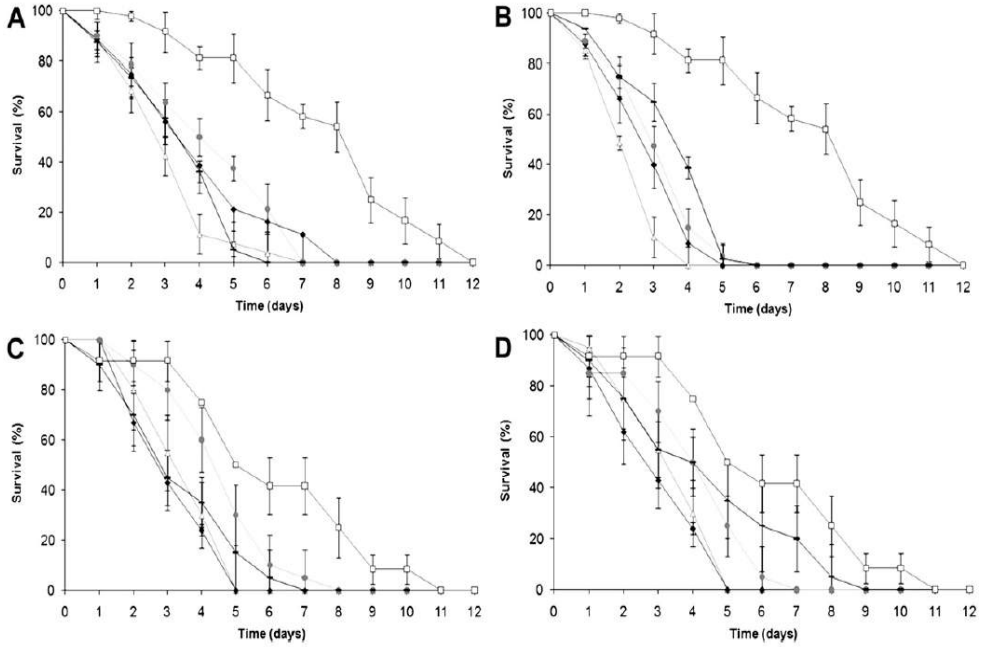

Fig. 2. Contact effect of OfiL (A and B) and E_{OfiL} (C and D) on *N. corniger* soldiers (A and C) and workers (B and D). Treatments used the protein concentrations 0.25 (-), 0.5 (●), 1.0 (◆), and 1.5 (Δ) mg ml^{-1}; 0.15 M NaCl was the negative control (□). Each dot represents the mean ± SD of five experiments.

