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Purificação, Imobilização e Avaliação de Propriedades Biológicas da Lectina da Entrecasca de *Crataeva tapia*

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“O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis.”

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Aos meus pais

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AH	Atividade hemaglutinante
AHE	Atividade hemaglutinante específica
AINES	Antiinflamatórios não esteroidais
AT	Antitrombina
CM	Carboximetil
CNBr	Brometo de cianogênio
Con A	Concanavalina A (lectina de *Canavalia ensiformis*)
Cra	Lectina de *Cratylia mollis*
CrataBL	Lectina da entrecasca de *Crataeva tapia*
CSF	Fator estimulante de colonização
DEAE	Dietilaminoetil
DL_{50}	Dose Letal (50%)
EDTA	Ácido etilenodiaminotetraacético
FV	Fator V da coagulação sanguínea
FVII	Fator VII da coagulação sanguínea
FVIII	Fator VIII da coagulação sanguínea
FIX	Fator IX da coagulação sanguínea
FX	Fator X da coagulação sanguínea
FXI	Fator XI da coagulação sanguínea
FXII	Fator XII da coagulação sanguínea
HMWK	Cininogênio de alto peso molecular
HPETE	5-Hidroxi-peroxi-6-8-11-14-eicosatetraenóico
INCA	Instituto nacional do câncer
ISI	Índice sensibilidade internacional
MTX	Metotrexato
PAGE	Eletroforese em gel de poliacrilamida
PEG 8000	Polietilenoglicol
PGE	Prostaglandina E
PGI	Prostaciclina
PL	Fosfolipídeos
PK	Pré-calicreína
PMNL	Leucócitos polimorfonucleares
PMSF	Fenilmetilsulfonilfluoreto
RIP-2	Proteína que inativa ribossomo tipo 2
RNI	Relação normatizada internacional
SBA	Aglutinina de soja
SDS-PAGE	Eletroforese em gel de poliacrilamida contendo dodecilsulfato de Sódio

SNC	Sistema nervoso central
SNP	Sistema nervoso periférico
TF	Fator tecidual
TFPI	Inibidor da via de fator tecidual
TNF	Fator de necrose tumoral
TP=PT	Tempo de protrombina
TTPA=APTT	Tempo de tromboplastina parcialmente ativada
VTE	Tromboembolismo venoso
WGA	Aglutinina de gérmen de trigo

RESUMO

Lectinas são proteínas ou glicoproteínas de origem não imune cuja ligação reversível e específica a carboidratos resulta em aglutinação celular. Estas proteínas, presentes em bactérias, invertebrados, vertebrados e plantas são detectadas por ensaio de hemaglutinação. *Crataeva tapia* L. pertence à família das Capparaceae. Uma lectina de entrecasca de *C. tapia*, CrataBL, foi purificada à homogeneidade através de fracionamento com sulfato de amônio (Fração 30-60%), seguida por cromatografia de troca iônica (CM-Celulose). CrataBL aglutinou eritrócitos de humanos, galinha e coelho (atividade hemaglutinante específica, AHE, 102) e principalmente inibida por glicoproteínas. CrataBL foi termoestável e tratamento com EDTA não afetou a atividade hemaglutinante (AH); atividade não foi alterada após adição de Ca^{2+}, Mn^{2+}, Mg^{2+} e após tratamento com as enzimas proteolíticas tripsina e quimiotripsina sua AH manteve-se estável. CrataBL migrou como uma única banda após eletroforese para proteínas nativas básicas e duas bandas polipeptídicas de massas moleculares 21.000 e 40.000 Da após SDS-PAGE com ou sem agente redutor; os polipeptídeos foram também detectados usando reagente para glicoproteína e sua porção carboidrato foi estimada em 12,8%. A natureza glicoprotéica de CrataBL foi também revelada por sua interação com lectina glicose/manose em gel de agarose. A massa molecular da lectina por cromatografia de filtração em gel foi de 40.000 Da. CrataBL imobilizada em Sepharose CL-4B adsorveu bioseletivamente caseína, fetuína, ovoalbumina e isolou glicoproteínas de plasma sanguíneo humano. O efeito da lectina na coagulação sanguínea foi avaliado através do tempo de tromboplastina parcial ativada (TTPA), tempo de protrombina (TP) e tempo de trombina (TT). CrataBL apresentou atividade anticoagulante somente na via intrínseca, onde o TTPA apresentou-se prolongado

(dose-dependente) a partir de uma concentração de 1,25 μg da lectina. CrataBL apresentou toxicidade para *Artemia salina* com LC_{50} de 71,7 μg/mL. A atividade antitumoral de CrataBL (20 mg/kg) foi avaliada utilizando Sarcoma 180 implantado em camundongos albinos Swiss (*Mus muscullus*) e foi detectada por redução do peso do tumor de 50,7%. A atividade antiinflamatória de CrataBL foi avaliada pelo modelo de edema de pata induzido por carragenina e a lectina apresentou efeito antiinflamatório significativo evidenciado pela redução (35,4%) do número de neutrófilos no exsudato inflamatório. CrataBL também apresentou atividade analgésica reduzindo em 34,8% o número de contorções induzidas por ácido acético.

Palavras-chave: purificação de lectinas, imobilização, coagulação sanguínea, atividade antitumoral, atividade antiinflmatória e analgésica.

ABSTRACT

Lectins are proteins or glycoproteins of no immune origin that interact specifically and reversibly with carbohydrates on diferent cellular surface to promote cell agglutination. This proteins, presents in bacterias, invertebrades, vertebrades and plants, are detected by hemagglutination activity. *Crataeva tapia* L. belong to the Capparaceae family. A lectin from *C. tapia* bark, CrataBL, was purified to homogeneity

by ammonium sulphate fractionation (30-60% Fraction) and ion exchange chromatography. CrataBL specific hemagglutinating activity (SHA, 102) was obtained with human, chicken and rabbit erythrocytes and was mainly inhibited by glycoproteins. CrataBL was thermostable and EDTA treatment did not affect HA; the activity did not changed after or Ca^{2+}, Mg^{2+} and Mn^{2+} addition or after proteolytic treatment with trypsin or chymotrypsin. CrataBL migrated as a single band after electrophoresis to native basic proteins and two polypeptide bands of molecular mass 21,000 Da and 40,000 Da after SDS-PAGE, with or without reducing agent; polypeptides stained using Schiff's reagent and its carbohydrate content was estimated to be 12.8%. Native CrataBL also interacted upon agarose gel with Cramoll 1,4, a glucose/mannose lectin, and showed molecular mass of 40,000 Da by gel filtration chromatography. CrataBL immobilized on Sepharose 4B bioselectively adsorbed casein, fetuin and ovalbumin and isolated human plasma glycoproteins. The effect of lectin on blood coagulation was performed through activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT). CrataBL showed anticoagulant activity just pathway intrinsic, where APTT was prolonged (dose-dependent) from 1.25 μg of lectin. The CrataBL toxicity on *Artemia salina* was detected and LC_{50} value of 71.7 μg/ml was

determined. Antitumoral activity of CrataBL (20 mg/kg) was evaluated using Sarcoma 180 bearing Swiss albino (*Mus muscullus*) mice and was detected inhibition by tumor growth (50.7%). The anti-inflammatory activity of CrataBL was evaluated by carrageenan-induced paw edema and the lectin showed significant anti-inflammatory effect by inhibiting (35.4%) migration of neutrophil in the inflammatory exsudate. CrataBL also shoewd antinociceptive activity inhibiting (34.8%) abdominal contractions induced by acetic acid.

Keywords: purification of lectins, immobilization, blood coagulation, antitumoral activity, anti-inflammatory and analgesic activity.

CAPÍTULO 1

1. Introdução

1.1. Lectinas

1.1.1. Generalidades

As proteínas estão entre as macromoléculas biológicas mais abundantes que ocorrem em todas as células sendo extremamente versáteis em suas funções. Elas participam da atividade celular, por exemplo, como enzimas, inibidores de enzimas, hormônios, proteínas de transporte, proteínas de reserva, proteínas contráteis e proteínas estruturais.

Dentre as proteínas está um grupo conhecido como lectinas que são amplamente distribuídas entre animais (Takahashi *et al.*, 2008), microorganismos (Leonidas *et al.*, 2007) e plantas (Sol *et al.*, 2007). Estas macromoléculas são conhecidas por sua capacidade de aglutinar células, especialmente eritrócitos de diferentes espécies animais (Paiva e Coelho, 1992; Correia e Coelho, 1995; Reynoso-Camacho *et al.*, 2003).

O termo lectina (originado do latim "*lectus*") foi introduzido por Boyd e Shapleigh no ano de 1954, em virtude da habilidade de se ligarem especificamente a carboidratos de uma forma não-covalente (Hong *et al.*, 2001). O termo aglutinina é usado como um sinônimo para lectina, porque se refere à habilidade de aglutinar eritrócitos ou outras células (Peumans e van Damme, 1995). Goldstein *et al*. (1980) definiram lectinas como proteínas ou glicoproteínas de origem não imunológica que se ligam a carboidratos através

de pelo menos dois sítios de ligação, aglutinam células vegetais e/ou animais e precipitam polissacarídeos, glicoproteínas e glicolipídeos. Avanços na análise estrutural e molecular das lectinas redefiniu o termo. De acordo com a nova definição, são consideradas lectinas as proteínas ou glicoproteínas que possuem pelo menos um sítio que se liga reversivelmente a mono ou oligossacarídeos específicos, sem apresentar função catalítica ou características imunológicas (Peumans e van Damme, 1995). Esta nova definição é mais abrangente, pois inclui uma série de proteínas com diferentes propriedades de aglutinação e/ou precipitação de glicoconjugados.

Baseada na estrutura global, lectinas de plantas são também classificadas em merolectinas, hololectinas, quimerolectinas e superlectinas (Peumans e van Damme, 1998). Merolectinas são pequenas e simples; devido à sua natureza monovalente são incapazes de precipitar glicoconjugados ou aglutinar células, sendo um exemplo deste grupo a proteína que se liga a quitina obtida do látex da seringueira (*Hevea brasiliensis*). Hololectinas contêm dois ou mais sítios de ligação para carboidratos, idênticos ou muito homólogos; devido à sua natureza di ou multivalente aglutinam células e/ou precipitam glicoconjugados. A maioria das lectinas 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. Exemplos de quimerolectinas são proteínas que inativam ribossomos tipo 2 (RIP-2). Superlectinas consistem de pelo menos dois sítios de ligação para carboidratos diferentes e podem ser consideradas como um grupo especial de hololectinas. Um exemplo de

superlectina é a lectina do bulbo de tulipa TxLCI, a qual possui dois sítios de ligação a carboidratos, sendo um específico para manose e outro para N-acetilgalactosamina.

Em lectinas, os sítios de ligação para carboidratos tendem a se localizar na superfície da molécula protéica e a seletividade da ligação é obtida através de ligações fracas, tais como: pontes de hidrogênio, interação de Van der Walls e interações hidrofóbicas entre o açúcar e a proteína (Cominetti *et al.*, 2002; Sharon e Lis, 2002).

A habilidade das lectinas em induzir o fenômeno de aglutinação celular (Figura 1) possibilita a detecção destas através de um ensaio de hemaglutinação. Nesse ensaio é feita uma diluição serial da lectina e em seguida, incubação com eritrócitos humanos ou de outras espécies animais. A atividade hemaglutinante é detectada pela formação de uma rede ou malha decorrente da interação entre a lectina e os carboidratos presentes na membrana dos eritrócitos (Naeem *et al.*, 2007). O ensaio possibilita, além da sua detecção, determinar a especificidade de uma lectina, para carboidratos e/ou eritrócitos.

A especificidade da lectina é definida em função do monossacarídeo que mais eficazmente inibe a atividade (Kennedy *et al.*, 1995). Lectinas são agrupadas de acordo com a ligação a carboidratos e os grupos de especificidade compreendem: fucose, manose, ácido siálico, N-acetilglicosamina, N-acetilgalactosamina-galactose e grupo glicanos complexos (Peumans e van Damme, 1998). A definição da especificidade da lectina pode ser feita por ensaios da inibição da atividade hemaglutinante (AH) com diferentes monossacarídeos, oligossacarídeos ou glicoproteínas ou por ensaios de precipitação de moléculas glicídicas (Sharon e Lis, 1990). Muitas lectinas possuem afinidade muito maior por oligossacarídeos do que por açúcares simples, devido ao fato de seus sítios de ligação para carboidratos serem mais complementares para essas moléculas. Lectinas

estruturalmente diferentes podem reconhecer os mesmos açúcares e muitas lectinas são específicas para glicanos que não são originados de plantas (Peumans e van Damme, 1998).

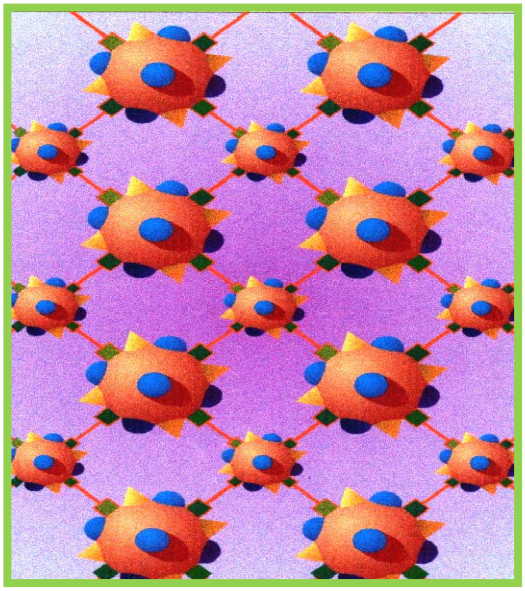

Figura 1. Representação esquemática de aglutinação por lectinas

■ = carboidrato específico; —⊏ = lectina

Fonte: Correia *et al.* (2008) *In Press*.

As plantas constituem ricas fontes de lectinas e sua distribuição ocorre principalmente nas raízes, folhas, flores, frutos, sementes, tubérculos, bulbos, rizomas e entrecascas. As lectinas são particularmente abundantes em sementes de leguminosas (Trindade *et al.*, 2006; Sol *et al.*, 2007), chegando a constituir até 10 % da proteína total (Sharon e Lis, 1990; Spilatro *et al.*, 1996). Porém tem crescido o interesse por lectinas presentes em tecidos como folhas (Coelho e Silva, 2000; Moriyama *et al*., 2003; Macedo *et al*., 2007), tubérculo (van Damme *et al*., 2004; Kaur *et al*.., 2006), entrecasca (Rojo *et al*., 2003; Ina *et al*., 2005; Nascimento *et al.*, 2008), raízes (Naeem *et al.*, 2001; Wang e Ng, 2006), rizomas (Tateno *et al*., 2003; Kaur *et al*., 2005), flores (Liu *et al*., 2002), cotilédones (Oliveira *et al*., 2002) e cerne (Sá *et al.*, 2008).

Várias evidências enfatizam uma forte semelhança entre as clássicas proteínas de reserva em sementes e suas homólogas funcionais em tecidos de entrecasca. Primeiro, as proteínas de reserva de entrecasca, definidas como proteínas presentes no tecido em grande quantidade durante o inverno e ausente durante o verão, estão sujeitas a rigoroso controle evolucionário. Segundo, as proteínas de reserva de entrecasca acumulam-se em células parenquimatosas depositadas em organelas similares aos corpos protéicos das sementes. Terceiro, a composição de aminoácidos destas proteínas se assemelha fortemente à das proteínas de reserva das sementes (van Damme *et al*., 1995).

Os papéis fisiológicos de lectinas de plantas não estão claramente definidos. Têm sido sugeridas diferentes funções, incluindo transporte de carboidratos, empacotamento e/ou mobilização de proteínas e carboidratos de reserva, alongamento da parede celular, interação entre plantas e microorganismos e defesa contra o ataque de fungos, vírus, pestes e insetos (Peuman e van Damme, 1995; Rudiger, 1998; Sá *et al.*, 2008).

As lectinas de entrecasca têm papel importante no metabolismo do nitrogênio de árvores da região temperada (van Damme *et al.*, 1995) e tem sido sugerida sua participação no mecanismo de defesa da planta (Rojo *et al.*, 1997). Outras funções propostas para estas proteínas incluem a participação no metabolismo dos carboidratos e estocagem de proteínas de reserva (van Damme *et al*., 2002; Nomura *et al.*, 2008).

1.1.2. Purificação e Caracterização

O crescente interesse na purificação e caracterização de lectinas deve-se à sua potencial utilização em diversas áreas da medicina clínica, bem como em pesquisa química e biológica (Matsui *et al.*., 2001; Banerjee *et al*., 2004; Vega e Pérez, 2006).

Lectinas de plantas têm sido isoladas e extensivamente investigadas em relação às suas propriedades química, físico-química, estrutural e biológica (Konozy *et al.*, 2003). Um grande número de lectinas tem sido clonado e homologias em suas seqüências de aminoácidos e similaridades em suas estruturas moleculares, estabelecidas (Goldstein, 2002). Lin *et al*. (2007) clonaram duas lectinas obtidas do veneno da cobra *Bungarus multicinctus* e identificaram similaridades em suas estruturas.

Métodos comuns utilizados na purificação de proteínas são aplicados para purificar as lectinas. Extratos podem ser feitos a partir de uma solução salina como no caso do isolamento da lectina das sementes de *Erythrina speciosa* (Konozy *et al*., 2003) ou usando tampões como na obtenção das lectinas de cotilédones de *Luetzelburgia auriculata* (Oliveira *et al*., 2002), dos tubérculos de *Helianthus tuberosus* L. (Suseelan *et al*., 2002), das sementes de *Cratylia floribunda* (Sol *et al.*, 2007) e de entrecasca de *Crataeva tapia* (Nascimento *et al*., 2008). Extratos apresentando atividade hemaglutinante (AH) podem ter suas lectinas purificadas parcialmente através de fracionamento salino com sulfato de amônio (Coelho e Silva, 2000; Sá *et al.*, 2008) ou pela utilização de polietilenoglicol (PEG 8000) que remove compostos polifenólicos, como os presentes na entrecasca de *Hevea brasiliensis* (Wititsuwannakul *et al*., 1998). Em alguns casos a precipitação salina da lectina com sulfato de amônio estabiliza a atividade hemaglutinante mesmo após longos períodos de armazenamento (Kennedy *et al*., 1995).

Após a extração e purificação parcial, diversos métodos cromatográficos são usados para purificação de lectinas à homogeneidade (Figura 2), tais como cromatografia de afinidade convencional (Sá *et al*., 2008) ou em membrana (Boi *et al.*, 2006; Sorci *et al.*, 2006), cromatografia de troca iônica (Yan *et al*.., 2005) e cromatografia de filtração em gel (Rojo *et al*., 2003).

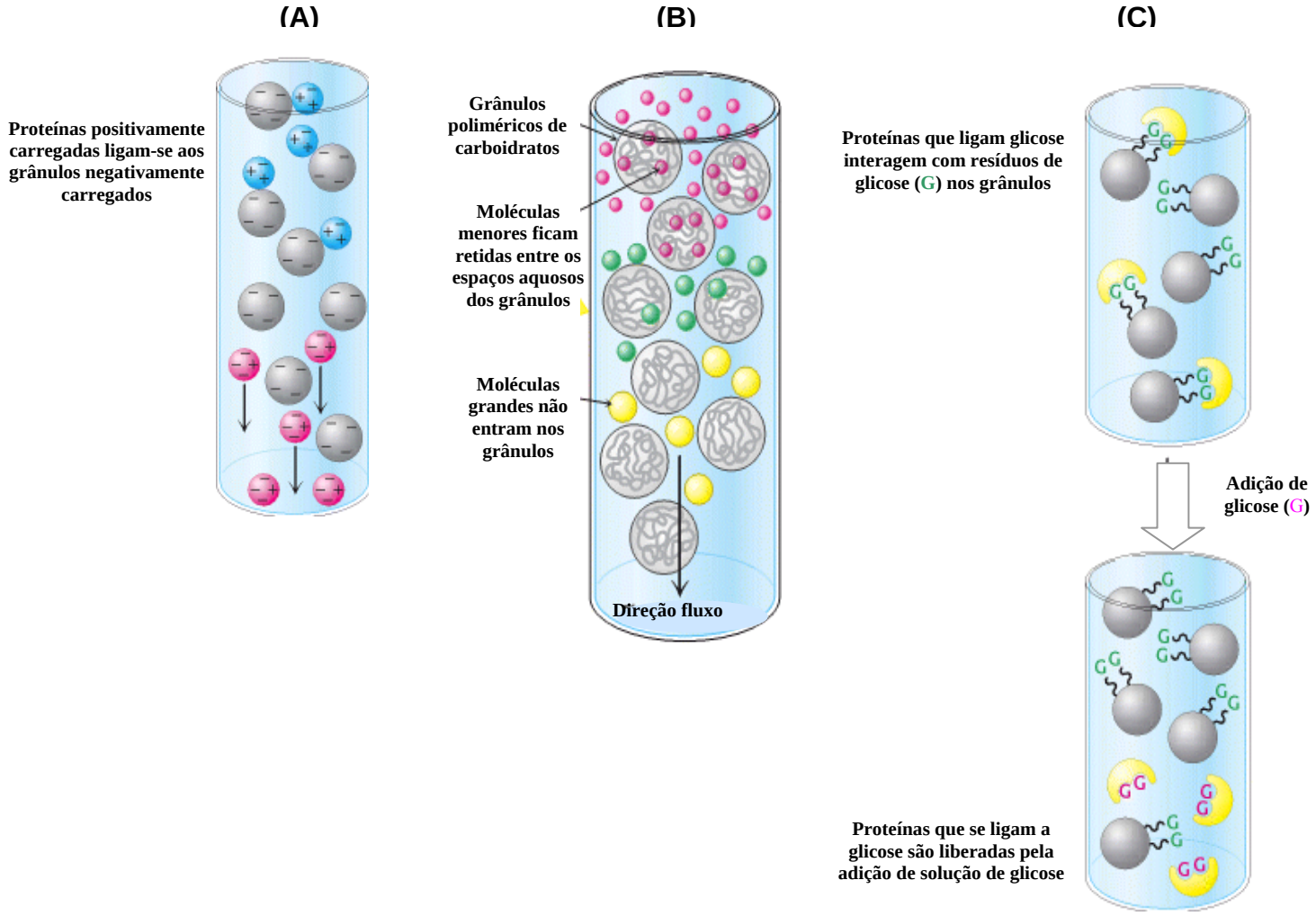

Figura 2. Métodos cromatográficos utilizados na purificação de proteínas: (A) Cromatografia de troca iônica, (B) Cromatografia de exclusão molecular e (C) Cromatografia de afinidade. Fonte: Stryer *et al*. (2004).

Na cromatografia de troca iônica (Figura 2A) a ligação da proteína ocorre com os grupos de carga de sinal contrário imobilizados na matriz. A coluna é lavada com solução tampão e as proteínas com nenhuma ou pouca interação com o trocador de íons são excluídas. As proteínas adsorvidas à matriz podem ser eluídas pelo aumento da força iônica ou alteração do valor de pH do meio (Datta *et al*., 2001). Uma das matrizes mais utilizadas

neste tipo de cromatografia é a celulose. Como exemplo tem a carboximetil (CM) celulose, um trocador catiônico carregado negativamente, e a dietilaminoetil (DEAE) celulose, um trocador aniônico (Li *et al*., 2008).

Na cromatografia de filtração em gel ou de exclusão molecular (Figura 2B), a separação ocorre de acordo com o tamanho da proteína. A coluna contém um polímero entrecruzado com os poros de tamanho selecionado. As proteínas maiores migram mais rapidamente que as menores; as proteínas grandes não penetram nos poros das esferas e, assim, tomam um caminho mais direto através da coluna. Este tipo de cromatografia é utilizado tanto para obter preparações protéicas homogêneas (Freire *et al*., 2002) como para definição da massa molecular da proteína (Kawagishi *et al*., 2001). Como exemplo de suporte utilizado na cromatografia de filtração em gel temos o Superdex 200 (Wong *et al.*, 2006; Takahashi *et al.,* 2008).

A cromatografia de afinidade (Figura 2C), técnica mais amplamente utilizada, tem como princípio de separação a habilidade das lectinas se ligarem especificamente a suportes polissacarídicos através seus sítios específicos. A proteína desejada é obtida com alto grau de pureza (Ye e Ng, 2002), alterando-se as condições de pH (Sá *et al*., 2008), força iônica (Freire *et al*., 2002) ou pela eluição com uma solução contendo um competidor (Oliveira *et al*., 2002). Exemplo de gel usado para cromatografia de afinidade é Sepharose 4B (Luo *et al.*, 2006).

Em geral, um protocolo de purificação de lectinas envolve um ou mais métodos cromatográficos. A lectina do cogumelo *Pleurotus citrinopileatus* foi obtida a partir de cromatografias de troca iônica, utilizando colunas de DEAE-Celulose, CM-Celulose e Q-Sepharose e cromatografia de gel filtração em coluna de Superdex 75 (Li *et al*., 2008).

Vale ressaltar que a purificação utilizando matrizes comerciais é onerosa, e, portanto a busca por matrizes alternativas com capacidade de ligar lectinas de diferentes especificidades e de baixo custo tem crescido. Um exemplo é o método desenvolvido para imobilizar proteínas da clara do ovo de galinha para obter uma matriz com ampla especificidade para diferentes lectinas (Zoccatelli *et al*., 2003). A partir de 12 ovos foram obtidas 35 a 40 g de resina seca que foi utilizada para a purificação de lectinas pertencentes a quatro classes de especificidade: hemaglutinina de *Phaseolus vulgaris* (sacarídeos complexos), aglutinina de *Lens culinaris* (manose e glicose), aglutinina de gérmen de trigo (ácido siálico, N-acetil-glicosamina e seus polímeros) e aglutinina de amendoim (galactose N-terminal). A matriz foi estável por pelo menos dois meses e eficiente para a purificação das lectinas e a pureza das preparações foi comparada com as lectinas disponíveis comercialmente.

Diversas propriedades físico-químicas são avaliadas para a caracterização de lectinas puras. A eletroforese em gel de poliacrilamida (PAGE) pode ser realizada usando um gel contendo dodecilsulfato de sódio (SDS-PAGE), que sob condições redutoras revela o grau de pureza, a composição e a massa molecular de subunidades (Reynoso-Camacho *et al*., 2003). A PAGE em condições nativas pode ser utilizada para caracterizar a proteína em relação à sua carga líquida. Proteínas ácidas, como a de *Erythrina speciosa* (Konozy et al., 2003), e proteínas básicas, como a de *Helianthu tuberosus* (Suseelan *et al*., 2002), foram reveladas. Os géis de poliacrilamida podem ser corados com azul de Coomassie ou negro de amido que detectam bandas polipeptídicas, ou com reagente de Sciff's específico para glicoproteínas (Konozy *et al*., 2003).

A avaliação da AH de lectinas em diferentes valores de pH, após serem submetidas a distintas temperatura ou ação de enzimas proteolíticas é importante para a determinação

da estabilidade destas moléculas quanto à sua propriedade de ligação a carboidratos e glicoconjugados. O pH tem efeito variado sobre as lectinas; em alguns casos não afeta a atividade (Wititsuwannakul *et al.,* 1998) e em outros a lectina perde sua atividade em determinada faixa de pH, como é o caso da lectina de *E. speciosa* (Konozy *et al.,* 2003). Quanto ao efeito da temperatura, algumas lectinas permanecem estáveis até 55-65° C e a partir de então, com a elevação da temperatura, a AH decai até ser abolida, como no caso das lectinas de *Luetzelburgia auriculata* e *E. speciosa* (Oliveira *et al*., 2002; Konozy *et al.,* 2003). Lectina ativa após aquecimento a 95° C também foi isolada (Suseelan *et al*., 2002). Após tratamento com enzimas proteolíticas, a AH da lectina pode ser alterada ou permanecer inalterada. A lectina de *H. brasiliensis* quando tratada com enzimas proteolíticas chegou ao mínimo de sua AH (Wititsuwannakul *et al.,* 1998).

Muitas lectinas contêm metais e, em alguns casos, existe evidência da necessidade de íons para sua atividade (Sharon e Lis, 1990). A lectina de *E. speciosa*, por exemplo, é uma metaloproteína que contém Ca^{2+} e Mn^{2+}, quando tratada com EDTA sua AH é totalmente abolida sendo a mesma restaurada após a adição de Ca^{2+} e Mn^{2+} (Konozy *et al.,* 2003). Por outro lado, a lectina de *H. tuberosus* não teve sua atividade abolida quando tratada com EDTA e não necessitou de íons metálicos tais como Ca^{2+}, Mn^{2+} e Mg^{2+} para a mesma (Suseelan *et al*., 2002).

Testes de inibição da AH, fazendo uso de monossacarídeos simples ou carboidratos complexos, também são freqüentes na caracterização de lectinas, desde que a especificidade é um critério para classificar lectinas de plantas em grupos de especificidade. Duas lectinas com diferentes propriedades de ligação a carboidratos foram isoladas da entrecasca de *Morus nigra* (Rougé *et al*., 2003); Morniga M e Morniga G foram específicas para manose e galactose, respectivamente. Lectinas de entrecasca também têm sido inibidas

com glicoproteínas, como é o caso da isolada de *H. brasiliensis* (Wititsuwannakul *et al*., 1998) que foi inibida com fetuína. A interação de lectinas com glicopeptídeos (Zeng *et al*., 2000) e glicoproteínas (Lima *et al*., 1997) tem sido também identificada através de ensaio de difusão dupla em gel de agarose.

As lectinas podem também ser caracterizadas pela determinação do ponto isoelétrico, eletroforese bidimensional, análise da composição de aminoácido, determinação da seqüência aminoacídica, estudos de fluorescência, dicroísmo circular e cristalização (Trindade *et al*., 2006; Naeem *et al*., 2007; Sol *et al*.,2007; Terada *et al.*, 2007; Parisi *et al.*, 2008).

1.1.3. Propriedades Biológicas

A ligação de lectinas às células pode resultar em uma variedade de propriedades biológicas. Devido a certas lectinas distinguirem entre células normais e malignas (Dhuna *et al.*, 2005; Kaur *et al.*, 2006), como também reconhecer diferentes tipos de grupos sanguíneos humanos (Sharon, 1993), estas proteínas têm sido extensivamente estudadas e usadas como ferramentas para estudos imunohistoquímicos e celulares (Vega e Pérez, 2006; Díaz *et al.*, 2008), fracionamento de células (Ohba *et al.*, 2002), como moléculas bioadesivas na entrega de drogas (Gabor *et al.*, 2001; Bies *et al.*, 2004) e no estudo de oligossacarídeos e glicoconjugados (Helmholz *et al*., 2003; Banerjee *et al*., 2004).

Várias lectinas são tóxicas para células de mamíferos *in vitro* e *in vivo*; inibem o crescimento quando incorporadas na dieta e são tóxicas quando injetadas em animais. Por outro lado, atividade citotóxica de lectinas (Reynoso-Camacho *et al.,* 2003) tem indicado seu uso como uma alternativa no tratamento do câncer. A lectina de *Musa basjoo* tem

propriedades de estimular macrófagos e inibir a proliferação de células leucêmicas (Wong e Ng, 2006). Lectina de *Pleurotus citrinopileatus* possui potente atividade antitumoral (Li *et al*., 2008).

Outras propriedades biológicas detectadas em lectinas isoladas foram: inseticida do cerne de *Myracrodruon urundeuva* (Sá *et al.*, 2008); atividade antiviral contra o vírus herpes tipo-1 e do vírus influenza das folhas de *Pandanus amarillifolius* (Ooi *et al.*, 2004); inibição da transcriptase reversa do HIV-1 (Li *et al*., 2008), atividade mitogênica (Wong *et al.*, 2006; Li *et al*., 2008) e atividade hipoglicemiante (Kavalali *et al*., 2003). Podemos encontrar, ainda, lectina com atividade aglutinante contra bactéria patogênica, como a encontrada em *Anguilla japonica* (Tasumia *et al*., 2004) e atividade antibacteriana no equinoderma *Holothuria scabra* (Oliveira *et al.*, 2008).

1.1.4. Imobilização para Suportes de Afinidades

A especificidade das lectinas com relação a diferentes carboidratos tem atraído o interesse na sua utilização em aplicações biotecnológicas (Monzo *et al.*, 2007; Optiz *et al.*, 2007). Técnica de separação por afinidade baseada nas interações moleculares bioespecíficas de lectinas são utilizadas para o isolamento de valiosas macromoléculas biológicas (Larsen *et al*., 2006; Monzo *et al.*, 2007). Além disso, a técnica pode ser empregada para analisar mudanças estruturais que ocorrem em oligossacarídeos presentes em glicoproteínas e glicolipídeos.

O desenvolvimento de um suporte de afinidade contendo lectina imobilizada é um pré-requisito crucial para o sucesso do isolamento do glicoconjugado (Rosenfeld *et al*., 2005; Larsen *et al*., 2006). A cromatografia de afinidade com lectinas imobilizadas facilita

o isolamento específico de compostos alvos de uma mistura complexa fazendo uso da habilidade das lectinas se ligarem especificamente a estruturas oligossacarídicas de glicoproteínas; a vantagem desta técnica não é apenas separar a proteína desejada, mas também preservar o padrão natural de glicosilação (Larsen *et al*., 2006; Optiz *et al*., 2007).

A eficiência de um adsorvente de afinidade é dependente da densidade do ligante, a qual é, por sua vez, uma função do método e condições escolhidas para o processo de ativação e imobilização. O adsorvente preparado é caracterizado de acordo com a sua especificidade, estabilidade e capacidade. Condições cromatográficas são definidas visando o isolamento do glicoconjugado entre elas tamanho da amostra, tamanho dos poros da matriz, velocidade do fluxo e comprimento da coluna (Rosenfeld *et al*., 2005; Anyulite *et al*., 2006).

As proteínas possuem um grande número de grupos reativos e podem ser imobilizadas sem a destruição de sua estrutura ou função. Dentre os suportes mais amplamente utilizados para imobilização de lectina está a agarose (Monzo *et al*., 2007), sendo os grupos amina das lectinas usados para sua imobilização. No método mais amplamente empregado (Figura 3), a imobilização de proteínas é feita por reação entre o grupo amina da lectina e o resíduo imidocarbonato da Sepharose ativada com brometo de cianogênio (CNBr).

A cromatografia de afinidade em colunas contendo lectinas imobilizadas tem sido amplamente utilizada no estudo de glicoproteínas humanas e de outras espécies animais. Células do vírus humano influenza A têm sido purificadas utilizando lectina de *Euonymus europaeus* como ligante de afinidade (Opitz *et al*., 2007). As glicoproteínas fetuína, de soro fetal bovino, e peroxidase, de rabanete, foram isoladas por cromatografia em colunas contendo WGA e Con A, respectivamente (Helmholz *et al*., 2003). A lectina do cogumelo

Marasmeus oreades imobilizada em Sepharose 4B ligou laminina de rato e tireoglobulina bovina, glicoproteínas que contêm o epítopo responsável por rejeição de transplantes em humanos (Loganathan *et al*., 2003). A lectina de soja imobilizada (SBA-Sepharose) foi capaz de isolar o polissacarídeo de *Streptococcus pneumoniae* (Franco-Fraguas *et al*., 2003). Cederfur *et al.* (2008) relataram o primeiro estudo sobre a ligação de glicoproteínas do soro humano a galectinas imobilizadas, utilizando cromatografia de afinidade.

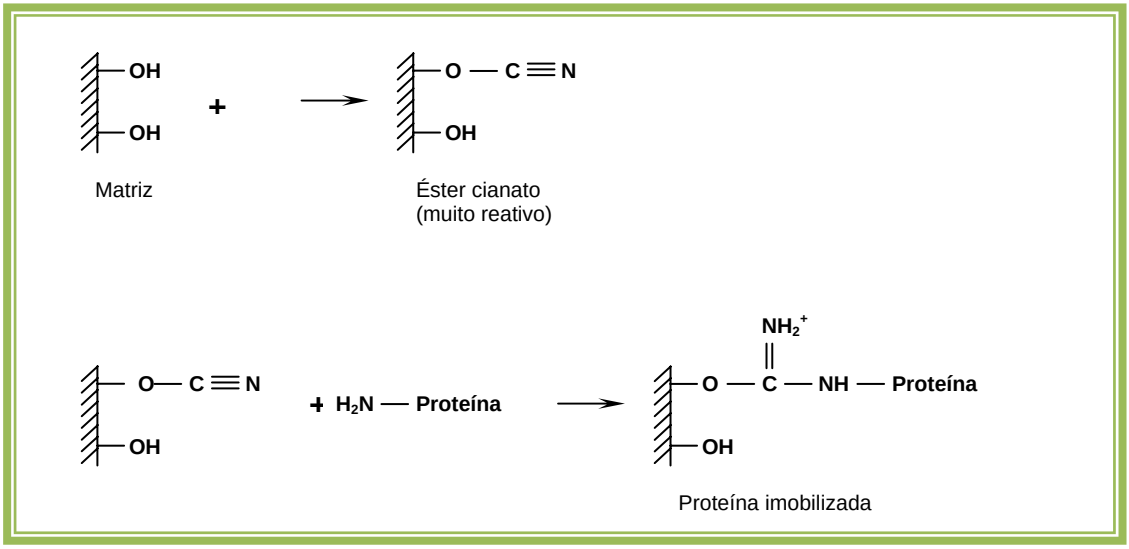

Figura 3. Imobilização de proteína em Sepharose-4B.
Fonte: Janson e Rydén (1989)

1.2. Bioensaios como Ferrramentas

Bioensaios podem ser utilizados para medir a atividade farmacológica de substâncias novas ou quimicamente indefinidas, medirem a concentração de substâncias conhecidas, investigar a função de mediadores endógenos, avaliar a eficiência clínica de

uma forma de tratamento ou ainda medir a toxicidade de uma substância (Silva *et al.*, 1999).

1.2.1. Coagulação Sanguínea

O sistema vascular exerce um papel fundamental no transporte de oxigênio e nutrientes para os tecidos, além de regular o extravasamento de fluidos, solutos, hormônios e macromoléculas. A manutenção da integridade do sistema vascular é absolutamente vital para o organismo humano. Quando a integridade da parede vascular é interrompida, ocorre a ativação do sistema hemostático. O mecanismo hemostático é um processo fisiológico e dinâmico que reconhece o dano vascular e recruta uma combinação apropriada de componentes celulares e proteínas plasmáticas solúveis, produzindo um "tampão" insolúvel, que cessa a perda sanguínea (Norris, 2003; van Doormaal *et al*., 2008; Aires, 2008). Este processo é regulado por diferentes mecanismos que envolvem diversas interações entre componentes endoteliais e subendoteliais, plaquetas e outras células sanguíneas periféricas e proteínas pró-coagulantes circulantes e inclui etapas como: vasoconstrição, adesão e agregação plaquetária, coagulação e reparação da parede vascular. Podemos dizer, ainda, que o processo hemostático possui dois componentes: hemostasia primária (formação do tampão ou trombo plaquetário) e hemostasia secundária (sistema de coagulação - formação de uma rede de fibrina) (Kolde, 2004).

A coagulação sanguínea envolve a ativação seqüencial de uma série de reações enzimáticas, na qual o produto de cada reação converte uma proteína plasmática inativa (zimogênio) em um produto ativo. Esta ativação ocorre através da hidrólise de uma ou duas ligações peptídicas culminando na geração de uma grande quantidade de trombina (Norris,

2003; Chatterjee *et al.*, 2006; Gopinath, 2007). Esta enzima cliva fibrinogênio em fibrina insolúvel e ativa as plaquetas através da ligação a receptores de trombina presentes na superfície destas células (Norris, 2003; Chatterjee *et al.*, 2006; Gopinath, 2007). A trombina também ativa os fatores da coagulação sanguínea V, VIII, XI e XIII amplificando, assim sua própria produção (Norris, 2003).

O mecanismo hemostático conduz a reações pró-coagulantes de extrema relevância envolvendo três complexos enzimáticos dependentes de vitamina K: o complexo protrombinase, o complexo tenase intrínseco e o complexo tenase extrínseco. Cada complexo é composto de serinoproteases e um cofator protéico não enzimático. Cerca de 30 proteínas interagem em todo esse processo (Gopinath, 2007).

De acordo com o sistema de nomenclatura internacional, as pró-enzimas e os co-fatores da coagulação são designados por algarismos romanos na ordem de sua descoberta e não corresponde à sua localização na sequência da cascata (Tabela 1). São gerados nas células do fígado com exceção do fator de von Willebrand que é produzido em diferentes órgãos, possivelmente em células endoteliais e megacariócitos (Mari *et al.*, 2008).

Tradicionalmente, dois caminhos principais têm sido descritos para a coagulação sanguínea: a via extrínseca ou de fator tecidual e a via intrínseca ou de contato (Norris, 2003). O modelo geralmente utilizado para descrever o mecanismo de coagulação é a cascata da coagulação (Figura 4).

Modelos de coagulação *in vitro* têm sido desenvolvidos na tentativa de definir o processo hemostático. Todos são baseados na reconstituição do sistema de coagulação, incluindo inibidores naturais como mecanismos de controle, e medidas de geração de trombina e/ou formação de fibrina. Estes experimentos *in vitro* essencialmente mostram que os fatores VIII e IX são dependentes do fator tecidual/fator VII e os caminhos

intrínsecos e extrínsecos não são independentes. Esta distinção, entretanto, permanece útil para "screening" clínico de sangramentos e coagulopatias (Gui *et al.*, 2007).

Na via extrínseca ocorre à participação de fatores tissulares, os quais são dependentes de co-fatores e proteínas encontradas no plasma. O fator tissular (glicoproteína de membrana com função de receptor celular) entra em contato com o sangue formando um complexo com o fator VII, ativando-o e em seguida o mesmo ativa o fator X, seguindo assim a via comum das vias intrínseca e extrínseca da coagulação (Norris, 2003; Mackman *et al.*, 2007).

Tabela 1. Características dos fatores da coagulação.

Fator	Sinônimo	Peso Molecular (Da)	Atividade Funcional	Meia-vida Biológica	Concentração Plasmática
Fator I	Fibrinogênio	340.000	-	90 h	300-400 mg/dL
Fator II	Protrombina	72.000	Serino protease	60 h	10-15 mg/dL
Fator V	Pró-acelerina	330.000	Cofator	12-36 h	0,5-1,0 mg/dL
Fator VII	Pró-convertina	48.000	Serino protease	4-6 h	0,1 mg/dL
Fator VIII:C	Fator Anti-hemofílico	70-240.000	Cofator	12 h	1-2 mg/dL
Fator IX	Fator Christmas	57.000	Serino protease	20 h	4 µg/mL
Fator X	Fator Stuart-Power	58.000	Serino protease	24 h	0,75 mg/dL
Fator XI	Precursor da tromboplastina palsmática	160.000	Serino protease	40 h	1,2 mg/dL
Fator XII	Fator de Hageman	80.000	Serino protease	48-52 h	0,4 mg/dL
Pré-calicreína	Fator Fletcher	80.000	Serino protease	48-52 h	0,29 mg/dL
Cininogênio de alto peso molecular	Fator Fitzgerald, Flaujaec ou Williams	120.000	Cofator	6,5 dias	0,7 mg/dL
Fator XIII	Fator estabilizador da fibrina (FSF)	320.000	Transglutaminase	3-5 dias	2,5 mg/dL
Proteína C	-	62.000	Serino protease	8-12 h	4-5 mg/dL
Proteína S	-	84.000	Cofator	-	25 mg/L

Fonte: Adaptada de Mari *et al.* (2008).