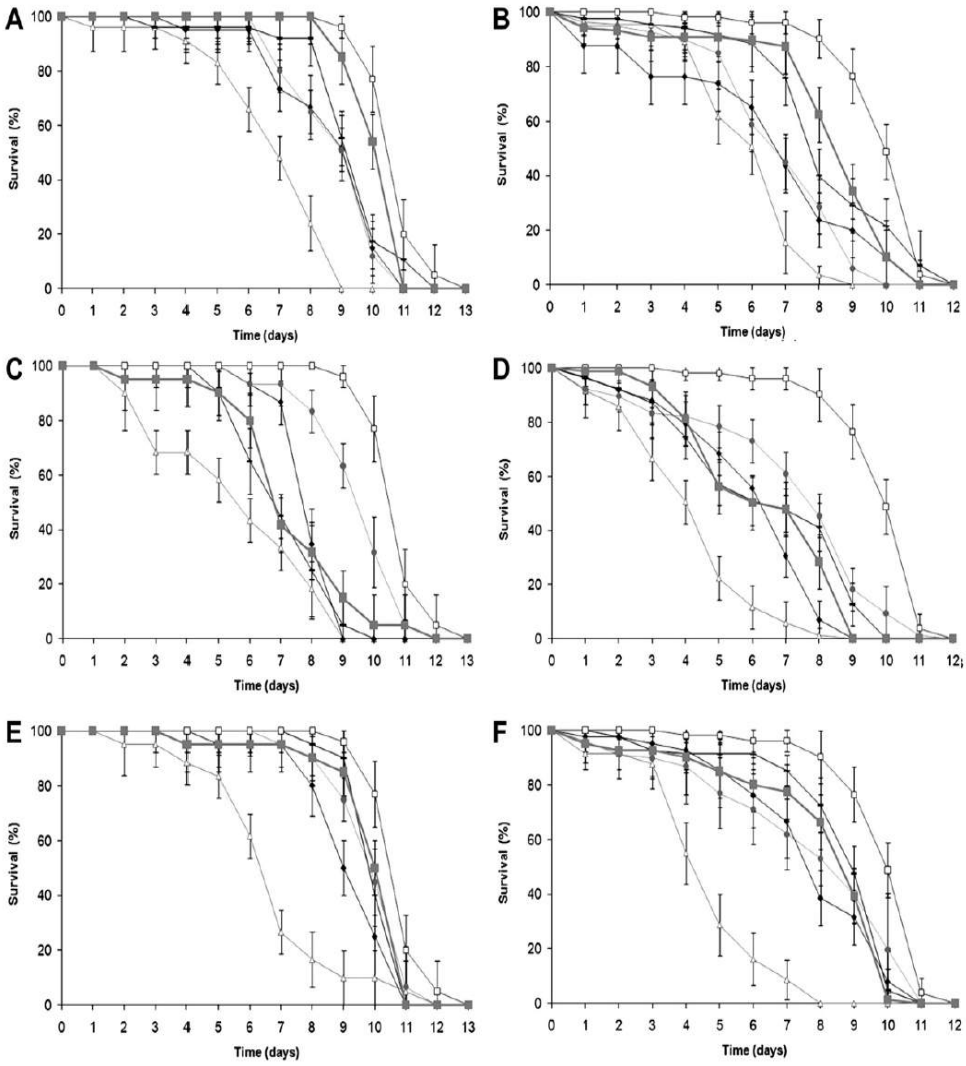

Fig. 3. Contact effect of E_{WSMoL} (A and B), F_{WSMoL} (C and D), and WSMoL (E and F) on *N. corniger* soldiers (A, C, and E) and workers (B, D, and F). Treatments used the protein concentrations 0.125 (■), 0.25 (-), 0.5 (●), 1.0 (◆), and 1.5 (Δ) mg ml^{-1}; 0.15 M NaCl was the negative control (□). Each dot represents the mean ± SD of five experiments.

Table 1
Properties of lectins from *Opuntia ficus indica* cladodes and *Moringa oleifera* seeds.

Property	OfiL	WSMoL[b]	cMoL[c]
Anionic nature	Yes	Yes	No[d]
Molecular weight of subunit (kDa)	8.4[a]	5[c]	26.5 and 14.9[d]
Chitin-binding lectin	Yes[a]	Yes[b,c]	Yes[e]
Inhibition of hemagglutinating activity			
Fructose	−[a]	+[b]	−[d]
Glucose	+[a]	+[b]	+[d]
Mannose	+[a]	+[b]	−[d]
N-Acetylglucosamine	−[a]	+[b,c]	−
Raffinose	−[a]	+[b]	+[d]
Fetuin	−[a]	+[b]	−

[a] According to Santana et al. (2009).
[b] According to Rolim et al. (2011).
[c] According to Coelho et al. (2009).
[d] According to Santos et al. (2009).
[e] According to Oliveira et al. (2011). Inhibited (+) and not inhibited (−).

termiticidal activity against *N. corniger*. The assays were performed using filter paper soaked in *O. ficus indica* and *M. oleifera* samples.

OfiL and WSMoL were isolated, and PAGE under native conditions revealed that they have an anionic nature, in contrast to cMoL, which is a cationic protein (Santos et al., 2009). Table 1 shows that OfiL, WSMoL, and cMoL have subunits with different molecular masses, and that they are able to interact with chitin — apart from inhibition of hemagglutinating activity by different carbohydrates.

The termiticidal assays revealed that OfiL had a deleterious effect against soldiers and workers, which was higher than that of E_{OfiL}, probably due to the lowest OfiL concentration in the extract. Similarly, the extract from *M. urundeuva* heartwood was not as effective against *N. corniger* as the preparation containing the isolated heartwood lectin (Sá et al., 2009).