A via extrínseca e a via comum da cascata da coagulação são avaliadas pelo Tempo de Protrombina (TP), o qual reflete alterações do fibrinogênio em três dos fatores dependentes da vitamina K (fatores II, VII e X) e do fator V (Zago *et al.*, 2004). A Organização Mundial de Saúde (OMS) para minimizar a discrepância entre as diferenças de resultados de TP entre os vários laboratórios sugeriu que as tromboplastinas fossem padronizadas segundo uma preparação de referência internacional, criando o Índice de Sensibilidade Internacional (ISI). Os resultados do TP podem ser expressos como Relação Normatizada Internacional (RNI), que representa a razão entre o TP do paciente e o TP de referência em segundos. A variação normal do RNI encontra-se na faixa entre 0,9 e 1,2. Um prolongamento do RNI pode ser devido à deficiência dos fatores I, II, V, VII e X, como também deficiência de vitamina K. Além da terapêutica com a administração de anticoagulantes orais em altas doses (heparina), casos clínicos de doenças hepáticas, coagulação intravascular disseminada e várias transfusões sanguíneas podem acarretar o prolongamento do TP (Zago *et al.*, 2004).

Todos os fatores participantes da via intrínseca (fatores XII, XI, IX , VIII, pré-calicreína e cininogênio de alto peso molecular) estão presentes no sangue circulante, e a reação em cadeia é desencadeada pelo contato com plaquetas ativadas ou componentes do tecido subendotelial. Pela via intrínseca, o fator contato ou fator XII é adsorvido sobre uma superfície carregada negativamente, tal como o colágeno *in vivo* e o vidro ou partículas de caolin *in vitro*, enquanto que a adsorção do fator XI e da pré-calicreína se faz indiretamente através do cininogênio de alto peso molecular. A pré-calicreína interage com o fator XII provocando sua ativação. O fator XII ativado ativa o fator XI que, por sua vez, ativa os fatores da coagulação seguinte (Norris, 2003; Gailani e Renné, 2007). Todas as etapas subseqüentes da cascata requerem íons cálcio e são fosfolipídio-dependentes (Kolde, 2004).

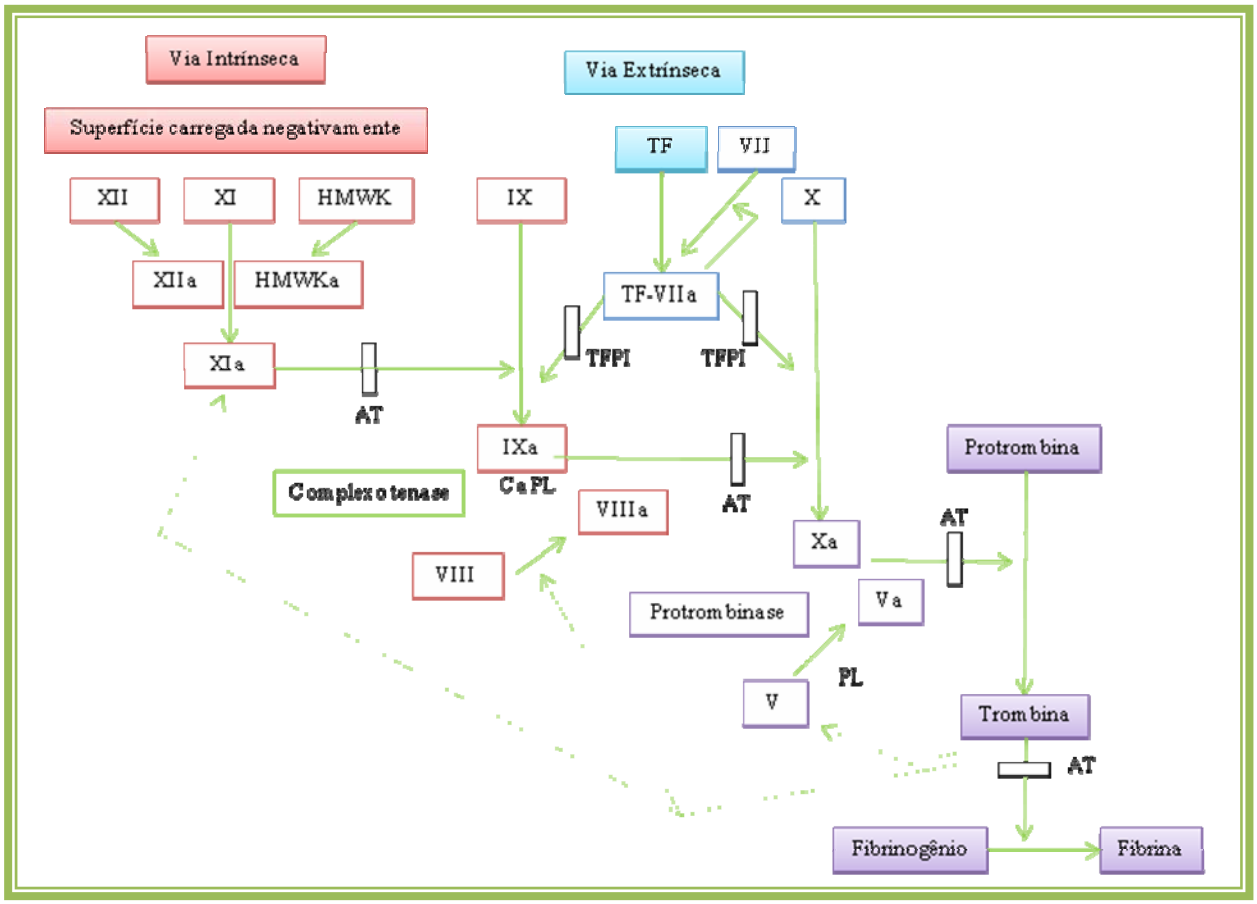

Figura 4. Cascata da Coagulação.
HMWK= cininogênio de alto peso molecular, TF = fator tecidual, TFPI = inibidor da via de fator tecidual, PL = fosfolipídios, Ca = cálcio, AT = antitrombina
Fonte: Adaptada de Norris *et al.*, 2003.

A via intrínseca da coagulação utiliza o Tempo de Tromboplastina Parcialmente Ativada (TTPA) para avaliar a detecção de anticorpos lúpicos e para monitorização laboratorial da heparina. Este teste também avalia a via comum (fatores X, V, II e I) da cascata da coagulação (Zago *et al.*, 2004). No TTPA utilizam-se substitutos de fosfolipídeos plaquetários como a cefalina ou a inositina que são tromboplastinas parciais, incapazes de ativar a via extrínseca. O tempo de tromboplastina parcial ativada pode estar prolongado em casos de deficiência dos fatores I, II, V, VIII, IX, X, XI, XII, pré-calicreína

e cininogênio de alto peso molecular ou na presença de inibidores destes fatores (Zago *et al.*, 2004). O TPPA pode ser prolongado pelas mesmas causas descritas para o prolongamento do TP. O registro do TTPA equivale ao tempo em que o plasma leva a coagular e o valor normal de referência está entre 26 e 33 segundos para formação do coágulo (Gilman *et al.*, 2007).

A fluidez do sangue é mantida pelo próprio fluxo sanguíneo, que reduz a concentração dos fatores pró-coagulantes, pela adsorção de fatores de coagulação e pela presença de múltiplos inibidores plasmáticos como, por exemplo, antitrombina, proteína C e proteína S, e o inibidor da via do fator tissular (Norris, 2003).

Fisiologicamente, o mecanismo hemostático requer plaquetas e fatores do plasma para sanar defeitos nas paredes dos vasos. Entretanto, excessiva atividade pró-coagulante pode resultar em trombose vascular (Renné *et al.*, 2006). Tromboembolismo venoso (VTE) é um importante problema clínico com incidência de 1-2 casos a cada 1000 indivíduos e acredita-se que o aumento nos níveis de fatores da coagulação esteja associado com o aumento do risco de VTE (Tripodi *et al.*, 2004). O tromboembolismo pode levar o indivíduo ao embolismo pulmonar ou ao infarto do miocárdio, doenças que são causas de morte e graves sequelas. O caminho intrínseco da coagulação é essencial para a estabilidade do trombo (Renné *et al.*, 2006). Os principais fatores de risco para o VTE são idade elevada, trombofilia, cirurgia, imobilização, cateter venoso, terapia de reposição hormonal, uso de contraceptivo oral e câncer (van Doormaal *et al.*, 2008). Pacientes com *diabetes mellitus* também têm um quadro de hipercoagulabilidade e, consequentemente, têm aumentado o risco de trombose e aterosclerose (Carr, 2001).

Anticoagulantes têm sido usados largamente em tratamentos sanguíneos durante diálises e cirurgia, bem como em estratégias para prevenção e tratamento de trombose

arterial e venosa (Tamada *et al.*, 2002). Estes anticoagualntes são capazes de inibir o sistema de coagulação mais seletivamente, inibindo específicos fatores de coagulação. A heparina, um anticoagulante comum e rotineiramente utilizado, promove formação de um complexo entre trombina e antitrombina para inibir a atividade enzimática da trombina. Todavia, a heparina tem potencialmente efeitos adversos tais como hemorragia e trombocitopenia (van Doormal *et al.*, 2008). Existe, então, uma grande pesquisa em busca por novos anticoagulantes, extraídos de variadas fontes. Peptídeos obtidos de extratos solúveis do equiuróide *Urechis unicinctus (*Jo *et al.*, 2008), polissacarídeos da alga verde *Monostroma latissimum* (Zhang *et al.*, 2008) e o látex de *Jatropha curcas* (Osoniyi e Onajobi, 2003) possuem atividade anticoagulante. Lectinas de veneno de cobra têm sido isoladas e utilizadas como convenientes ferramentas envolvidas nas reações da cascata de coagulação, já que o veneno pode alterar a hemostasia ativando ou inibindo fatores da coagulação (Lu *et al.*, 2005; Ogawa *et al.*, 2005). A lectina do veneno de *Agkistrodon acutus* exibe atividade anticoagulante (Li *et al.*, 2005) e esta mesma lectina teve suas subunidades clonadas e expressadas na levedura *Pichia pastoris* com o intuito de melhor estudar sua função anticoagulante (Hu *et al.*, 2005).

1.2.2. Avaliação de Toxicidade

A determinação da toxicidade de um composto geralmente utiliza animais invertebrados como modelo (Caldwell *et al.*, 2003). O bioensaio com *Artemia salina* e *Daphinia magna* provou ser um adequado modelo para "screening" rápido e barato da toxicidade dos metabólitos de fungos que são utilizados como agentes de biocontrole para pestes agrícolas, ervas daninhas e doenças (Favilla *et al.*, 2006).

Artemia salina L. (Artemiidae), uma larva de camarão, é um invertebrado componente da fauna dos ecossistemas aquáticos salinos e marinhos (Parra *et al.*, 2001). Possui de 8 a 10 mm de comprimento, pertence à classe *Brachiopoda*, subclasse *Sasostraca* e ordem *Anostraca* (Figura 5). Nada sempre de dorso, com o ventre para cima, para a luz ou claridade do ambiente em que se encontra (telotaxia ventral) e em direção à luz (orientação fotopositiva). Reproduz-se com bastante facilidade e rapidez e é muito utilizado na indústria da aqüicultura como alimento vivo para peixes, tendo seus ovos comercialmente disponíveis em lojas de animais (Barahona e Sánchez-Fortún, 2006). Seus ovos, quando secos, podem ser conservados durante 10 anos, estando sempre aptos a eclodirem, desde que sejam colocados em água (Barnes e Ruppert, 1996). *A. salina* tolera uma ampla faixa de salinidade (4 - 250 g/L), e, assim, têm sido largamente utilizadas para a avaliação da contaminação de águas salinas e/ou marinhas por agentes químicos (Barahona e Sánchez-Fortún, 2006).

A letalidade de organismos simples tem sido utilizada para monitoramento rápido e simples de resposta biológica, onde existe apenas um parâmetro envolvido: morte ou vida. O ensaio de letalidade permite a avaliação da toxicidade geral e, portanto, é considerado como ensaio preliminar no estudo de compostos com potencial atividade biológica (Cavalcante *et al.,* 2000; Taylor *et al.,* 2005).

Bioensaio com *A. salina* tem sido considerado ideal para avaliar o efeito de agentes sobre taxa de sobrevivência por ser um organismo simples que pode sentir o efeito de qualquer substância biologicamente ativa, requerer uma pequena quantidade de amostra (2 - 20 mg) e apresentar facilidade na obtenção de sua larva (Pimenta *et al.,* 2003). A avaliação é considerada uma das ferramentas mais úteis para a preliminar avaliação da toxicidade geral (McLaughlin *et al.*, 1991).

Figura 5. *Artemia salina.*
http://www.wfu.edu/biology/faculty/brownera/artemia.jpg
(endereço eletrônico acessado dia 21 de maio de 2008)

A dose letal (DL_{50}) é a quantidade de uma substância a qual causa a morte de 50% de um grupo de animais teste e a determinação da DL_{50} é uma indicação da intoxicação potencial (toxicidade aguda) de um composto (Graminha *et al.*, 2008). Meyer et al. (1982) classificaram extratos brutos e substâncias puras em tóxicas (valor $DL_{50} < 1000$ µg/mL) e não-tóxicas (valor $DL_{50} > 1000$ µg/mL).

Parra *et al.* (2001) testaram toxicologicamente extrato de 20 plantas usando métodos *in vivo* e *in vitro* e os resultados mostraram boa correlação, sugerindo que o bioensaio com o camarão marinho (nome normalmente utilizado) é um útil modelo alternativo. Extratos de folhas, cascas e ramos de *Gochnatia polymorpha* ssp *floccosa* foram avaliados quanto à sua letalidade empregando a *A. salina* e foi sugerido que a ausência de toxicidade nesses extratos é um indicador de que a planta pode ser bem tolerada frente ao sistema biológico (Stefanello *et al.*, 2006). A alta toxicidade revelada para o látex *in natura* e de frações derivadas dos mesmos extraídos da folha de *Euphorbia conspicua* indicou o seu potencial uso como fonte alternativa de moluscicida natural (Santos *et al.*, 2007). A toxicidade das tiossemicarbazonas e seus complexos de ferro também foi avaliado contra estes

microcrustáceos e como este bioensaio tem uma boa correlação com atividade citotóxica em tumores humanos sólidos, estes compostos apresentam potenciais aplicações biotecnológicas (Graminha *et al.*, 2008).

1.2.3. Atividade Antitumoral

Os primeiros relatos da ocorrência de câncer em seres humanos foram encontrados nos papiros do Egito antigo por volta de 1500 a.C., e são referentes à descrição de úlceras cutâneas resistentes a tratamentos (Cowldry, 1955).

O câncer é uma das doenças que mais causam temor na sociedade, por ter se tornado um estigma de mortalidade e dor. Na verdade, a palavra câncer de origem latina (*cancer*) significando "caranguejo" deve ter sido empregada em analogia ao modo de crescimento infiltrante, que pode ser comparado às pernas do crustáceo, que as introduz na areia ou lama para se fixar e dificultar sua remoção. Atualmente, a definição científica de câncer refere-se ao termo neoplasia, especificamente aos tumores malignos, como sendo uma doença caracterizada pelo crescimento descontrolado de células transformadas (Figura 6A). Existem quase 200 tipos de neoplasias que correspondem aos vários sistemas de células do corpo, os quais se diferenciam pela capacidade de invadir tecidos e órgãos, vizinhos ou distantes (Almeida *et al.*, 2005).

Há alguns anos, o câncer tem sido apontado como a maior causa de mortes e o número de indivíduos com esta doença continua se expandido (Dongre *et al.*, 2007). No Brasil, as estimativas para o ano de 2008, válidas também para o ano de 2009, apontam que ocorrerão 466.730 casos novos de câncer (INCA, 2007).

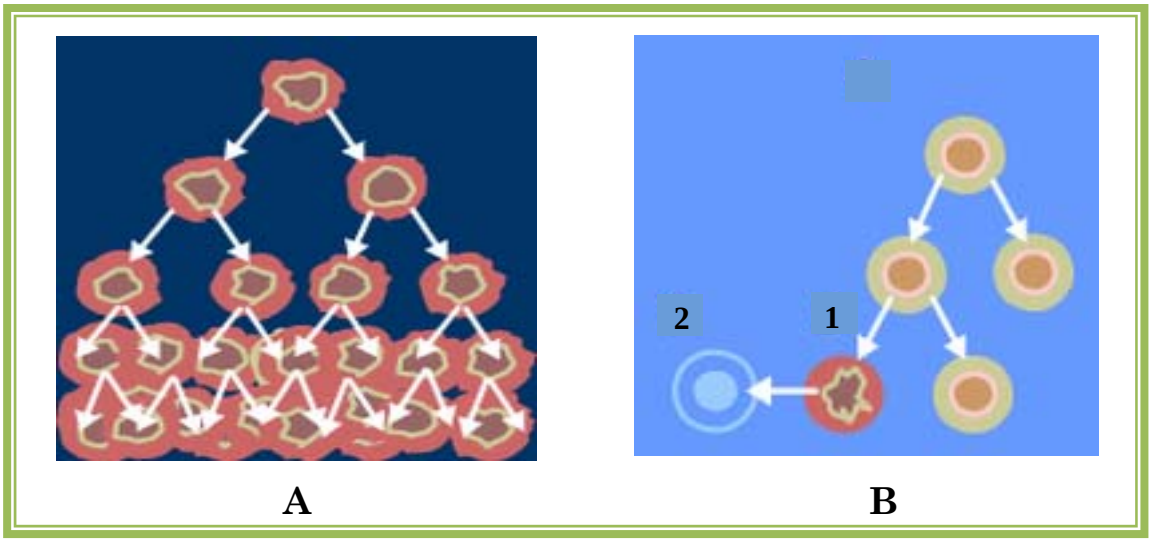

Figura 6. Células cancerosas: (A) Células transformadas multiplicando-se, (B) Células normais diferenciando-se em células cancerosas (1) e sofrendo morte celular (2).
Fonte: Adaptada de http://www.territorioscuola.com/wikipedia/pt.wikipedia
(endereço eletrônico acessado dia 17 de junho de 2008)

As causas de câncer são variadas, podendo ser encontradas no meio ambiente ou ser internas ao organismo, estando ambas inter-relacionadas (Almeida *et al.*, 2005). A nutrição é um aspecto importante podendo estar associado com o processo patológico do câncer (Ray, 2005). As causas internas são, na maioria das vezes, geneticamente pré-determinadas e estão ligadas à capacidade do organismo de se defender das agressões externas. Esses fatores causais podem interagir de várias formas, aumentando a probabilidade de transformações malignas nas células normais. Hormônios podem modular a expressão de fatores de crescimento, oncogenes, citocinas e outros componentes envolvidos na transdução de sinal, contribuindo assim para transformação celular e progressão do tumor (Li *et al*, 2008).

O número de células nos organismos multicelulares é regulado tanto pelo controle de taxa de divisão celular como também pelo controle de morte celular. Um dos objetivos

da terapia anticâncer é destruir as células neoplásicas (Almeida *et al.*, 2005). A morte celular (Figura 6B) é a perda irreversível da estrutura e funções vitais da célula que ocorre por dois processos morfologicamente distintos: necrose e apoptose (Parolin e Reason, 2001). Necrose se caracteriza tanto pela perda da integridade da membrana plasmática quanto pela alteração de sua permeabilidade. Neste processo de necrose ocorre a liberação dos constituintes intracelulares para o meio extracelular estimulando a resposta inflamatória e ampliando a lesão tecidual (Anazetti *et al*., 2004). Ao contrário, apoptose é um processo ativo que tem como principal característica a autodigestão controlada dos constituintes celulares, devido à ativação de proteases endógenas que comprometem a integridade do citoesqueleto. Os restos celulares serão fagocitados pelos macrófagos teciduais ou células adjacentes (Anazetti et al., 2004).

O câncer é classificado de acordo com o tipo de célula normal que o originou, e não de acordo com os tecidos para os quais se espalhou. Isso é o que pode se chamar de classificação primária e quase todos os tipos podem ser colocados em um dos seguintes grupos, onde o sufixo - *oma* significa literalmente tumor: carcinomas, sarcomas, linfomas, leucemia, mielomas, tumores das células germinativas, melanomas, gliomas e neuroblastomas (Almeida *et al.*, 2005).

O sarcoma refere-se a neoplasias do tecido mesenquimal, a palavra deriva do grego *sar* que significa carnoso, visto que possuem pouco estroma de tecido conjuntivo e, portanto, são carnosos (Contran *et al.*, 2000). O Sarcoma 180, a princípio conhecido como tumor de Crocker, é um tumor indiferenciado, descoberto em 1914 no Godcer Laboratory (Columbia University, NY). Foi encontrado inicialmente como uma massa sólida localizada na região axilar de um camundongo albino, sendo inicialmente classificado como carcinoma mamário, mas após vários transplantes subcutâneos, observou-se que suas

características morfológicas e seu comportamento eram peculiares de um tumor sarcomatoso, passando então a ser chamado Sarcoma 180 (Sugiura, 1994).

O Sarcoma 180 e outras células cancerosas, como o carcinoma de Erlich, têm sido muito utilizados em pesquisas para avaliação de potenciais atividades antitumorais de produtos naturais. As células sarcomatosas são de fácil obtenção e após sua inoculação no camundongo, desenvolve-se o tumor em 90% a 100% dos casos (Buchi, 2002; Sato *et al.*, 2005). As células sarcomatosas são mantidas pela inoculação intraperitoneal do líquido ascítico ($5x10^6$ células) em camundongos (Sato *et al*., 2005; Li et al., 2008).

De acordo com Sato *et al.* (2005), o comportamento de camundongos tratados com sarcoma 180 demonstrou uma exacerbação em granulócitos e supressão em linfócitos B. O fator estimulante de colonização (CSF) produzido por células tumorais em camundongos tratados com sarcoma 180 aumenta o número de células periféricas polimorfonucleares (PMN).

O campo da pesquisa terapêutica vem se desenvolvendo no sentido de testar novas formas de tratamento, bem como, novas substâncias potencialmente eficazes contra as neoplasias (Saad-Hossne *et al.*, 2004). Plantas usadas na popular e tradicional medicina têm sido consideradas como as principais fontes de descoberta e desenvolvimento de drogas antitumorais para o câncer (Abdullaev, 2001).

As plantas medicinais apresentam substâncias que podem desencadear reações adversas, seja de seus próprios componentes, ou até mesmo, resultado das preparações caseiras duvidosas e também os adulterantes contidos em fitoterápicos já comercializados (Turolla e Nascimento, 2006). No entanto, os efeitos adversos dos agentes fitoterápicos são menos freqüentes quando comparados com as drogas sintéticas (Calixto, 2000).

Vários produtos de plantas têm sido testados para atividade anticâncer e alguns deles como a vincristina (obtidos da *Catharanthus roseus*) e o taxol (retirado de *Taxus brevifolia*) já se encontram disponível comercialmente como droga de escolha (Gupta *et al*., 2004; Mongelli *et al.*, 2005).

Muitas lectinas têm sido alvos de pesquisa na bioterapia do câncer por seu efeito de estimular o sistema imune (Andrade *et al.*, 2004). O sistema imune tem a função de prevenção do desenvolvimento e controle do crescimento tumoral. Avanços no entendimento do mecanismo fundamental de regulação do sistema imune estão levando ao desenvolvimento de novas estratégias para promover uma resposta imune mais efetiva contra os tumores. Dentre as células envolvidas na resposta imune, os macrófagos têm a função de promover um mecanismo de defesa contra as células tumorais, agem como coordenadores, eles expõem os antígenos e secretam citocinas, tais como fator de necrose tumoral (TNF-α) e interleucinas (Sato *et al.*, 2005).

Lectinas têm recebido muita atenção por sua potencial atividade antitumoral em aplicações terapêuticas contra o câncer (Sharon e Lis, 2002). Lectina de *Cratyllia mollis* encapsuladas em lipossomas apresentaram atividade antitumoral quando testada em camundongos tratados com Sarcoma 180 (Andrade *et al.*, 2004). Efeito anti-câncer em diferentes linhagens celulares de câncer humano foi detectado na lectina de tubérculo de *Arisaema helleborifolium* (Kaur *et al.*, 2006). Con A, lectina de *Canavalia ensiformis,* e a lectina do cogumelo comestível *Pleurotus citrinopilestus* (Li *et al.*, 2008) apresentam atividades citotóxica ou inibitória para numerosas linhagens celulares de câncer (Chang *et al.*, 2007; Lei e Chang, 2007. Lectinas também têm sido relatadas induzir apoptose em células cancerosas como a lectina de *Sophora flavescens* (Liu *et al*., 2008).

1.2.4. Atividade Antiinflamatória

O termo inflamação deriva do latim *eflamare* que significa "atear fogo". Os egípcios há cerca de 3.500 anos a.C. a simbolizaram com um curioso símbolo hieroglífico em um papiro, representado por um braseiro. Os gregos, por volta de 2000 anos atrás, utilizaram o termo *phlogosis* enquanto os romanos usaram a palavra *inflammation,* todos com o mesmo propósito, descrever o fenômeno atualmente conhecido como inflamação (Almeida e Menezes, 2002).

Inflamação é uma resposta complexa do tecido conjuntivo vascularizado ao agente agressor, caracterizando-se pelo extravasamento de líquidos e de células do sangue para o interstício, levando a um acúmulo de fluidos e neutrófilos nos tecidos extravasculares. Esse processo é uma resposta fundamental de proteção cuja finalidade é neutralizar e/ou destruir o agente causador da lesão celular (microorganismo, toxina) e as conseqüências dessa lesão, como células e tecidos necrosados (Pereira *et al.*, 1998).

As causas para as reações inflamatórias são muito variadas, entretanto os mecanismos de surgimento deste processo são comuns. As causas podem ser classificadas em endógenas e exógenas. As endógenas seriam aquelas derivadas de degenerações ou necroses tissulares e as derivadas de alterações na resposta imunológica (por imunocomplexo ou autoimune). Enquanto, que as exógenas poderiam ser causadas por agentes físicos (calor e frio, eletricidade, radiações, sons e ultra-sons, magnetismo, gravidade, traumas mecânicos e atritos), agentes químicos (inorgânicos e orgânicos), ou até mesmo agentes biológico (infecciosos ou parasitários).

Na maioria dos casos, o processo inflamatório age como uma defesa orgânica a uma injúria, isolando e/ou destruindo o agente agressor com o intuito final de facilitar o

processo de reparação do tecido lesado seja por cicatrização ou por regeneração. Porém em algumas situações a inflamação pode causar sérios danos ao organismo, visto que uma resposta exacerbada pode resultar em um mecanismo de auto lesão (Lenz *et al.*, 2007). A resposta orgânica a uma injúria, interna ou externa, pode-se dar pela liberação, ativação ou produção de uma série de mediadores químicos ou farmacológicos da inflamação que resultam em várias alterações locais, tais como: dilatação de vasos da microcirculação, aumento do fluxo sanguíneo e da permeabilidade vascular, extravasamento de líquido plasmático, formação de edema, diapedese de células para o meio extravascular, fagocitose, aumento da viscosidade do sangue e diminuição do fluxo sanguíneo (Andrade *et al.*, 2007; Szanto e Rószer, 2007).

Mesmo a inflamação sendo local, todo o organismo é envolvido através do sistema nervoso e do sistema endócrino que agem na regulação do processo e na determinação do aparecimento de manifestações gerais, como a febre, leucocitose, taquicardia, fibrinólise e alterações bioquímicas do sangue (Potvin *et al.*, 2007).

A inflamação pode ser aguda ou crônica. Na resposta inflamatória aguda, as modificações do calibre e do fluxo, aumento da permeabilidade e migração dos leucócitos são mais intensos e ocorrem concomitantemente com o apareciemtno de dor, calor, rubor, edema e perda de função, considerados como os cinco sinais cardinais da inflamação (Michel *et al*., 2007; Tirosh *et al*., 2007).

A inflamação aguda é de curta duração, podendo ser de alguns minutos ou horas a um ou dois dias, dependendo do estímulo causal, e suas principais características são a exsudação de fluidos e proteínas do plasma e migração de leucócitos, predominantemente neutrófilos. Por ser praticamente uniforme, pressupõe-se a participação de substâncias comuns no processo que são os mediadores químicos da inflamação (Kluft e Maat, 2002).

Dentre os mediadores químicos, destacam-se: as aminas vasoativas, proteases plasmáticas e cininas plasmáticas. As aminas histamina e a serotonina estão relacionadas com a primeira fase (aumento da permeabilidade capilar) e são normalmente estocadas em grânulos citoplasmáticos de mastócitos, basófilos e plaquetas, podendo causar vasodilatação. As proteases, como as do sistema complemento, aumentam a permeabilidade vascular pela liberação da histamina de mastócitos e plaquetas representadas pelas anafilotoxinas C3a e C5a. As cininas plasmáticas, formadas pela ativação do fator XII da coagulação sanguínea, ou de Hageman, levam à formação da bradicinina, potente agente vasodilatador responsável pelo aumento da permeabilidade vascular na inflamação aguda, e conseqüente migração de células (polimorfonucleares) (Michel *et al.*, 2007; Tirosh *et al.*, 2007).

Os metabólitos do ácido araquidônico, pela via ciclooxigenase, também liberam mediadores químicos importantes na inflamação aguda, dentre eles destacam-se: a prostaciclina (PGI2), que promove vasodilatação; o tromboxano A2, que produz vasoconstrição e a prostaglandina E (PGE), que induz vasodilatação. Pela via lipoxigenase existem os endoperóxidos HPETE (5-Hidroxi Peroxi 6-8-11-14 Eicosa Tetra Enóico), que promovem vasoconstrição e aumento da permeabilidade vascular; os leucotrienos C4, D4, E4, responsáveis por vasoconstrição e aumento da permeabilidade vascular; e os leucotrienos B4, que produzem aumento da permeabilidade vascular (Groenewegwn *et al.*, 2007; Mantovani *et al.*, 2007).

A inflamação crônica inclui eventos proliferativos e alterações histológicas e é caracterizada por migração celular e mitose intensa, presença de fibroblastos, angioblastos e a necrose tecidual. Caracteriza-se por ser menos uniforme e de duração mais longa, que pode ser de semanas a meses e decorrente devido à persistência do estímulo causador da

inflamação aguda ou por exposição continuada a agentes tóxicos, interferência no processo normal de cicatrização e sob certas condições, como nas doenças auto-imunes (Kluft e Maat, 2002; Martindale *et al*., 2007).

Existe acentuado interesse quanto á descoberta de fármacos antiinflamatórios. Assim vários testes têm sido realizados, baseados no modelo de edema de pata de rato, causado por injeção de um agente inflamatório. Este agente pode ser a carragenina, dextrana, kaolin, dentre outros.

A carragenina é um polissacarídeo sulfatado extraído de uma alga marinha, *Chondrus chrispus* (Dayrens *et al.*, 1980). Desde que Winter *et al*. (1962) introduziu a carragenina como agente flogístico para o modelo de edema de pata do rato, este se tornou um dos testes mais utilizados por ser um método rápido, estável e servir de "screening" para descoberta de novas drogas antiinflamatórias (Mascolo *et al*., 1997; Henriques *et al.*, 1987).

O edema de pata induzido pela injeção subplantar de carragenina em ratos é bifásico (Xie et al., 2008). A primeira fase (0-2,5 horas) da inflamação é devida á liberação de histamina, serotonina e cininas na permeabilidade vascular. A segunda fase está correlacionada com a elevada produção de prostaglandinas, radicais livres derivados de oxigênio e produção de ciclooxigenase (Panthong *et al*., 2004).

A terapia de doenças inflamatórias está usualmente direcionada aos processos inflamatórios e desenvolvimento de novos antiinflamatórios não esteroidais (AINES) tem sido efetuado (Osadebe e Okoyé, 2003). Entretanto, estas drogas são conhecidas por provocar irritação gastrointestinal (Nguemfo *et al*., 2007).

Vários estudos estão sendo realizados na tentativa de descobrir novos medicamentos contra a inflamação, mais eficazes e com menos efeitos colaterais (Silva e Carvalho, 2004).

Desde quatro décadas atrás, tem ressurgido o interesse no estudo e uso de plantas medicinais. Este renovado interesse global tem levado à caracterização e identificação de novas moléculas e isolamento dos princípios ativos de compostos derivados de plantas de interesse terapêutico (Ojewole, 2007).

Vários compostos extraídos de entrecascas têm sido investigados e atividades antiinflamatórias identificadas, como no extrato da entrecasca de *Thespesia populnea* (Vasudevan *et al*., 2008), em extratos e frações da entrecasca de *Allanblackia monticola* (Nguemfo et al., 2007) e *Austroplenckia populnea* (Andrade *et al.*, 2007).

Lectinas também têm sido identificadas como potentes agentes antiinflamatórios. A lectina de semente de *Araucaria angustifolia* (Araucariaceae) interfere na liberação da cascata de mediadores inflamatórios (Santi-Gadelha *et al.*, 2006). Napimoga *et al*. (2007) relatam que a lectina de semente de *Lonchocarpus sericeus* atenuou a interação leucócito-endotélio e a transmigração de neutrófilos, além de inibir a produção de citocinas e quimocinas. Bitencourt *et al*. (2008) obtiveram resultados similares com a lectina da alga marinha vermelha *Hypnea cervicornis*.

1.2.5. Atividade Analgésica

A dor não é apenas uma modalidade sensorial, mas uma experiência. A Associação Internacional para o Estudo da Dor (IASP) conceitua dor como "uma experiência sensorial e emocional desagradável associada à lesão tissular ou potencial, ou descrita em termos desta lesão". Esta definição reconhece a interpelação entre os aspectos sensoriais fisiológicos, objetivos da dor e seus componentes subjetivos, emocionais e psicológicos (Teixeira, 2001).

A classificação do processo doloroso é diversificada e depende do critério adotado. A dor pode ser classificada em três tipos distintos: nociceptiva, neuropática e psicogênica (Serpell *et al.*, 2008). A dor nociceptiva é um mecanismo crucial de defesa que protege um organismo de um dano potencial ou eminente e é produzido, assim como suas conseqüências fisiológicas, pelo funcionamento normal tanto do Sistema Nervoso Central (SNC) quanto do Sistema Nervoso Periférico (SNP). A dor neuropática, em contraste, é reflexo do funcionamento patologicamente modificado desses dois sistemas. Na dor psicogênica é difícil determinar se a causa da dor é secundária ou atual, podendo está associada com ansiedade e depressão (Serpell *et al.*, 2008).

No início do século XX, Sherrington (1906) introduziu o termo "nocicepção", palavra derivada do latim *nocere* que significa "danificar" e "prejudicar". O sistema nociceptivo é um componente totalmente ajustável de controle da hemostasia. A nocicepção refere-se à percepção e à resposta do corpo à dor, ou seja, é atividade do sistema nervoso aferente, induzida por estímulos nocivos.

Os neurônios que respondem preferencialmente a estímulos nocivos são chamados de "nociceptores". Eles conduzem as informações nociceptivas ao sistema nervoso central através de fibras nervosas periféricas. Os sinais nocivos são gerados em fibras aferentes tipo Aδ (A-delta, pequenas e pouco mielinizadas, de condução rápida) e tipo C (pequenas e amielínicas, de condução mais lenta), que respondem a uma variedade de estímulos fisiológicos intensos tais como calor, frio, compressão e substâncias potencialmente nocivas (Brooks e Tracey, 2005). Praticamente todos os tecidos são inervados por fibras aferentes.

Normalmente, a estimulação nociceptiva periférica ativa os nociceptores e desencadeia reação de defesa com liberação de mediadores químicos (Teixeira, 2001). Dentre os diversos mediadores pode-se destacar: íons hidrogênio, norepinefrina, bradicinina, histamina, íons potássio, citocinas, serotonina, óxido nítrico e produtos do metabolismo do ácido araquidônico (Calixto *et al.*, 2001).

Um episódio doloroso também pode ser classificado de acordo com o critério temporal em agudo e persistente ou crônico. A dor aguda é caracterizada pela injúria substancial de um tecido com a subseqüente ativação dos nociceptores locais. É fundamental para a preservação da integridade do indivíduo porque é um sintoma que alerta para a ocorrência de lesões no organismo. A injúria altera a resposta característica dos nociceptores, suas conexões centrais e o sistema nervoso autônomo (Teixeira, 1999). A dor persistente ou crônica, característica de neuropatias, é geralmente iniciada a partir de uma injúria ou patologia e pode ser perpetuada por fatores alheios aos que disparam o processo doloroso. Esse tipo de dor tem a duração de meses e é uma importante causa de incapacidade, e acarreta alterações de comportamento psicomotor que são prejudiciais ao bem estar físico e mental (Teixeira, 1999).

Terapias analgésicas para a dor aguda e crônica estão normalmente relacionadas com três classes de fármacos: os antiinflamatórios não esteroidais (AINEs), os opióides e um grupo de fármacos conhecidos como coadjuvantes, tais como antidepresssivos, anticonvulsivos e analgésicos locais (Giublin, 2002; Sawynok, 2003).

Há diferentes testes para avaliar o sistema nociceptivo dos animais com variados tipos de estímulos como elétricos, térmicos ou químicos (Bars *et al.*, 2001).

No modelo do ácido acético, a administração intraperitoneal de agentes químicos irritantes provoca um comportamento estereotipado nos camundongos, que é caracterizado

por contrações abdominais, movimentos do corpo como torção da musculatura dorsoabdominal e redução na atividade motora e coordenação (Bars *et al.*, 2001).

Este modelo, apesar de ser relativamente simples e de pouca especificidade, é de fácil observação, apresenta boa sensibilidade a vários fármacos analgésicos e AINEs, bem como os fármacos semelhantes à morfina e outros analgésicos que atuam centralmente (Blane, 1967). Os resultados obtidos com várias classes de drogas analgésicas, neste modelo, mostram boa correlação com a ação analgésica encontrada em outros modelos pré-clínicos, bem como em estudos clínicos (Blane, 1967).

O comportamento de contorção em camundongo pela injeção de ácido acético, na nocicepção química, é utilizado para avaliar essencialmente atividade analgésica central e periférica. Ácido acético causa algesia pela liberação de substâncias endógenas e muitas outras que excitam terminações nervosas de dor (Khanna *et al*, 2003; Trongsakul *et al.*, 2003). A resposta nociceptiva induzida pelo ácido acético pode envolver a estimulação direta das fibras aferentes nociceptivas devido à redução do pH e a síntese de mediadores inflamatórios (França *et al*., 2001). Ribeiro *et al.* (2000) mostraram que a nocicepção induzida pelo ácido acético depende da liberação de citocinas, que são proteínas reguladoras produzidas principalmente por células imuncompetentes como macrófagos e basófilos.

Agentes analgésicos convencionais representam um importante papel na terapia moderna da dor, porém apresentam uma série de efeitos adversos (Otero, 2004). Desta forma, é crescente a procura por novos e melhores agentes analgésicos que possuam ação seletiva e, conseqüentemente, uma menor quantidade de efeitos colaterais.

Os metabólitos secundários derivados de plantas têm contribuído em muito para o nosso conhecimento sobre importantes mecanismos relacionados com o processo de

transmissão e o tratamento da dor, como a caracterização dos tipos de receptores e dos ligantes endógenos envolvidos no processo nociceptivo (Calixto *et al.*, 2000).

Muitas plantas de uso corrente na medicina popular têm apresentado propriedades analgésicas experimentalmente comprovadas, como o extrato das folhas de *Aquilaria sinensis* (Lour.) Gilg. (Zhou *et al.*, 2008), extratos das sementes de *Peganum harmala* L. (Farouk *et al.*, 2008), o extrato etanólico das folhas de *Taxus wallichiana* Zucc. (Nissar *et al.*, 2008) e o extrato aquoso de raízes de *Glycine tomentella* (Lu *et al.*, 2007).

Lectinas também têm sido amplamente investigadas quanto à sua atividade antinociceptiva. A lectina isolada da alga marinha *Amansia multifida* foi investigada através do modelo do ácido acético e demonstrou ser um potente composto antinociceptivo; quando pré-tratada com carboidrato específico D-manose seu efeito foi quase totalmente abolido, indicando que o sítio de ligação a carboidrato está envolvido de alguma forma na ação antinociceptiva da lectina (Neves *et al.*, 2007). A lectina da alga marinha *Hypnea cervicornis*, no teste do modelo do ácido acético, reduziu as contorções dos camundongos sugerindo que seu efeito antinociceptivo pode está relacionado à inibição da liberação de mediadores na resposta ao ácido acético (Bitencourt *et al.*, 2008).

1.3. *Crataeva tapia L.*

Crataeva tapia L. é uma planta da família Capparaceae, conhecida vulgarmente como trapiá (Figura 7). É encontrada de Pernambuco até São Paulo e Minas Gerais (Zona da Mata), na mata pluvial Atlântica e no Pantanal Matogrossense (Lorenzi, 1998). Em Pernambuco foi encontrada no litoral, na zona da mata e em vegetação de caatinga (Tabarelli *et al.*, 2002).

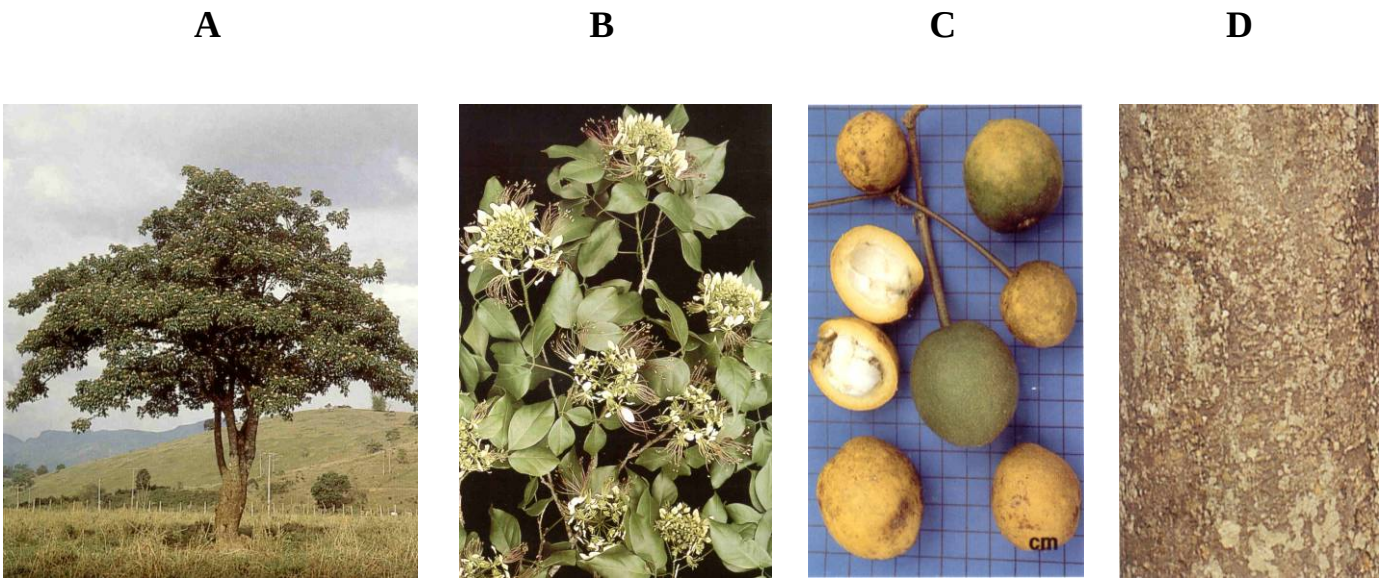

Figura 7. Visão geral de *Crataeva tapia*: árvore (A), folhas e flores (B), frutos (C) e entrecasca (D). Fonte: Lorenzi, 1998.

É uma planta de 5-12 m de altura, dotada de copa arredondada e densa. Tronco geralmente tortuoso e mais ou menos cilíndrico, com casca rugosa, de 20-40 cm de diâmetro. Folhas compostas trifolioladas, folíolos membranáceos, glabros em ambas as faces. Flores apícolas e frutos com polpa carnosa, contendo muitas sementes. Sua madeira é empregada em alguns locais para obras internas em construção civil, para forros, caixotaria e confecção de canoas. Os frutos são comestíveis, ingeridos apenas como refresco e bebida vinosa, além de serem muito apreciados por aves, peixes, macacos e outros animais silvestres, sendo inclusive usado como isca para o peixe "Pacu" no pantanal mato-grossense. A árvore possui ainda, atributos ornamentais que a recomendam para arborização paisagística (Lorenzi, 1998).