The termiticidal activity of *O. ficus indica* preparations was higher than that of *M. oleifera* seed preparations (Table 2), and OfiL was more toxic against *N. corniger* workers than the lectins isolated from *M. urundeuva* bark, heartwood, and leaf, as well as from *C. verticillaris*, which showed LC_{50} levels of 0.974, 0.248, 0.374, and 0.196 mg ml^{-1}, respectively (Sá et al., 2008; Silva et al., 2009;

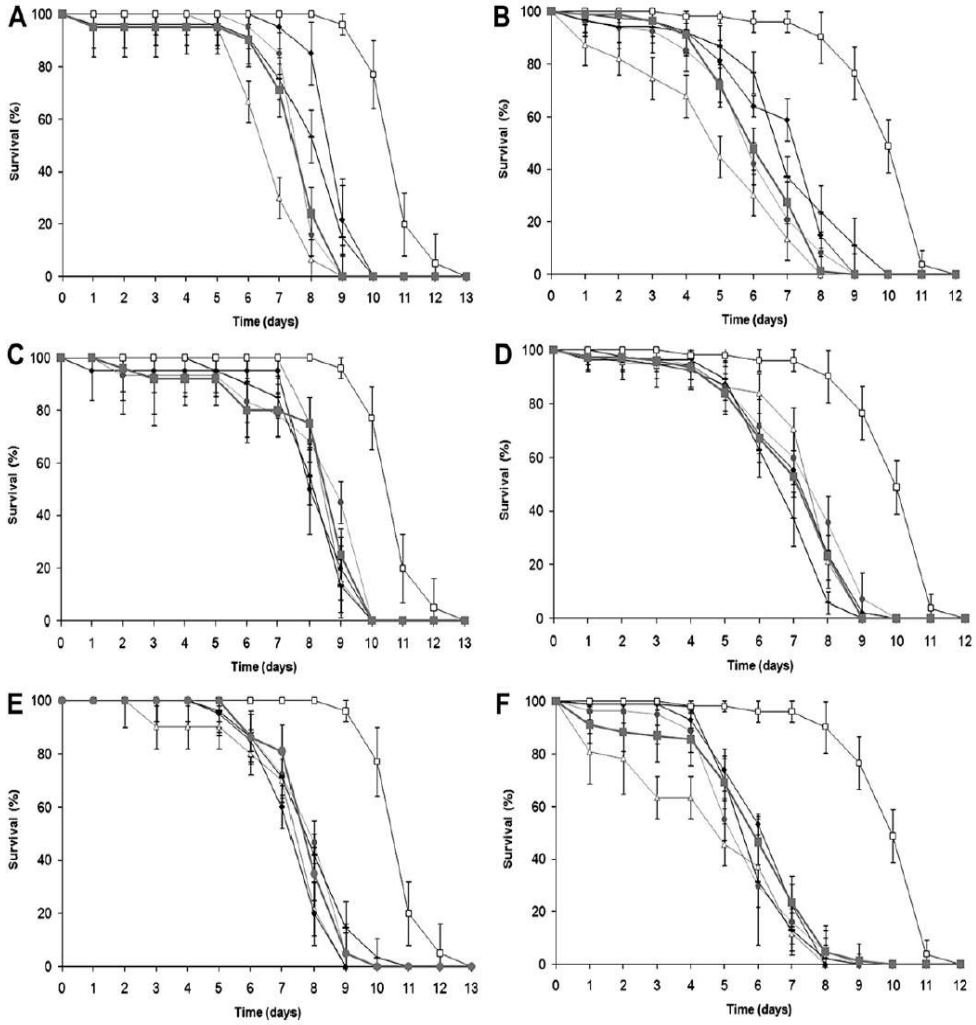

Fig. 4. Contact effect of E_{cMoL} (A and B), F_{cMoL} (C and D), and cMoL (E and F) on *N. corniger* soldiers (A, C, and E) and workers (B, D, and F). Treatments used the protein concentrations 0.125 (■), 0.25 (-), 0.5 (●), 1.0 (◆), and 1.5 (Δ) mg ml^{-1}; 0.15 M NaCl was the negative control (□). Each dot represents the mean ± SD of five experiments.

Table 2
Specific hemagglutinating and termiticidal activities of lectin preparations from *O. ficus indica* cladodes and *M. oleifera* seeds.