Esta planta é também muito utilizada na medicina caseira, que a empregam em emplastros nos panarícios e unheiros. As cascas são usadas como tônico, estomáquico,

antidisentérico e febrífugo. Os frutos, no combate às infecções do trato respiratório. A entrecasca do caule vem sendo amplamente utilizada pela população para diminuir o nível de glicose no sangue, ou seja, tem sido utilizada como hipoglicemiante.

1.4. Objetivos

1.4.1. Geral

Purificar, imobilizar e avaliar propriedades biológicas da lectina de entrecasca de *Crataeva tapia L.*.

1.4.2. Específicos

- Isolar a lectina de entrecasca de *C. tapia*;
- Caracterizar parcialmente a lectina;
- Imobilizar a lectina de *C. tapia* em Sepharose CL-4B e avaliar a eficiência da matriz CrataBL-Sepharose na ligação de glicoproteínas comerciais;
- Purificar glicoproteínas do plasma sanguíneo utilizando cromatografia em CrataBl-Sepharose;
- Determinar o efeito da lectina sobre a coagulação sanguínea;
- Examinar a toxicidade da lectina em bioensaio com *A. salina*;
- Avaliar a atividade antitumoral da lectina usando Sarcoma 180;
- Investigar a atividade antiinflamatória da lectina através do modelo de peritonite induzido por carragenina;
- Analisar a atividade analgésica da lectina utilizando o modelo do ácido acético.

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CAPÍTULO 2

Purification in milligram quantities of *Crataeva tapia* bark lectin and its use as a biospecific adsorbent for glycoprotein isolation

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Purification in milligram quantities of *Crataeva tapia* bark lectin and its use as a biospecific adsorbent for glycoprotein isolation

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Abstract

A new thermostable lectin has been purified from *Crataeva tapia* bark by ion exchange chromatography on CM-Cellulose yielding 10 ± 0.8 mg (90.9%) of pure lectin (CrataBL) per 11 mg of active 30-60% ammonium sulphate fraction. CrataBL, a basic glycoprotein showed hemagglutinating activity (AH) with different erythrocytes, was mainly inhibited by glycoproteins and not stimulated by divalent ions. Proteolytic treatment of CrataBL did not abolish AH. The native molecular mass of CrataBL determined by gel filtration on a Sephacryl S-300 column was 40 kDa and its carbohydrate content was estimated to be 12.8%. Native CrataBL also interacted with a glucose/mannose lectin from *Cratylia mollis* seeds upon agarose gel. Pure CrataBL was immobilized on Cyanogen bromide-activated Sepharose 4B; this affinity matrix (1 ml) bound pure glycoprotein (casein, fetuin and ovalbumin) and it was able to isolate glycoproteins from human plasma.

Keywords: Bark lectin; *Crataeva tapia*; lectin immobilization; glycoprotein isolation.

1. Introduction

Lectins are proteins that interact specifically and reversibly with carbohydrates on different cellular surfaces to promote erythrocyte agglutination (Correia et al., 2008). Plant lectins comprise a structurally versatile group of proteins that are composed of multiple subunits and are thermostable at high temperatures (Sá et al., 2008). Legume seeds have been the main source of plant lectins, but its presence has also been detected in other tissues such as tuber (Suseelan et al., 2002) and bark (Ina et al., 2005). From the later tissue lectins were isolated from species belonging to different families including Moraceae, genus *Morus* (Rougé et al., 2003), Leguminosae, genus *Maackia* (van Damme et al., 1997a), *Cladrastis* (van Damme et al., 1995a), *Robinia* (van Damme et al., 1995b), *Sophora* (van Damme et al., 1997b) and *Laburnum* (Lutsik and Antoniuk, 1982); Euphorbiaceae, genus *Hevea* (Wititsuwannakul et al., 1998) and Caprifoliaceae, genus *Sambucus* (Nsimba-Lubaki et al., 1986).

Bark lectins, in general, were not human blood group specific, were inhibited by glycoproteins, had multiple subunits and were thermostable, remaining active at high temperatures (Broekaert et al., 1984; van Damme et al., 1995a,b; van Damme et al., 1997a,b; Wititsuwannakul et al., 1998). Evaluation of bark lectin biological properties revealed the ineffective action of *Sambucus nigra* lectin upon tobacco-mosaic virus translation (Broekaert et al., 1984) and mitogenic activity of *M. amurensis* lectin (van Damme et al., 1997a).

Lectins play an important role in affinity separations as bioaffinity ligand among its versatile applications. Biologically active glycoproteins have been applied for laboratory study or diagnosis of human diseases (Marcipar et al., 2003). The interest in lectin affinity

chromatography is due to the fact that it can be carried out under very mild conditions with preservation of biological activities from target molecules (Wen and Niemeyer, 2007). The method has been extensively employed for the isolation, fractionation and structure characterization of glycoproteins and other biologically important glycoconjugates (Mechref et al., 2008; Wen and Niemeyer, 2007; Aniulyte et al., 2006; Ina et al., 2005; Fraguas et al., 2004; Paiva et al. 2003).

In this paper we describe the purification in milligram quantities and partial characterization of *C. tapia* bark lectin named CrataBL. The lectin was also immobilized on Sepharose 4B and the affinity matrix CrataBL-Sepharose 4B was analyzed for binding of casein, fetuin, ovalbumin and glycoproteins from human plasma. This seems to be the first report of a bark lectin purification from the Capparaceae family.

2. Experimental

2.1. Chemicals

CM-Cellulose, fetuin and ovalbumin were obtained from Sigma (USA). CNBr activated-Sepharose 4B was obtained from Pharmacia (Sweden). Casein was obtained from Inlab (Brazil). Glutaraldehyde, Schiff´s reagent (analytical grade) were obtained from Merck (Germany). All other chemicals were of analytical grade.

2.2. Preparation of extract

Dried bark from *C. tapia* was collected in the city of Olinda (state of Pernambuco, Northeast of Brazil). The bark was powdered and an extract was obtained by agitation of a

10 % (w/v) mixture in 150 mM NaCl, overnight at 4 ºC. This was followed by filtration through a gauze and centrifugation at 4,000 g for 15 min. The supernatant was termed crude extract (CE).

2.3. Lectin purification

CE was fractionated with ammonium sulphate according to Green and Hughes (1955). The obtained 30-60% fraction (F30-60%) was dialyzed against distilled water (2 h) followed with 10 mM citrate-phosphate buffer at pH 5.5 (2 h). F30-60% sample (11 mg of protein) was applied to a CM-Cellulose column (5.2 x 1.6 cm) equilibrated (flow rate of 20 ml/h) with 10 mM citrate-phosphate buffer, pH 5.5. The column was washed with the equilibrium solution until the absorbance (measured at 280 nm) was less than 0.05. Afterwards, the lectin was eluted from CM-Cellulose column with 500 mM NaCl and termed CrataBL.

2.4. Hemagglutinating activity and effect of ions, temperature and inhibitors on activity

Hemagglutinating activity (HA) was determined in microtitre plates (Paiva et al., 1992) using human A, B, O type and rabbit erythrocyte suspensions (2.5% v/v) treated with glutaraldehyde (Bing et al., 1967). The HA titer was defined as the lowest lectin concentration able to produce visible hemagglutination. Specific HA (SHA) was defined as the ratio between the titer and the protein concentration (mg/ml).

To evaluate the effect of different divalent ion concentrations on HA CrataBL was dialyzed against 5 mM EDTA for 16 h at 4 ºC followed by 150 mM NaCl for 6 h at 4 ºC to eliminate EDTA. Aliquots (50 μl) of dialyzed CrataBL were serially diluted with 150 mM

NaCl containing 5 mM Ca^{2+}, Mg^{2+} or Mn^{2+} before erythrocyte addition. To evaluate the effect of temperature CrataBL was heated at different temperatures (30, 40, 50, 60 and 100 ºC) for 30 min before the HA assay. For to determine the effect of inhibitors on HA the lectin was incubated (45 min) with a number of monosaccharides (100 mM) and glycoproteins (500 μg) before erythrocyte suspension addition.

2.5. Effect of Proteolytics Enzymes on CrataBL HA

The assay was made according to Rios et al. (1996). CrataBL (12.5 μg) was incubated (37°C, 3h) with bovine trypsin (5.0 μg) or chymotrypsin (5.0 μg) in 0.1 M Tris-HCl, pH 8.2 (50 μl) and the enzyme reaction was stopped by addition (13 μl) of phenylmethylsulponylfluoride (PMSF) in 0.1 M Tris-HCl, pH 8.2 buffer at 3:1 proportion (v/v). The effect of enzymes on CrataBL was evaluated by measurement of HA.

2.6. Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed on 10% (w/v) gel according to the method described by Laemmli (1970). Polypeptide bands of CrataBL (50 μg of protein) and molecular mass standards (bovine serum albumin, 66,000 Da; ovalbumin, 45,000 Da, glyceraldehyde-3-phosphate dehydrogenase, 36,000 Da; carbonic anhydrase, 29,000 Da; trypsinogen, 24,000 Da; trypsin inhibitor, 20,000 Da and α-lactalbumin, 14,200 Da from Sigma (USA) were stained with Coomassie Brilliant Blue. Glycoprotein staining (Pharmacia, 1980) was performed with Schiff´s reagent. PAGE for native basic [7.5% (w/v) gel] and acidic [12% (w/v) gel] proteins were carried out according to the protocols of Reisfeld et al. (1962) and Davis (1964), respectively.

2.7. Carbohydrate content

The amount of neutral sugars in the purified lectin (12.5 μg) was estimated by the phenol-sulfuric acid method (Dubois *et al*., 1951) using mannose as standard.

2.8. Gel filtration chromatography

CrataBL was chromatographed by gel filtration on a Hiprep 16/60 Sephacryl S-300 column (16 mm x 60 cm)/Äkta FPLC system (Amersham Pharmacia Biotech, Sweden) pre-equilibrated at 24 ºC with 500 mM NaCl. Samples (2.0 ml containing 4 mg of protein) were injected and eluted (3.0 ml fraction) with NaCl at a flow rate of 0.5 ml/min. Bovine serum albumin (66,000 Da), fetuin (64,000 Da), ovalbumin (45,000 Da) and the trypsin inhibitor type III-O chicken (28,000 Da) standards were similarly chromatographed.

2.9. Gel diffusion

A gel diffusion assay was performed according to Ashford et al. (1982). The central well was charged with glucose/mannose-specific isolectins (15 μg) from *Cratylia mollis*, Cramoll 1,4 (Correia and Coelho, 1995). The sample wells were filled with 100 μg of CE, F30-60%, or 26 μg of CrataBL. The samples were allowed to diffuse in agarose gel [1% (w/v) in 150 mM NaCl] in a humid chamber at 4 ºC for 24 h after this the gel was exhaustively washed and stained with Coomassie Brilliant Blue [0.4% (w/v) in 25% (v/v) ethanol and 8% (v/v) acetic acid] for 15 min.

2.10. CrataBL immobilization on Sepharose 4B

CNBr-activated Sepharose 4B was used for CrataBL immobilization according to Paiva et al. (2003). CNBr-activated Sepharose 4B (3 g) was washed with 500 mM NaCl at pH 2.5, followed by 100 mM $NaHCO_3$/500 mM NaCl, pH 8.2. The incubation (24 h at 4 ºC) was performed with CrataBL (10 mg of protein). After filtration and washing with $NaHCO_3$ solution the CrataBL-Sepharose 4B matrix was washed with 100 mM $NaHCO_3$/500 mM NaCl at pH 8.2 followed by 100 mM sodium acetate/500 mM NaCl at pH 4.0 and distilled water. CrataBL retention was determined by calculation of the difference between the concentration (mg) of loaded CrataBL and the amount of protein found before filtration and washing.

2.11. Evaluation of glycoprotein binding on CrataBL-Sepharose 4B

Casein, fetuin and ovalbumin glycoproteins (1 mg) were chromatographed on CrataBL-Sepharose 4B column (2.3 x 1.2 cm) equilibrated with 10 mM citrate-phosphate buffer at pH 5.5 (flow rate of 10 ml/h). The lectin affinity support was then washed with the same buffer until the absorbance (measured at 280 nm) was less than 0.05. The bound proteins were then eluted with buffer containing 1000 mM NaCl. The procedure was performed three times for each glycoprotein.

Human plasma (0.5 ml) in 10 mM citrate-phosphate buffer (0.5 ml) was recycled ten times at 10 ml/h on a CrataBL-Sepharose 4B column (1 ml; 2.3 x 1.2 cm). The lectin affinity support was then washed with the same buffer until the absorbance (measured at 280 nm) was less than 0.05. The bound proteins were then eluted with buffer containing 50 mM, 100 mM or 1000 mM NaCl and finally with 1000 mM acetic acid. The procedure was performed 15 times.

2.12. Protein assays

The concentration of protein was determined using serum albumin (0 to 500 μg/ml) as the standard according to the method of Lowry et al. (1951). The relative protein concentration was also estimated by absorbance at 280 nm.

2.13. Statistical analysis

The computer package GraphPad Prism, version 4.02 was used for statistical analysis. Data were expressed as a mean ± standard deviation.

3. Results and discussion

Bark is rich in phenolic compounds which can promote erythrocyte dispersion and thus interfere with hemagglutination assays (Wititsuwannakul et al., 1998). The inhibition of extract HA by ovalbumin glycoprotein indicates that a lectin was present in used sample.

The CE was found to contain a total HA of 40,960 and the 72% of this activity was detected in F30-60%. Chromatography of F30-60% (11 mg of protein with 1,024 of HA) on CM-Cellulose produced a single 500 mM NaCl active peak (Figure 1) containing 10 mg of purified CrataBL and corresponding to HA yield of 100%; the lectin SHA had increased 1.2 fold in relation to CE (Table 1).

The HA of CrataBL did not discriminate between human and rabbit erythrocytes. The lectin was found to be thermostable within a temperature range of 30 – 60 °C (SHA of 51) and inactivated at 100 ºC. CrataBL HA was not affected after dialysis against EDTA

and ions addition. This may be because EDTA treatment did not completely eliminate Ca^{2+}, Mg^{2+} or Mn^{2+} binding to the lectin as was observed by Oliveira et al. (2002).

CrataBL was partially inhibited by monosaccharides (N-acetylglucosamine, glucose, mannose > fructose and galactose) and glycoproteins (casein > ovalbumin), but fetuin completely abolished HA. Bark lectin inhibition by monosaccharides, glycoproteins or both, has been previously reported (Kim et al., 2004). In the case of *Morus nigra* bark lectin it has been speculated that its inhibition by glycoproteins could be due to the existence of an extended carbohydrate-binding site on this lectin (Rougé et al., 2003).

Proteolytic treatment of CrataBL with either trypsin or chymotrypsin did not eliminate HA. Some lectins from different sources were resistant to proteolytic enzymes, as the lectin from *Artocarpus integrifolia* seeds (Rios et al., 1996) and the ACL-I, a lectin from the marine sponge Axinella corrugate (Dresch et al., 2008). However, the bark *H. brasiliensis* lectin when treated with trypsin, protease K and pronases have its HA destroyed (Wititsuwannakul *et al.*, 1998). In general, native proteins showed low digestibility related to their highly compacted three dimensional structures, stabilized by numerous hydrophobic interactions and hydrogen bridges (Ramos et al., 1999).

Native PAGE and ion-exchange chromatography have been used to detect isolectins and determine their size (Guzmán-Partida et al., 2004). PAGE for native basic CrataBL revealed a single protein band (Figure 2A). None band was detected by PAGE for native acidic proteins. The electrophoretic pattern and the detection of only one HA peak after separation on a CM-Cellulose column indicated that CrataBL is a single protein. This is unusual because isolectins are common in bark (van Damme et al., 1997). SDS-PAGE revealed two polypeptide bands of molecular mass 21,000 Da and 40,000 Da (Figure 2B).

The same pattern was obtained with or without β-mercaptoethanol reducing agent. Gel filtration revealed one protein peak of 40,000 Da indicating that native CrataBL could be an oligomeric lectin, as other bark lectins (Kim et al., 2004).

Glycoprotein staining on SDS-PAGE revealed that the 21,000 Da and 40,000 Da polypeptide bands were glycosylated. Carbohydrate analysis by the phenol-sulphuric acid method showed that CrataBL is a glycoprotein with 12.8% carbohydrate content, similar to *Robinia pseudoacacia* bark lectin which is also a glycoprotein (Ina *et al.*, 2005). Interaction of CrataBL with glucose/mannose isolectins, Cramoll 1,4, indicated that the carbohydrate moiety of CrataBL contains these sugar residues (Figure 3); Cramoll 1,4 is not a glycoprotein (Correia and Coelho, 1995) and can be used to investigate the presence of glucose, mannose or its derivate residues in glycoconjugates. Binding of *Erythrina speciosa* seed lectin to glucose/mannose lectin from *Canavalia ensiformis* has yielded similar results (Konozy et al., 2003). Indeed, mannose is a common constituent of lectin glycan residues (Nsimba-Lubaki et al., 1986).

Casein, fetuin and ovalbumin glycoproteins could be recognized and bound (0.54 mg) by CrataBL-Sepharose 4B (Figure 4). Immobilized CrataBL was used for at least 9 cycles of adsorption, washing, elution and regeneration, with the same yield.

CrataBL-Sepharose 4B was efficient for separation of glycoproteins from human plasma. The elution with subsequent increase of the ionic strength showed different proteic patterns by SDS-PAGE (Figure 5). After the regeneration, the adsorbent can be used again with the same performance. The adsorbent is applicable for several cycles without significant loss of its activity. Cederfur et al. (2008) reported the first survey of galectins binding glycoproteins of human serum using lectin affinity column.

4. Conclusion

This cheap protocol of ionic exchange chromatography with CM-Cellulose matrix provided milligram amounts of purified lectin from *Crataeva tapia* bark. The thermal stability and proteolytic resistance of the lectin, and its glycoprotein binding capacity on CrataBL-Sepharose 4B suggest potential biotechnological applications such as a use as a cell surface marker in histochemical studies or as an affinity matrix to evaluate structural characteristics of the glycan moiety in glycoconjugates. The isolation of CrataBL stimulates the evaluation of its biological properties.

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Figure captations

Figure 1. CrataBL isolation by ion exchange chromatography.

A sample of F30-60 % (11 mg in 10 mM citrate-phosphate buffer, at pH 5.5) was applied to the column equilibrated with the same buffer. Arrows indicate the addition of eluents. Fractions of 2.0 ml were collected. Absorbance at 280 nm (●), HA (○). Each bar represents the mean ± S.D. of four experiments.

Figure 2. Electrophoretic patterns of CrataBL.

CrataBL (1) and cytochrome c (2) were loaded onto a 7.5 % gel for native basic proteins [Reisfeld et al. 1962] and the gel stained with Amido Black (A). CrataBL (1) was loaded onto a 10 % gel [Laemmli, 1970] and stained with Coomassie Brilliant Blue (B).

Figure 3. Diffusion in agarose gel.

CE, F 30-60% and CrataBL were allowed to diffuse in agarose gel against Cramoll 1,4 (15 μg) from *C. mollis* (A). The precipitation lines were stained with Coomassie Brilliant Blue. Wells: 1- CE (100 μg); 2- F 30-60% (100 μg) and 3- CrataBL (26 μg).

Figure 4. Chromatography on CrataBL-Sepharose 4B.

The column (2.3 x 1.2 cm) was equilibrated with 10 mM citrate-phosphate buffer at pH 5.5. The samples (1 mg) of casein (A), fetuin (B) and ovalbumin (C) were applied to the column. The lectin affinity support was then washed with the same buffer. Elution was carried out at 24° C at a flow rate of 10 ml/h. Arrows demonstrate the points at which

eluent was added. Fractions of 2.0 ml were collected. Each bar represents the mean ± standard deviation of three experiments.

Figure 5. Electrophoretic patterns of glycoproteins isolated from human plasma by chromatography on CrataBL-Sepharose 4B. Sample of human plasma (1), samples of column elutions with NaCl: 50 mM (2), 100 mM (3) and 100o mM (4) and sample of column elution with 1000 mM acetic acid (5), were loaded onto a 12 % gel [Laemmli, 1970] and stained with Coomassie Brilliant Blue.

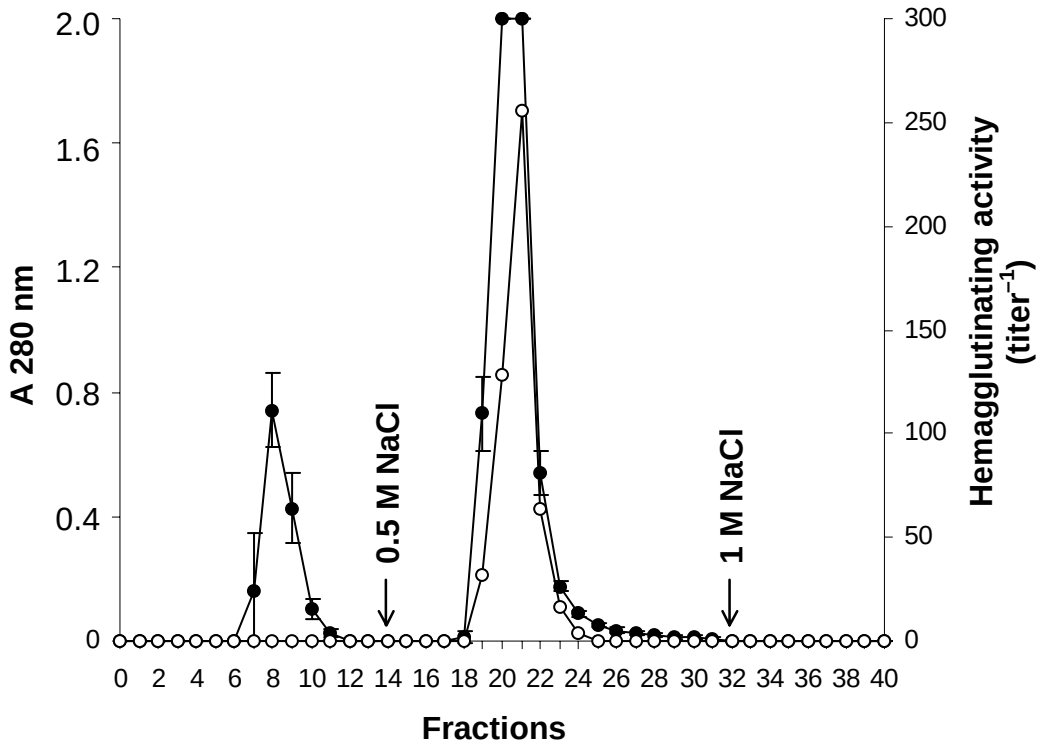
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Figure 1

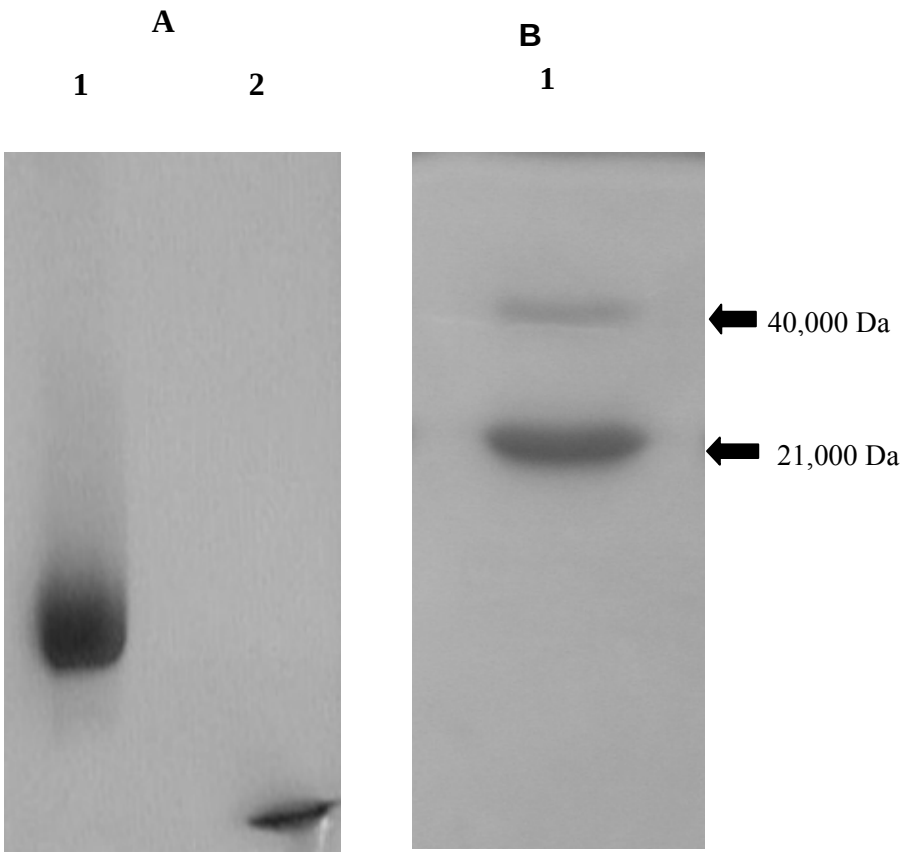

Figure 2

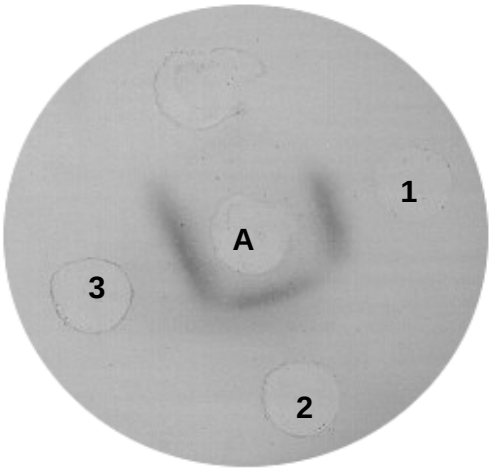

Figure 3

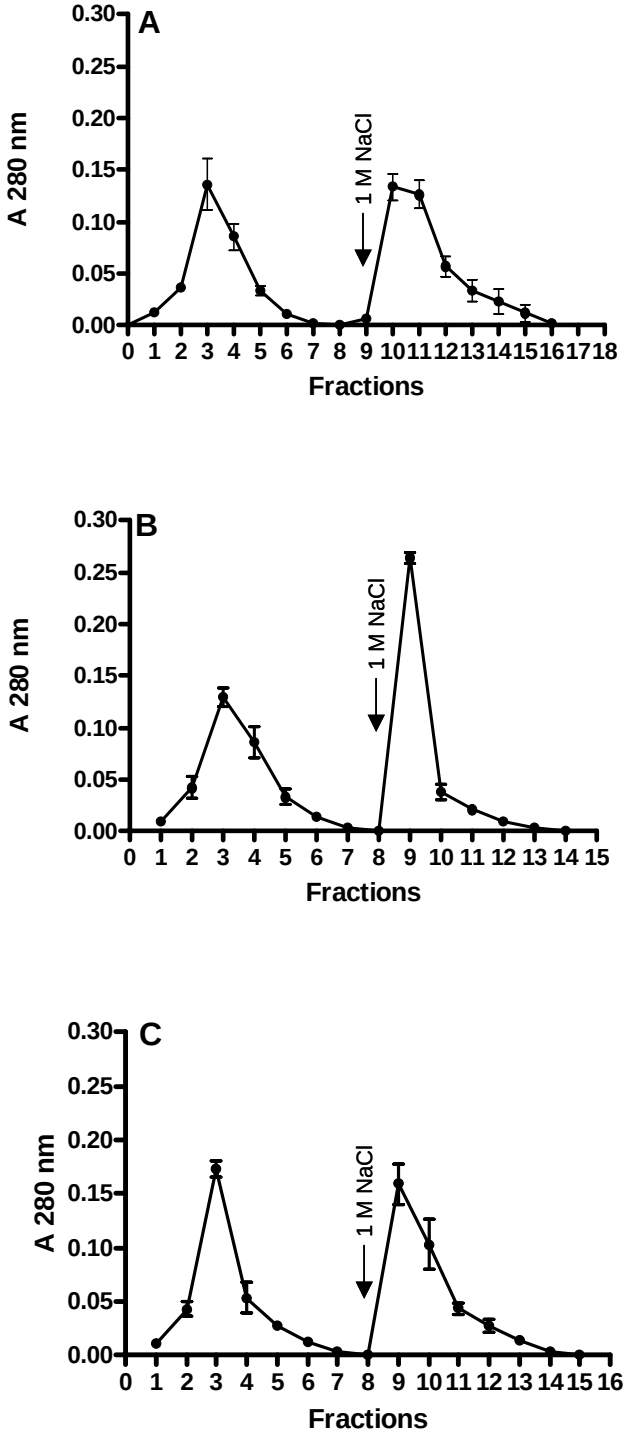
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Figure 4

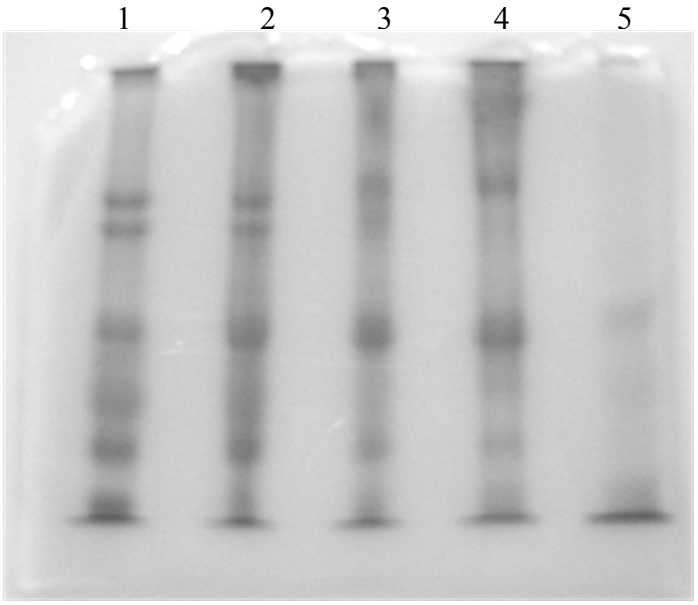

Figure 5

Table 1. Summary of CrataBL purification.

Preparation	Total protein (mg)	Total HA	SHA	Purification (fold)	Yield (%)
CE	473	40,960	87	1.0	100
F30-60%	310	29,696	96	1.1	72*
CrataBL	10	1,024	102	1.2	100**

Hemagglutinating activity (HA) titre was defined as the lowest lectin concentration able to produce visible hemagglutination. SHA (specific HA) was calculated from the ratio of titer to protein concentration (mg/ml). Purification was measured as the ratio between the SHA in the stage and SHA of CE (crude extract). Similarly yield was measured by the ratio of HA values. * Percentage of CE total HA recovered in the ammonium sulphate fraction. ** Percentage of F30-60 % (total HA) applied on CM-Cellulose and isolated in 500 mM NaCl (eluted fraction).

CAPÍTULO 3

Anticoagulant Activity of *Crataeva tapia* bark lectin

Artigo a ser submetido ao periódico International Journal of Laboratory Haematology

Anticoagulant activity of *Crataeva tapia* bark lectin

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Abstract

In this paper the effects of *Crataeva tapia* bark lectin, CrataBL, on coagulation activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) were evaluated. Although changes with a statistical significance were observed, the PT and TT remained within normal limits at all concentrations of CrataBL. In contrast, the APTT was significantly prolonged by CrataBL at 1.25 to 40 μg in a dose-dependent manner. The specific factor innibitory assay was also evaluated. CrataBL dose-dependent interacted with blood coagulation factors VIII, IX, XI and XII. These results revealed CrataBL as the first plant lectin with anticoagulant activity that impairs only the intrinsic pathway and thus *C. tapia* bark could be a source of potent exogenous anticoagulant.

Keywords: *Crataeva tapia*, lectin, Blood coagulation, Intrinsic pathway, Coagulation factors (F VIII, IX, XI and XII).

1. Introduction

Venous thromboembolism (VTE) is an important clinical problem with an incidence of 1-2 per 1000 persons and the consequences can be life-threatening (van Doormaal *et al.*, 2008). Although the causes are not always identified it is believed that the increasingly hypercoagulability can alter the balance of hemostasis, explaining the occurrence of VTE in apparently healthy individuals (Tripodi *et al.*, 2004). Hypercoagulability may be due to defective naturally occurring anticoagulant mechanisms or to heightened levels of procoagulant factor (Anderson e Spencer, 2003). In particular, increased levels of such coagulation factors such as VIII (Kyrle *et al.*, 2000), IX (van Hylckama *et al*., 2000) and XI (Meijers *et al.*, 2000) emerged as independent risk factors of VTE. These factors belong to the clinical intrinsic pathway of blood coagulation, which are cumulatively explored by the coagulation and activated partial thromboplastin time (APTT) tests, used over the last 50 years as a standard screening test in clinical laboratories throughout the world (White II, 2003). It is believed that the shortening of APTT may reflect the procoagulant imbalance and consequently increase the levels of coagulation factors leading an increased risk of VTE (Tripodi et al., 2004).

Traditionally, anticoagulant therapy is used for prevention and treatment of VTE. As endogenous or exogenous anticoagulants interfered with the coagulation factors, the blood coagulation can be prolonged or stopped (Jo *et al*., 2008). These anticoagulants have been convenient tools for exploration of the complex mechanisms of coagulation cascade, and coincidentally, as a source of research of anticoagulants that also have therapeutic purposes, as a cure for hemophilia (Jung *et al*., 2001).

In the search for new anticoagulant agents for anticoagulant therapy has been described a number of C-type lectin-like proteins isolated from venoms of snake that affect thrombosis and hemostasis by inhibiting or activating of specific platelet membrane receptors and blood coagulation factors (Ogawa et al, 2005). Anticoagulant lectins has been isolated from venom of snake. CrataBL, a lectin from *Crataeva tapia* bark, was purified and characterized in our laboratory and in this study the lectin prolonged the blood clotting time and seems to act as an anticoagulant protein.

2. Materials and Methods

2.1. Isolation of Crataeva tapia bark lectin

Powder of bark (10g) was mixed to 150 mM NaCl (100 ml). Extract was obtained by agitation of mixture overnight at 4 ºC. Followed filtration through gauze and centrifugation at 4,000 g for 15 min the extract was fractionated with ammonium sulphate according to Green and Hughes (1955). The resultant 30-60% fraction (F30-60%) was dialyzed against distilled water (2 h) followed with 10 mM citrate-phosphate buffer at pH 5.5 (2 h). F30-60% sample (11 mg of protein) was applied to a CM-Cellulose column (5.2 x 1.6 cm) and equilibrated (flow rate of 20 ml/h) with 10 mM citrate-phosphate buffer at pH 5.5. The column was washed with the equilibrium solution until the absorbance (measured at 280 nm) was less than 0.05. Afterwards, the lectin was eluted from CM-Cellulose column with 500 mM NaCl and termed CrataBL.

2.2. Prothrombin time and activated partial thromboplastin time

Activated partial thromboplastin time (APTT) and prothrombin time (PT) were determined in an automated coagulometer ST4 (Diagnostica Stago, France). The plasma utilized was obtained commercially (Instrumentation Laboratory, Milano, Italy). In the PT, the control was made with 50 µl of plasma, incubation for 60 s, with subsequent addition of 100 µl of reagent (recombinant thromboplastin) from Instrumentation Laboratory (Milano, Italy). CrataBL at different concentrations (1.25 µg; 2.5 µg; 5 µg; 10 µg; 20 µg and 40 µg) was incubated (60 s) in 50 µl of plasma, with subsequent addition of 100 µl of reagent. In the APTT, the control tube containing 50 µl of plasma and 50 µl of APTT reagent from Instrumentation Laboratory (Milano, Italy) or test tubes containing CrataBL (same concentrations used to PT), 50 µl of plasma and 50 µl of APTT reagent were incubated by 120 s at 37 °C After this, 50 µl of 0.025 M calcium chloride was added to tubes. The assays were made in triplicate and the results expressed by the average of the determinations of each sample.

2.3. Thrombin clotting time

For thrombin time (TT) measurement, 50 µl of plasma was incubated for 120 s in 100 µl of bovine thrombin (15 UNIH – International Unit of Hematology) from Instrumentation Laboratory (Milano, Italy). CrataBL in different concentrations (1.25 µg; 2.5 µg; 5 µg; 10 µg; 20 µg and 40 µg) were incubated (120 s) in 50 µl of plasma, with subsequent addition of 100 µl of thrombin (15 UNIH). The tests were made in triplicate and the results express by the average of the determinations of each sample.

2.4. Effects of CrataBL on the activities of blood coagulation factors VIII, IX, XI and factor XII

The APTT was determinated on factor VIII (Diagnostica Stago, France), IX (Dade Behring, USA), XI or XII (Diamed, Switzerland) deficient plasma. The control was made with 50 µl of deficients plasmas (FVIII, FIX, FXI, FXII), 50 µl normal plasma and 50 µl APTT reagent (Instrumentation Laboratory, Milano, Italy), incubation for 180 s at 37 °C. Then, subsequent addition of 0.025 M calcium chloride (50 µl). CrataBL (50 µl) in differents concentrations (1.25 µg; 2.5 µg; 5 µg; 10 µg; 20 µg and 40 µg) were pre-incubated with 50 µl of FVIII, FIX, FXI or FXII deficient plasma, 50 µl of normal plasma and 50 µl of APTT reagent for 180 s at 37 °C. The 0.025 M calcium chloride (50 µl) was then added and the clotting time recorded. The experiments were performed in triplicate.

2.5. Statistical analysis

The results were expressed as mean ± S. E. Data for the various paramaters were compared and statistically assessed using one-way ANOVA (Origin® 6.0). *P* values less than 0,05 were considered significant.

3. Results and discussion

A variety of new anticoagulants are being developed and tested to inhibit the various steps in the coagulation cascade (Gopinath et al., 2007; Jo et al., 2008; van Doormaal et al., 2008). Traditionally, two main pathways have been described for blood coagulation: the intrinsic or contact pathway and the extrinsic or tissue factor pathway (Norris, 2003). Experimental *in vitro* models have been developed based on reconstitution of the

coagulation system. These *in vitro* experiments essentially show that the factors VIII and IX are dependent on extrinsic pathway activators, tissue factor/factorVIIa (TF/FVIIa) and therefore there is no independence between extrinsic or intrinsic pathways of blood coagulation (Gui et al., 2007).

However, these distinctions remain useful for clinical screening of bleeding and clotting disorders (Gui et al., 2007). Like this the CrataBL effects on 'intrinsic', 'extrinsic' and 'common' coagulation, we performed PT, APTT and TT assays on citrated plasma in the presence and absence of the lectin (Table 1). The APTT was significantly prolonged by CrataBL at all tested concentrations in a dose-dependent response. These results indicated that the lectin inhibited the intrinsic pathway and had negligible effects on the extrinsic pathway, and suggested that CrataBL had a dominant effect on one or more of the intrinsic factors rather than those of the extrinsic pathway. As reported in previous studies (Li *et al*., 2005), a C-type lectin-like protein from snake venom of *Agkistrodon acutus* (5.6 μM) also prolonged APTT from 30 s (control time) to 300 s, concentration higher than that obtained with 1.0 μM CrataBL (40 μg) which prolonged more than 300 s.

Although changes with a statistical significance were observed, the PT and TT remained within normal limits (McPherson and Pincus, 2007) at all concentrations of CrataBL. Sakurai et al. (2003) evaluating the effect of the L-amino acid oxidase from *Agkistrodon halys blomhoffii* snake venom (M-LAO) on blood coagulation observed that the enzyme inhibited the intrinsic pathway and had negligible effects on the extrinsic pathway.

There results indicated that the CrataBL potently prolonged the normal clotting time on APTT. Aiming to identify interaction between CrataBL and intrinsic factors, the specific factor innibitory assays were evaluated at CrataBL presence. CrataBL interacted with blood

coagulation factors (F) VIII, IX, X and XI and prolonged the APTT time of coagulation (Figure 1) and consequently reduced all factors tested (Figure 2) confirming that the anticoagulant activity of CrataBL was due to interaction with all factors of intrinsic pathway. Usually, in the literature, differently from CrataBL, blood coagulation inhibition occurs by either coagulation factor. A C-type lectin-like from venom of Chinese green tree viper *Trimeresurus stejnegeri* dose-dependent interacted with FIX (Lee et al., 2003). Sakurai et al. (2003) investigated the effects of the enzyme (M-LAO) purified from venom of *Agkistrodon halys blomhoffii* snake on each coagulation factor and detected that the II, V, VII and X factors remained unchanged in the presence of enzyme, the VII and XI factors appeared to be slightly reduced remainning within normal limits and the IX factor activity was significantly reduced. A peptide from the marine echiuroid *Urechis unicinctus* prolonged the APTT but not PT and TT; only the FIX activity was potently inhibited (Jo et al., 2008). On the other hand, the peptide inhibited both coagulation pathways. Additionally a high efficient anticoagulant protein so called 'Factor Ixbp' was reported with irreversible anticoagulant activity by binding with both IX and X factors (Gopinath et al., 2007).

4. Conclusion

Crataeva tapia bark lectin showed a significant anticoagulant activity *in vitro* and inhibitory activity against all blood coagulation factors of the intrinsic pathway. The experimental results revealed that CrataBL could be a potent source of exogenous anticoagulant agent. This work represents the first plant lectin with anticoagulant activity.

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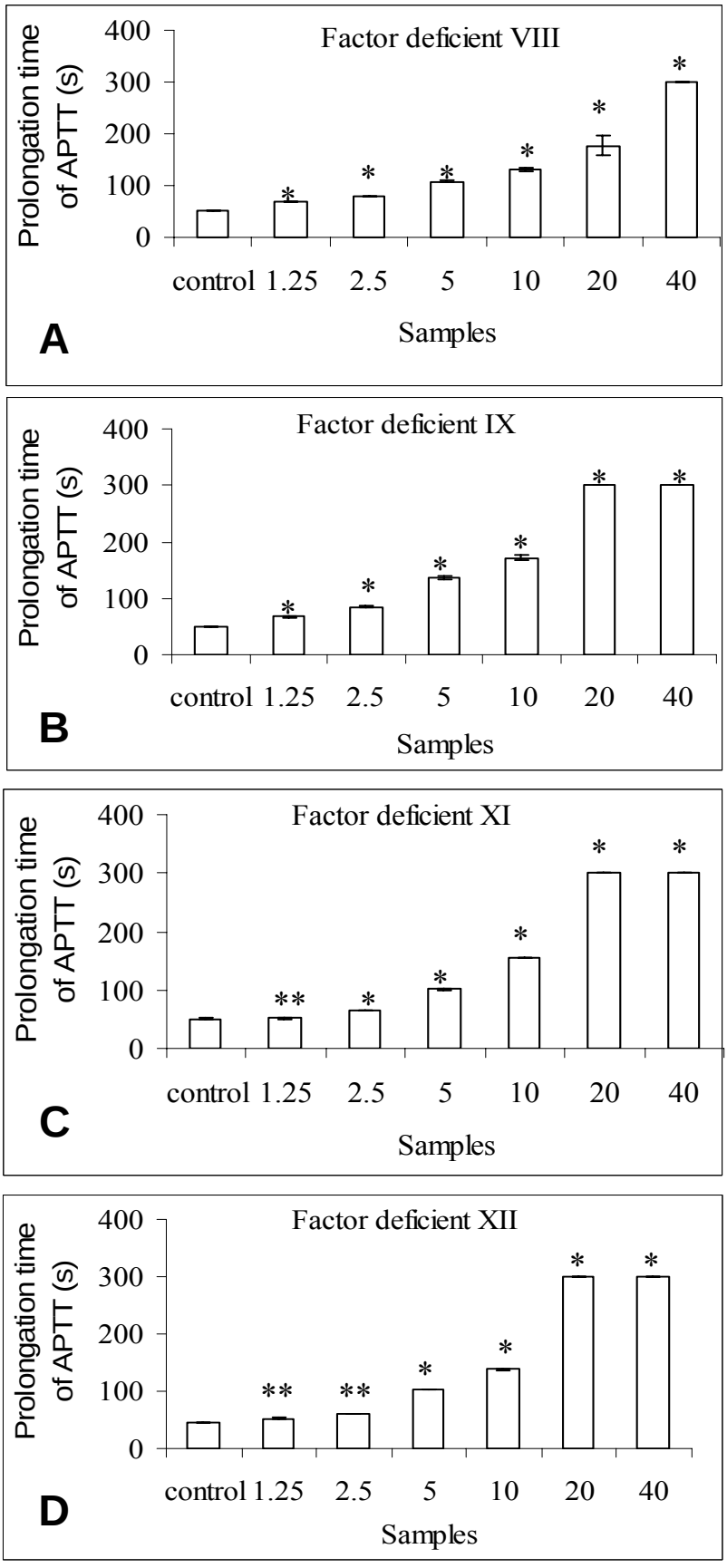
Figure capitation

Figure 1 – Effect of CrataBL on the activities of blood coagulation factors VIII (A), IX (B), XI (C) and XII (D), of deficient human plasma. Samples: control group and CrataBL at concentrations of 1.25µg, 2.5 µg, 5 µg, 10 µg, 20 µg and 40 µg. APTT = activated partial thromboplastin time. Each column represents the mean of three experiments and vertical lines show the S.E.M. Asterisks denotes the significance level in comparison to control value: ***** $P<0.001$ and ****** $P<0.01$.