Lectin preparation	Specific hemagglutinating activity[a]	Termiticidal concentrations (mg ml^{-1})[b]	
		Soldier	Worker
O. ficus indica			
E_{OfiL}	3.0	ND	1.5
OfiL	40.0	1.5	0.5, 1.0, 1.5
M. oleifera			
E_{WSMoL}	0.73	1.5	1.0, 1.5
F_{WSMoL}	1.17	1.5	1.5
WSMoL	6826	1.5	1.5
E_{cMoL}	7.96	1.5	1.0, 1.5
F_{cMoL}	17.06	ND	ND
cMoL	200	ND	1.5

ND: not detected.
[a] Hemagglutinating assay using rabbit erythrocytes; specific hemagglutinating activity corresponds to the ratio between titer and protein concentration (mg ml^{-1}).
[b] Protein concentration that showed significant ($p < 0.05$) effect different to negative control.

Napoleão et al., 2011). The lectin isolated from *B. monandra* roots showed an LC_{50} of 0.09 mg ml^{-1} (Souza et al., 2011), a value lower than for OfiL. Nevertheless, it should be remembered that this LC_{50} was determined after 12 days, while the LC_{50} of OfiL was determined after 4 days through the course of the present experiment.

Table 2 shows that F_{WSMoL} and WSMoL preparations presented higher specific hemagglutinating activity than E_{WSMoL}, with identical effect on the survival of workers and soldiers; thus, WSMoL purification did not result in increased termiticidal effect. Preparations of WSMoL were not good termiticidal agents, since they showed toxicity against termites only when used at high concentrations, in comparison with the previously described termiticidal lectins. Also, the termiticidal activity of crude preparation (E_{WSMoL}) on workers was detected at the protein concentration of 1.0 mg ml^{-1}, while F_{WSMoL} and WSMoL induced worker mortality only at a concentration of 1.5 mg ml^{-1}. These results indicate that WSMoL is not the only termiticidal agent present in *M. oleifera* seeds.

Activity against workers was shown with E_{cMoL} and cMoL, while F_{cMoL} was inactive. This may be due to the presence of a compound able to inhibit the termiticidal activity of lectin in *M. oleifera* seeds.

Probably, this inhibitor is present at low concentration in E_{cMoL}, though it higher levels were observed in F_{cMoL}, being eliminated after chromatography on guar gel. Suzuki and Mori (1989) also reported the presence of a lectin inhibitor in *Pinctada fucata martensii* hemolymph that was detected during isolation of lectin.

The mechanisms of action of insecticidal lectins include binding to glycoconjugates present at epithelial cells along the digestive tract of insects, interaction with chitin components in the insect gut and *N*-acetylglucosamine residues from peritrophic matrix, and binding to the sugar moiety of glycosylated digestive enzymes or assimilatory proteins (Macedo et al., 2007; Fitches et al., 2008). Napoleão et al. (2011) reported that the mechanisms of termiticidal lectins from *M. urundeuva* involve a chitin-binding property, resistance to degradation by termite proteases, and antibacterial activity against symbiotic bacteria of *N. corniger* gut. OfiL may promote termite mortality due to its chitin-binding ability.

Repellent activity was evaluated by observing the building of tunnels and closing of galleries by *N. corniger*, since this insect closes tunnels and galleries in the presence of toxic substances, to avoid physical contact with them (Su et al., 1982; Sá et al., 2009). The preparations from *O. ficus indica* cladodes and *M. oleifera* seeds were not repellent against termites, similarly to the lectins from *M. urundeuva*, *C. verticillaris*, and *B. monandra* (Sá et al., 2008; Silva et al., 2009; Napoleão et al., 2011; Souza et al., 2011). Non-repellent termiticides are advantageous, since they do not cause termites to migrate elsewhere.

Worker termites are responsible for foraging, storage of food, and construction of nests. Although OfiL was not a good termiticidal agent against *N. corniger* soldiers, this lectin was highly toxic to workers, which may promote impairment of foraging dynamics, resulting in considerable disturbance in the organization, structure, and maintenance of termite colonies.

5. Conclusions

The chitin-binding lectins OfiL, WSMoL, and cMoL differ in the specificity of carbohydrate-binding site, charge nature, and molecular mass. These lectins showed different termiticidal activity and thus their chitin-binding ability is probably not the main reason for the toxicity of these lectins on *N. corniger*. The termiticidal assays revealed that OfiL, WSMoL, and cMoL showed different effects on castes of *N. corniger* and this fact indicates that the biological characteristics of soldiers and workers interfere in the sensitiveness to the toxic effect of lectins. The lectin from *O. ficus indica* cladodes possesses biotechnological potential for use in controlling the pest termite *N. corniger* since it was highly toxic for workers.

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