Figure 2 – Effect of CrataBL (20 µg) on APTT of human plasma: (1) Control, (2) factor VIII deficient, (3) factor IX deficient, (4) factor XI deficient and (5) factor XII deficient. APTT = activated partial thromboplastin time. Each column represents the mean of three experiments and vertical lines show the S.E.M. Fold = time obtained/time control. Asterisks denotes the significance level in comparison to control value: ***** $P<0.001$.


Factor deficient VIII
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100
0
control 1.25 2.5 5 10 20 40
Samples
B
Factor deficient XI
Prolongation time of APTT (s)
400
300
200
100
0
control 1.25 2.5 5 10 20 40
Samples
C
Factor deficient XII
Prolongation time of APTT (s)
400
300
200
100
0
control 1.25 2.5 5 10 20 40
Samples
D

Figure 1

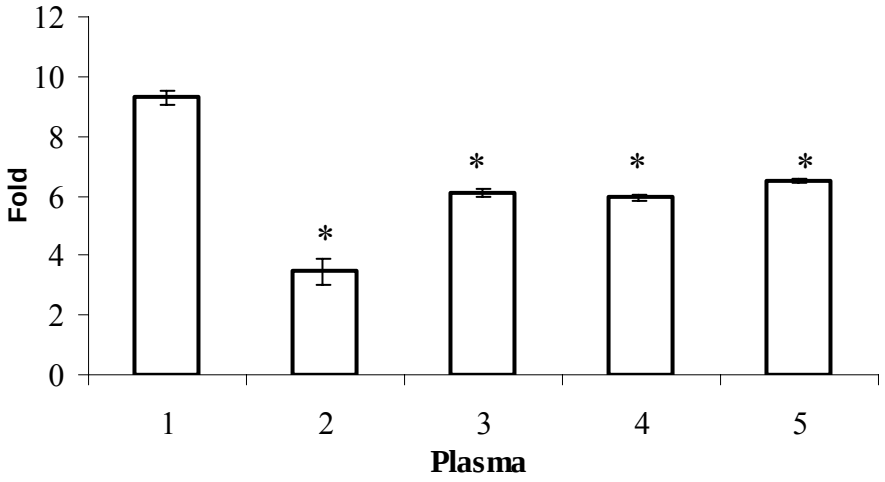

Figure 2

Table 1 - Effects of CrataBL on blood coagulation

Sample	Dose	APTT (s)	PT (s)	TT (s)
Control	_	26.9 ± 0.4	13.3 ± 0.5	23.4 ± 0.1
CrataBL	1.25 µg	37.2 ± 0.4*	14.0 ± 0.5**	23.5 ± 0.1
	2.5 µg	44 ± 0.4*	14.1 ± 0.5**	23.6 ± 0.1
	5 µg	70.2 ± 0.4*	14.2 ± 0.6**	23.8 ± 0.1***
	10 µg	96.6 ± 0.4*	14.7 ± 0.5*	23.9 ± 0.1***
	20 µg	250.2 ± 0.4*	15.3 ± 0.5*	24.0 ± 0.1**
	40 µg	> 300 ± 0*	16.7 ± 0.4*	24.9 ± 0.1**

***** $P<0.001$, ****** $P<0.01$ and ******* $P<0.05$ vs. control

APTT = activated partial thromboplastin time, PT = prothrombin time and TT = thrombin time.

CAPÍTULO 4

Crataeva tapia Bark Lectin: Toxicity, Antitumoral, Anti-inflammatory and Analgesic Properties

Artigo a ser submetido ao periódico Journal of Pharmacological Sciences

Crataeva tapia Bark Lectin: Toxicity, Antitumoral, Anti-inflammatory and Analgesic Properties

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Abstract

The *Crataeva tapia* bark lectin (CrataBL) was evaluated for its toxicity on brine shrimps and antitumor activity against sarcoma 180 bearing Swiss albino mice. The anti-inflammatory and analgesic properties of CrataBL were investigated in models of inflammation and nociception. The CrataBL toxicity on *Artemia salina* was detected and LC_{50} value of 71.7 μg/ml was determined. The lectin showed significant antitumor activity causing inhibition of tumor growth (50.7%) when administered intraperitonealy at 20 mg/kg daily for 7 days. CrataBL at 30 mg/kg also inhibited (35.4%) migration of neutrophil induced by carrageenan. Concerning the antinociceptive property, the lectin (30 mg/kg) inhibited (34.8%) abdominal contractions induced by acetic acid. The results revealed CrataBL as a lectin with significant antitumoral, anti-inflammatory and antinociceptive activities.

Keywords: *Crataeva tapia*, bark, lectin, *A. salina*, antitumoral, anti-inflammatory, antinociceptive

1. Introduction

Plants have been a source of medicines for humans and it is notable, in the last four decades, the resurgence of interest in the study and use of medicinal plants. Natural products derived from plants have been characterized and identified as new chemical compounds of therapeutic importance. This crescent interest in plant-derived drugs is mainly due to the belief that "natural medicines" are cheaper, safe and reliable than the synthetic drugs, many of which are toxic and have adverse side-effects (Ojewole, 2007).

Lectins are (glyco)proteins that can recognize and reversibly bind to carbohydrates or other substances derived from sugars (Correia et al., 2008). Lectins comprise many different families featuring different structures and specificities. They have been widely identified in nature, but plant lectins have been studied far more extensively.

In the past, sugar–protein interactions were neglected in favor of other interactions. Nowadays, it is well known that despite their small size, sugars play roles in storage or in relaying information within or between cells. Lectin–carbohydrate interactions have gained attention, since they may be employed to improve delivery and targeting of active compounds to their site of actions (Neumann et al., 2004). Owing to the fine specificity, lectins display a diversity of important biological activities including anticancer, anti-inflammatory and antinociceptive activities (Andrade et al., 2004; Saint-Gadelha et al., 2006; Bitencourt et al., 2008).

Currently, to block the development of cancer, a promising strategy is to chemoprevention, what is defined as the use of natural or synthetic agents (alone or combination) aiming inhibit the tumor growth. Approximately 60 % of currently used anticancer agents are derived from natural sources, including plants, marine organisms, and

microorganisms (Newman et al., 2003; Cragg et al., 2005). Plants, vegetables and herbs used in the folk and traditional medicine have been accepted as one of the main source of discovery and development of cancer chemoprevention drugs (Abdullaev, 2001).

Inflammation is usually associated with pain as a secondary process resulting from the release of algesic mediators (Osadebe and Okoye, 2003). Therapy of inflammatory diseases is usually directed at the inflammatory processes. Through years of syntheses and structural modifications, which usually accompany design and development of new drug substances, many non-steroidal anti-inflammatory agents (NSAIDS) have been prepared and marketed (Osadebe and Okoye, 2003). However, these drugs are known to provoke gastrointestinal irritation (Nguemfo et al., 2007). This makes them unacceptable; hence, the search for alternative anti-inflammatory drugs and medicines plants is stimulated (Saint-Gadelha et al., 2006).

The evaluation of plants as source of analgesic agents generally use to acetic acid-induced abdominal writhing, which is the visceral pain model. The writhing response of the mouse to an intraperitoneal injection of noxious chemical is used to screen for both peripherally and centrally acting analgesic activity. Acetic acid causes algesia by releasing of endogenous substances, which then excite the pain nerve endings; the abdominal constriction is related to sensitization of nociceptive receptors to prostaglandins (Chen et al., 1993). Aqueous extract of *Sida cordifolia* leaves at the oral dose of 400 mg/kg inhibited the number of writhes produced by acetic acid and was suggested that the effect was due to inhibition of prostaglandin synthesis (Franzotti et al., 2000).

The aim of the present study was to investigate the antitumoral, anti-inflammatory and antinociceptive properties of bark lectin from *Crataeva tapia* on experimentally induced tumor, inflammation and pain.

2. Materials and Methods

2.1. Isolation of Crataeva tapia bark lectin

Powder of bark (10g) was mixed to 150 mM NaCl (100 ml). Extract was obtained by agitation of mixture overnight at 4 ºC. Followed filtration through gauze and centrifugation at 4,000 g for 15 min the extract was fractionated with ammonium sulphate according to Green and Hughes (1955). The resultant 30-60% fraction (F30-60%) was dialyzed against distilled water (2 h) followed with 10 mM citrate-phosphate buffer at pH 5.5 (2 h). F30-60% sample (11 mg of protein) was applied to a CM-Cellulose column (5.2 x 1.6 cm) and equilibrated (flow rate of 20 ml/h) with 10 mM citrate-phosphate buffer at pH 5.5. The column was washed with the equilibrium solution until the absorbance (measured at 280 nm) was less than 0.05. Afterwards, the lectin was eluted from CM-Cellulose column with 500 mM NaCl and termed CrataBL.

2.2. Animals

Swiss albino male mice (*Mus musculus*) weighting approximately 33 g (± 50-days-old) were obtained from the Bioterium of the Centro de Pesquisa Ageu Magalhães/FIOCRUZ and maintained under constant conditions (temperature: 23 ± 2 °C, humidity: 40-60%, 12-h light/12-h dark cycle). The mice were allowed to access standard rodent chow diet (Purina®) and water *ad libitum.* All experiments involving Swiss albino male mice were approved by the Ethics Committee for Animal Experimentation of the Biological Sciences Center of the Federal University of Pernambuco, Brazil (CEEA – UFPE - Ofício n° 40/06).

2.3. Brine Shrimp Lethality Activity

The assay was according to Meyer et al. (1982) modified. *A. salina* eggs (25 mg) were hatched in seawater natural at temperatures of 20 to 30 °C. The pH was adjusted between 8.0 and 9.0 using Na_2CO_3 to avoid risk of death to the larvae by decrease of pH during incubation (Lewan et al., 1992). The eggs were placed in container with two compartments. The compartment with the eggs was covered in order to keep the eggs in a dark ambient. The other compartment of the container was illuminated in order to attract *A. salina* through perforations at the boundary plate. After 24 h, the phototropic brine shrimp, which went to the illuminated compartment, were collected by Pasteur pipette. Stock solution (5,000 ppm) was prepared in seawater. *A. salina* bioassay used a 5,000 ppm CrataBL stock solution prepared in seawater: stock solution was diluted (110-50 ppm) in vials containing 5 ml of seawater and 15 *A. salina*. The control group was set with vehicle used for dilutions and the time of exposition of the target organisms was of 24 h. Survival rate was determined and used to estimate LC_{50}. The assays were carried out in triplicate. The collected data were computerized and the LC_{50} value estimated using the statistical method of probity (Finney, 1971).

2.4. Antitumor Activity

The *in vivo* antitumor activity of CrataBL against Sarcoma-180 was carried out in male mice (six animals per cage). CrataBL and MTX (methotrexate) dosages were calculated according to animal body mass index (20 mg/kg for CrataBL and 2.5 mg/kg for MTX). Malign tumor cells (Sarcoma-180) from tumor carrier animals with 8 days of implanting were used. All animals were previously higienized in an experimental surgery room. Donor mice were anaesthetized for tumor suctioning and the ascetic form of the

tumor was introduced under the right axilla of the receptor animals. Therapeutics, by i. p. route, was begun 24 h after implanting for 7 days. The negative control group received only the vehicle (saline solution) and the standard one (positive) received MTX as referential antitumor drug. In the eighth day, the animals were sacrificed by cervical dislocation and solid tumors excised and weighed. Tumor inhibition was expressed as the mean of tumor weight for the treated animal group (T) in comparison to the untreated control group (C). The tumor inhibition was then calculated according to: percentage tumor inhibition = [(C-T)/C]×100. Animal experiments were performed according to the NCI protocol (Geran et al., 1972).

2.5. Anti-inflammatory assay – carrageenan-induced peritonitis

Saline (0.15 M NaCl) solution (control), dexamethasone (2 mg/kg), piroxican (10 mg/kg) and indomethacin (10 mg/kg) as standard drugs and CrataBL (30mg/kg) as test drugs were administered by i. p. route to the correspondent groups (6 animals per group). After 1 h, 0.25 ml carrageenan (1% in saline solution) injected intraperitoneally was used as phlogistic agent. After 4 h the animals were sacrificed by cervical dislocation and immediately submitted to surgery for abdominal opening (Gupta et al., 2005). Peritoneal cavity was washed with 2 ml of saline solution with EDTA. Exsudates were collected and the polymorphonuclear leukocytes (PMNL) count was determined in a Neubauer chamber after sample dilution in Turk solution (1:200).

2.6. Analgesic activity – acetic acid-induced writhing response

The response to an intraperitoneal injection of acetic acid solution, which manifests as a contraction of the abdominal muscles and stretching of the hind limbs, was evaluated

using a method adapted from Young et al. (2005). Animals (6 per group) were pretreated i.p. with CrataBL (30 mg/kg), vehicle (saline solution) or piroxican (10 mg/kg) and dipyrone (150 mg/kg) as standard drugs. 1 h later, a dose of 0.1 ml/10g body weight of 1 % acetic acid was injected i.p. After 10 min, the number of writhes during the following 20-min period was counted. Inhibition percentage was calculated through the decrease of total writhes number in the treated groups against control group.

2.7. Statistical Analysis

The results were expressed as mean ± S. E. M. (n=6) and statistically assessed using one-way ANOVA (Origin® 6.0). *p* values less than 0.05 were considered significant.

3. Results and Discussion

Crataeva tapia bark extract is used by people as medicine to treat hypoglicemy, stomach problems and fever. Definition of biological activities of CrataBL isolated from bark in quantities milligrams (Nascimento et al., 2008) can direct its popular use.

Despite several traditional drugs derived from plants have lost much space for those of synthetic origin, others have appeared and received special attention and terapeutic status, such as taxol, a plant product now available as a drug of choice for treatment of cancer (Cragg and Newman, 2005).

CrataBL to promoted *A. salina* mortality at 50 and 110 ppm (Table 1) and the low C_{50} value 71.73 µg/ml indicated its high toxicity. According to Meyer et al. (1982) crude extracts and pure substances with LC_{50} value < 1000 µg/ml and LC_{50} value > 1000 µg/ml are toxic and non-toxic, respectively. LC_{50} values determined for the latex and fractions

from aerial parts of *Euphorbia conspicua* were all considered to be toxic for *A. salina* with irritant fraction II showing the highest activity with LC_{50} 0.068 μg/ml (Santos et al., 2007). The low value of LC_{50} determined by *A. salina* bioassay indicates compounds with potential antineoplastic properties since there is good correlation between artemicide activity and citotoxic activity (Santos et al., 2003).

Treatment of Swiss albino mice with CrataBL (20 mg/kg) significantly ($p<0.05$) reduced (50.7 %) the Sarcoma 180 volume as compared to that tumor volume of control group (Figure 1). Antitumor activity of lectin was already described. *Cratylia mollis* lectin (Cra) promoted 41% of tumor inhibition and encapsulation of Cra in lipossomes increased this activity to 71% (Andrade et al., 2004).

We have evaluated the putative anti-inflammatory activity of CrataBL using carrageenan-induced paw edema as an *in vivo* model of inflammation and evaluating the exsudate from CrataBL treated and control groups. Our data showed that the lectin promoted significant reduction (35.4 %) in the number of PMNL in the inflammatory exsudate when compared with the control group (Figure 2). The paw edema model has been frequently used to assess the antiedematous effect of natural products since carrageenan induces multi- mediated edema formation is characterized by intense neutrophil infiltrate and the release of inflammatory mediators such as polypeptide kinins, prostaglandins, and nitric oxide (Morris et al., 2003; Ojewole et al., 2007). It is possible that CrataBL anti-inflammatory activity may be due to interfering with the release of a cascade of inflammatory mediators since inhibition of leukocyte recruitment might prevent inappropriate inflammation (Kelly et al., 2007). Anti-inflammatory lectins were already revealed using the same experimental model used here. The lectin from marine alga *Hypnea cervicornis* significantly decreased neutrophil migration when injected into the

peritoneal cavity of mices (Bitencourt et al., 2008) and Santi-Gadelha et al. (2006) demonstrated that the anti-inflammatory effect of *Araucaria angustifolia* seed lectin involved its carbohydrate binding sites.

The results of acetic acid-induced writhing responses in mice treated with CrataBL and commercial antinociceptive drugs are showed in Table 2. It was found that CrataBL and drugs at the doses assayed caused a significant inhibition on the writhing responses when compared with the control. Researchers lead their efforts to elucidate different pharmacological effects of lectins related to its antinoceptive activity. Study with *Amansia multifida* lectin demonstrated potent antinoceptive effects of both central and peripheral origin and was suggested that the analgesic effect possibly involved the participation of the opioid system (Neves et al., 2007). Bitencourt et al. (2008) demonstrated that the antinociceptive activity of lectin from marine alga *Hypnea cervicornis* could be related to inhibition of the release of mediators in response to acetic acid and that the activity involved the lectin carbohydrate-binding site.

It is clear that there is a strong association between the inflammatory process and the development of pain (Osadebe and Okoye, 2003). Experimental studies have demonstrated that the inhibition of neutrophil migration reduces the hypernociception induced by different inflammatory stimuli (Verri et al. 2004; Cunha et al. 2005; Santodomingo-Garza et al. 2006). *Lonchocarpus sericeus* lectin decreased leukocyte migration and mechanical hypernociception by inhibiting cytokine and chemokine production (Napimoga et al. 2007). CrataBL antinociceptive activity may be associated to its anti-inflammatory activity here demonstrated.

Conclusion

In summary, our work describes interesting biological properties of lectin from *Crataeva tapia* bark that not only display a significant antitumor effect but also shows anti-inflammatory and antinociceptive activities.

Acknowledgements

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Figure Caption

Figure 1 – Effect of CrataBL and MTX (methotrexate) on growth of Sarcoma-180 in Swiss albino male mice. Each column represents the mean of six animals and vertical lines show the S.E.M. Asterisks denotes the significance level in comparison to control value: $*P<0.05$.

Figure 2 – Effect of pre-treatment with dexamethasone, piroxican, indomethacin (standard drugs) and CrataBL on migration of polymorphonuclear leukocytes (PMNL) (number of PMNL/ml exsudate) in carrageenan-induced peritonitis in mice. Each column represents the mean of six animals and vertical lines show the S.E.M. Asterisks denotes the significance level in comparison to control value: $*P<0.05$.

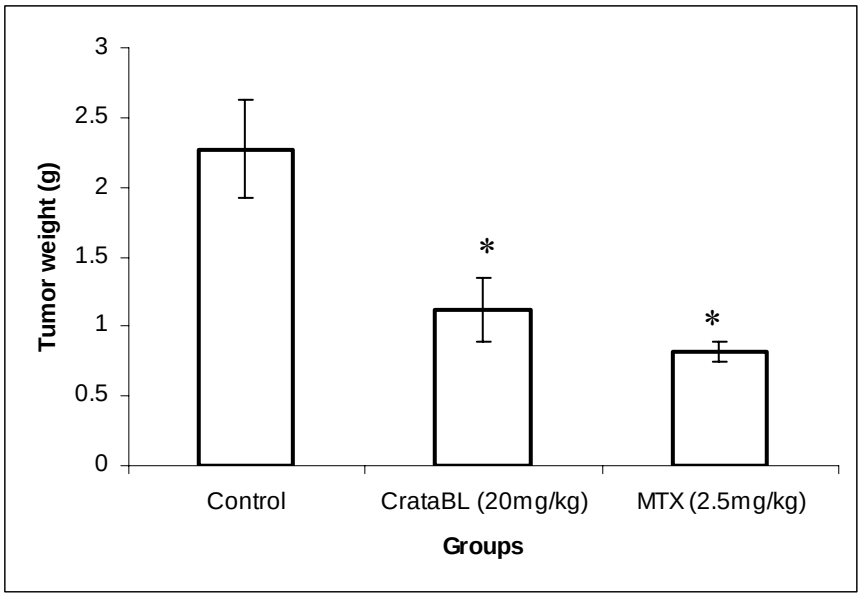
3
2.5
2
1.5
1
0.5
0
Tumor weight (g)
*
*
Control
CrataBL (20mg/kg)
MTX (2.5mg/kg)
Groups

Figure 1

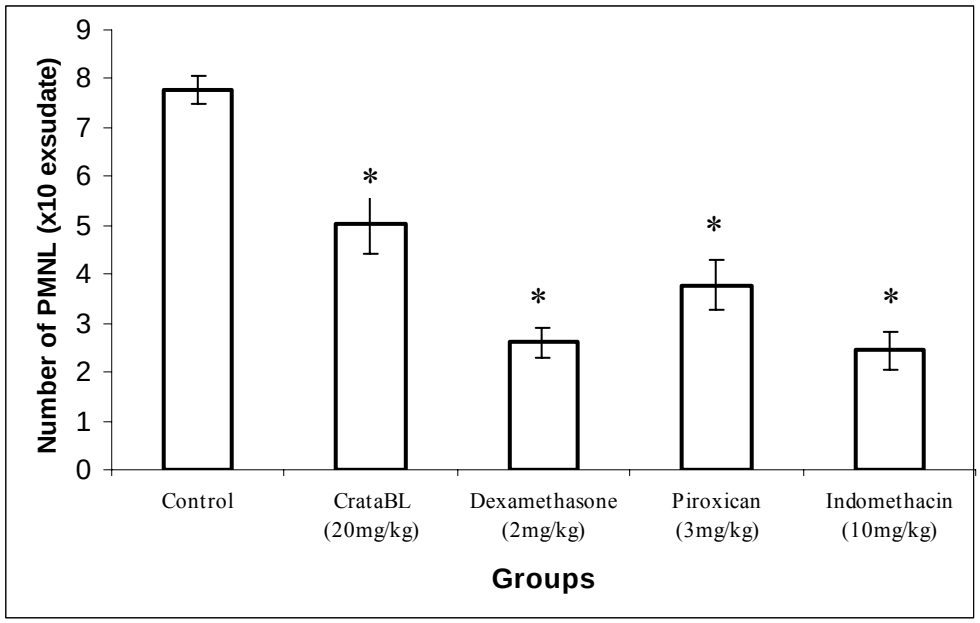
Number of PMNL (x10 exsudate)
9
8
7
6
5
4
3
2
1
0
*
*
*
*
Control
CrataBL (20mg/kg)
Dexamethasone (2mg/kg)
Piroxican (3mg/kg)
Indomethacin (10mg/kg)
Groups

Figure 2

Tables

Table 1 – The activity of CrataBL against *Artemia salina*

Sample (μg/ml)	**Mortality (%)**	**Lethal Concentration (μg/ml)**		
		$LC_{10}[IC_{95}]$	$LC_{50}[IC_{95}]$	$LC_{90}[IC_{95}]$
CrataBL		40.4	71.73	103.4
		[38.79 42.16]	[70.0 73.47]	[102.54 103.54]
110	*93.3*			
90	*82.2*			
70	*46.6*			
50	*20*			

Table 2 – Antinociceptive activity of CrataBL and analgesic drugs

Compound	**Dose**	**Medium ± S.E.M.**	**Protection (%)**
Control	_	64.4 ± 4.1*	_
Piroxican	10 mg/kg	44.7 ± 1.6*	32.7
Dipyrone	150 mg/kg	19.8 ± 1.9*	73.6
CrataBL	30 mg/kg	42.0 ± 4.1*	34.8

n=6. *P<0.05 vs control group.

CAPÍTULO 5

Conclusões

CONCLUSÕES

1. Uma lectina (CrataBL) foi purificada da entrecasca de *Crataeva tapia* com elevado rendimento em quantidades miligrama. CrataBL, uma lectina inibida por glicoproteína, foi eficientemente imobilizada à Sepharose CL-4B e o suporte foi hábil em isolar glicoproteínas do plasma humano.

2. CrataBL possui atividade anticoagulante, inibindo os fatores VIII, IX, XI e XII da via intrínseca da coagulação sanguínea.

3. CrataBL foi tóxica para *Artemia salina* e apresentou atividade antitumoral sobre Sarcoma 180. As atividades antiinflamatória e analgésica de CrataBL foram detectadas usando modelos animais.

ANEXOS

ANEXO 1

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Short communication

Optimized extraction of a lectin from *Crataeva tapia* bark using AOT in isooctane reversed micelles

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ABSTRACT

Crataeva tapia bark lectin was extracted from a crude extract into a reversed micelle phase of the anionic surfactant AOT in isooctane and back-extracted, to a final aqueous phase by addition of butanol. The effects of pH, ionic strength and surfactant concentration on the protein transfer process from the aqueous to the organic phase were characterized, being the best results obtained after 5 min of contact, under agitation, between the two phases, at pH 5.5 (10 mM citrate-phosphate buffer), 30 mM NaCl, and 5 mM AOT. Recovery to a new aqueous phase was performed with 5 min of contact, under agitation, 10 mM citrate-phosphate buffer at pH 5.5, 500 mM KCl and 5% of butanol. The overall yield obtained for the process was 80% for lectin activity and 56% for protein recovery. The efficiency of the process was confirmed by SDS-PAGE analysis.

1. Introduction

Lectins occur ubiquitously in nature and are important in medicine and technology. Most lectins are basically non-enzymic in action and non-immune in origin. They may bind to a carbohydrate moiety that is in solution or to one that is a part of protein/particulate body. They can agglutinate cells and/or precipitates glycoconjugates [1]. Plant seeds and bark provide a major source from which lectins are isolated. Some plant lectins appear to have pharmacological activity [2]. For example, in the northeast of Brazil *Crataeva tapia* bark infusions have been used in popular medicine as hypoglycemic agent.

Protein extraction from aqueous solution by reverse micelles is a process that utilizes basic techniques of chemical engineering such as classical liquid–liquid extraction, and thus has the potential for industrial application [3]. This process can be used to separate biological products such as, proteins, which have been solubilized in organic solvents using surfactants, without affecting their functional properties [4]. The process can be performed by a forward extraction of the target protein or contaminants, from an aqueous solution to a reversed micellar organic phase, followed by back-extraction, during which the biomolecules are released from micelles and transferred to a new aqueous phase [5], recent examples being the extraction of a lysozyme and ovalbumin [6], chitanases [7], α-amylase [8] and nattokinase [9].

In this work, the extraction and purification of a new thermostable plant lectin from *C. tapia* bark was studied using a reversed micelle system of the anionic surfactant sodium di(2-ethylhexyl)sulfosuccinate (AOT) in isooctane. Process optimization was performed by addition of butanol and manipulation of pH, ionic strength and surfactant concentration.

2. Materials and methods

2.1. Chemicals

Sodium di(2-ethylhexyl)sulfosuccinate was obtained from Sigma (USA); isooctane, butanol and glutaraldehyde were obtained from Merck (Germany); bicinchoninic acid (BCA) was obtained from Pierce. All other chemicals were of analytical grade.

2.2. Preparation of extract

C. tapia bark was collected in the region of Recife city (Pernambuco, Brazil) and the extract was obtained by pulverizing dried bark [10% (w/v) in 150 mM NaCl] followed by agitation overnight at 4 °C. Afterwards, the extract was filtered through

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a gauze and the filtrate centrifuged at 4000 × g for 15 min. The supernatant was termed crude extract (CE).

2.3. Extraction and back-extraction of protein with reversed micelles

The reversed micellar system was constituted using the anionic surfactant, sodium di(2-ethylhexyl)sulfosuccinate in isooctane. Extraction and back-extraction procedures were performed as follows: (1) to buffered lectin preparations (3 ml) at different pH (10 mM citrate-phosphate, pH 3.0–6.0, 10 mM sodium-phosphate, pH 7.0, 10 mM Tris–HCl, pH 8.0, 10 mM carbonate–bicarbonate, pH 9.0–10.0 and 10 mM glycine–NaOH, pH 11.0–12.0) containing 30 mM NaCl, an equal volume (3 ml) of micellar phase (AOT in isooctane at a concentration range of 0.125–100 mM) was added and both phases were stirred for 5 min for protein extraction. The mixture was then centrifuged for 5 min at 3000 × g, for phase separation. (2) After extraction, the separated micellar phase (2 ml), containing solubilized protein, was added to an equivalent volume of buffered aqueous solution at different pH (10 mM citrate-phosphate, pH 5.0–6.0 and 10 mM sodium-phosphate, pH 7.0) containing 50–1000 mM KCl and 5% (v/v) of butanol was added to the system. The mixture was stirred for 5 min, centrifuged for 5 min at 3000 × g for phase separation and the lectin recovered to the new aqueous phase. Agitation speed (700 rpm), temperature (25 °C) and initial protein concentration (0.374 mg/ml) were kept constant in all experiments.

2.4. Protein assays

The protein content in the aqueous and organic phases was spectrophotometrically determined using the bicinchoninic acid according to Smith et al. [10], with bovine serum albumin as standard, at a range of 0–600 μg/ml.

2.5. Determination of the hemagglutinating activity

The determination of the hemagglutinating activity (HA) in the aqueous phase was performed in microtiter plates according to Correia and Coelho [11]. Lectin preparations (50 μl) were two-fold serially diluted with 0.15 M NaCl before addition of 50 μl suspension of rabbit erythrocytes treated with glutaraldehyde 2.5% (v/v). The HA was expressed as the log of the highest dilution exhibiting hemagglutination. The determination of the activity in the back-extraction aqueous phase was carried out after dialysis due to the interference of salt concentration used. The protein hemagglutinating activity was not measured in organic phase, due to interference of the organic solvent (micellar phase). The specific HA (SHA) was given by the ratio between HA and protein content (mg/ml). The purification factor (PF) as follows:

$$PF = \frac{\text{SHA in new aqueous phase after back-extraction}}{\text{SHA in initial aqueous phase}} \quad (1)$$

2.6. Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed on 10% (w/v) gel according to Laemmli [12]. Polypeptide bands of lectin (150 μg of protein) and standards [bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; lysozyme, 14.3 kDa from Sigma (USA)] were stained with Coomassie Brilliant Blue. PAGE for native basic [7.5% (w/v) gel] and acidic [12% (w/v) gel] proteins were performed according to Reisfeld et al. [13] and Davis [14], respectively.

2.7. Gel filtration chromatography

Lectin extracted by reversed micelles was chromatographed on gel filtration on a Hiprep 16/60 Sephacryl S-300 column (16 mm × 60 cm)(Akta FPLC system (Amersham Pharmacia Biotech, Sweden) pre-equilibrated at 24 °C with 0.5 M NaCl. Samples (2.0 ml; 1 mg of protein) were injected and eluted with the same solution at a flow rate of 3.0 ml/min. The standards (Sigma, USA) similarly chromatographed were bovine serum albumin (66 kDa), fetuin (64 kDa), ovalbumin (45 kDa) and trypsin inhibitor type III-O chicken (28 kDa).

3. Results and discussion

3.1. Effect of pH on the extraction

The major determining factors on protein solubilization in reversed micellar systems are electrostatic interactions among biomolecules and charged surfactant heads as well as the aggregation properties of surfactant. pH influence on lectin extraction was evaluated for a 30 mM NaCl aqueous phase and 5 mM AOT/isooctane. The pH of the aqueous phase (CE) was varied between 3.0 and 12.0, using different buffer systems according to their pK_a value (Fig. 1). The results showed a high transfer of protein to the micellar phase within the pH range of 3.0–6.0, with a maximum of ca. 70% at pH 5.5. A similar situation has been reported for *Cratylia mollis* seed lectin [15], with a maximum extraction at pH 5.0. This behaviour suggests that pH 5.5 was more selective, probably due to favorable attractive electrostatic interactions between positively charged lectin molecules at pH 5.5 (lectin has a p*I* 9.5 as previously determined in our laboratory) and negatively charged surfactant heads. For smaller proteins, using anionic surfactants at pH conditions below the protein p*I* their transfer from aqueous to micellar solution is favoured [16]. As the pH of aqueous phase increased from 5.5 to 12, the extracted protein decreased ca. of 34% probably due to the proximity of the isoelectric point of the lectin. This phenomenon was observed for different proteins at distinct isoelectric points [17]. The protein hydrophobic patches exposed may interact with anionic surfactant hydrophobic tails minimizing extraction. Besides the protein charge, the density of surface charge is an important factor on protein solubilization. Therefore, the selected pH to be used in further experiments was 5.5.

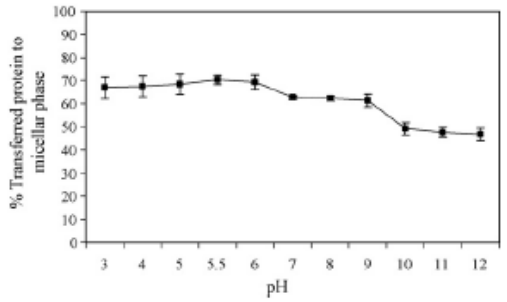

Fig. 1. Effect of pH on lectin extraction with 30 mM NaCl, 5 mM AOT in isooctane reversed micelles. Buffers: pH 3.0–6.0 (10 mM citrate-phosphate); pH 7.0 (10 mM sodium-phosphate); pH 8.0 (10 mM Tris–HCl); pH 9.0–10.0 (10 mM carbonate–sodium bicarbonate); pH 11.0–12.0 (10 mM glycine–NaOH). Each data point is an average of three experiments and the error bars show the standard deviation.

3.2. Effect of AOT concentration on the extraction

Protein solubilization is strongly dependent on the concentration of surfactant and on the size of the micelle relative to that of the protein. Before analysing the effect of AOT concentration on lectin purification the critical micellar concentration was determined as being 1 mM AOT, which is in agreement with results presented by Yan-ching and Ache [18]. For a AOT concentration of 0.125 mM, no phase separation occurred and for AOT concentrations of 0.25 and 0.50 mM an apparent phase separation (aqueous phase/micellar phase) was observed, but no protein was transferred to the micellar phase while on the other hand, 78.8 and 82.11% of protein formed a clearly visible precipitate at the interface, respectively. For AOT concentrations higher than the cmc (1–100 mM AOT), the influence of AOT concentration on lectin extraction under 30 mM of NaCl and pH 5.5, was evaluated (Fig. 2). The results showed that protein extraction was ca. 60% at AOT concentration between 1 and 3 mM, remaining constant at 70% for AOT concentrations between 5 and 50 mM with a 5% decrease for 100 mM AOT. It is well known that the increase in the amount of surfactant in organic phase leads to an increase of protein solubilization due to the increase of the amount of surfactant aggregation and/or the increase of size of reverse micelles [19]. Nevertheless, no significant differences were found on extraction over AOT concentration range of 5–50 mM. These results could be explained by the size of lectin associated to a strong electrostatic

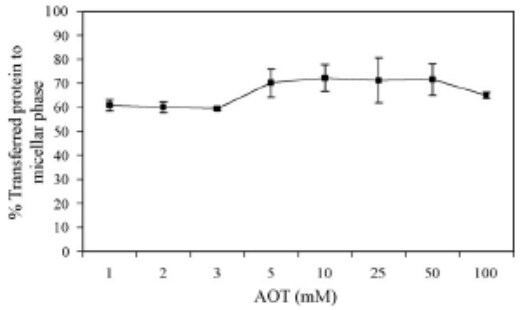

Fig. 2. Effect of AOT concentration on lectin extraction under 30 mM of NaCl and pH 5.5 conditions during forward transfer to micellar phase. Each data point is an average of three experiments and the error bars show the standard deviation.

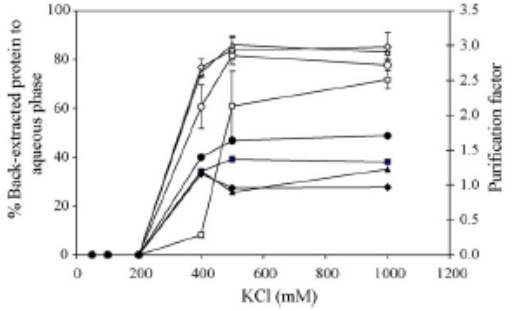

Fig. 3. Effect of pH and ionic strength on back-extraction at pH 5.5, with 5 mM AOT in isooctane reversed micelles to a new 10 mM buffered aqueous phase at pH 5.0 (□), 5.5 (○) and 6.0 (△) (with citrate-phosphate) and pH 7.0 (◇) (with sodium-phosphate) with addition of KCl concentrations of 50–1000 mM. Initial concentration of protein in micellar phase = 0.260 mg/ml and initial log HA = 1.5. Key: open symbols, % back-extracted protein to aqueous phase; closed symbols, purification factor. Each data point is an average of three experiments and the error bars show the standard deviation.

interaction between protein and surfactant due to the basic nature of the lectin. A similar situation has been reported for the extraction of a lipase from *Penicillium citrinum* across an AOT concentration range of 100–350 mM [20]. Taking in account these results, the 5 mM AOT concentration was chosen for the following experiments since it might be expected that a high surfactant concentration makes difficult the backward transfer of proteins into a second aqueous phase [4].

3.3. Effect of pH and ionic strength on the back-extraction

The back-extraction step was more difficult to accomplish, probably due either to the strong electrostatic interactions between positive lectin charge and negative charge of AOT or possibly the high affinity between the protein and the surfactant. A small amount of alcohol added to an organic solution can improve the back-extraction behaviour of proteins depending on the concentration and alcohol species used [21]. For example, the presence of butanol in the system may change the water properties inside reversed micelles [22] and may affect protein–micelle interactions in a fundamental manner [23]. For this reason 5% butanol was used during back-extraction. Fig. 2 shows that the amount of protein back-extracted to the new aqueous phase increased with the increase of pH value and KCl concentration with a maximum recovery (85%) at pH 7.0 containing 500 mM KCl. A further increase in the KCl concentration did not result in a higher content of back-extracted protein or a higher purification factor. This electrostatic screening effect may also be responsible for the decrease of the surfactant head group repulsions, leading to smaller reversed micelles [4], which in turn, could be a factor responsible for the higher back-extraction of proteins to the aqueous solution. However, we found that KCl concentrations higher than 500 mM did not increase the recovery of the protein. This may well be due to the fact that the electrostatic effect reached a maximum at 500 mM KCl. While the higher back-extraction (85%) was found at pH 7.0, a better purification factor (1.7) was obtained at pH 5.5 (Fig. 3) with 500 mM KCl. These results suggest that the increased percentage of protein transferred into a new aqueous phase when pH was increased from 5.5 to 7.0 was not due to higher lectin content but to other contaminant proteins. Similar purification factors of protein, 1.8 and 1.5, using micellar systems have been reported for an extracellular alkaline protease from fermentation broth [22] and for a xylanase from fermentation broth [24], respectively. The presence of butanol in the system permitted the back-extraction of lectin from a reversed micellar phase to an aqueous phase, as a consequence of a structural factor change of the micelles, corroborated by Liu et al. [9], and Lee et al.

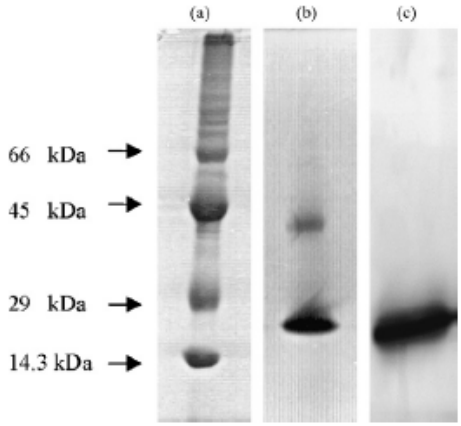

Fig. 4. PAGE of back-extracted sample at pH 5.5, containing 500 mM KCl with the addition of 5% (v/v) butanol at 25 °C. SDS-PAGE: (a) molecular weight markers and (b) lectin purified by reversed micelles; (c) PAGE for native basic protein: lectin purified by reversed micelles.

[21]. The optimal condition for direct extraction of lectin from crude extract led to a recovery of 56% with retention of, at least, 80% of the lectin HA.

3.4. Structural characterization of purified lectin by reversed micellar system

Electrophoresis was applied to the purified lectin as previously described. SDS-PAGE showed two polypeptide bands of molecular mass 21 and 40 kDa (Fig. 4a and b) while PAGE for native basic protein revealed a single lectin band (Fig. 4c) and no acidic protein band was detected. The evaluation of purified lectin by gel filtration chromatography (Fig. 5a) revealed two protein peaks corresponding to 40 and 29 kDa. When the crude extract was applied to the chromatography column (Fig. 5b) one main peak of 40 kDa and additional peaks of 26, 19 and 7.6 kDa were detected. At 29 kDa, a very small peak was also observed. The chromatographic profiles obtained indicate the efficiency of the reversed micellar system for lectin purification as none of the contaminants detected on the crude extract were observed in the purified

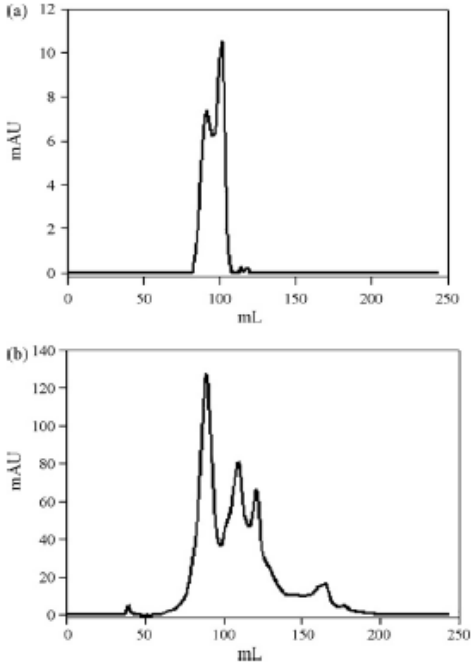

Fig. 5. Gel filtration chromatography on Sephacryl S-300: (a) purified lectin (2 ml; 0.3 mg) applied on column (16 mm × 60 cm) equilibrated with 500 mM NaCl and (b) crude extract (2 ml; 4 mg). Fractions (3 ml) were collected at 1.0 ml/min.

preparation. The two peaks detected by gel filtration chromatography under non-denaturing conditions for the purified lectin reflect the presence of aggregated and non-aggregated protein as already observed in SDS-PAGE (Fig. 4b). The obtention of a 29 kDa fraction by gel filtration chromatography, as compared to the 21 kDa fraction obtained by SDS, may be due to the incomplete unfolding of the non-aggregated lectin that occurs on the presence of SDS denaturing conditions.

4. Conclusion

The utilization of reverse micelles on lectin extraction and purification from a crude extract was successfully applied using an AOT/isooctane/water biphasic system with the addition of butanol on the back-extraction step and adjustment of pH, ionic strength and AOT concentration. Although the maximum recovery (85%) was found to be at pH 7.0 with 500 mM KCl, the highest purification factor (1.7) was found to be at the same pH level (5.5) found on the extraction step. Being so, the pH value of 5.5 was established as a compromise between the maximum activity (80%) and protein (56%) recovery. These results were obtained using a 10 mM citrate-phosphate buffer solution, pH 5.5 containing 30 mM NaCl; 5 mM AOT and 5 min agitation for the extraction step and a 10 mM citrate-phosphate buffer solution, pH 5.5 containing 500 mM KCl plus 5% of butanol and 5 min agitation for the back-extraction. This purification procedure allows for the obtention of a high purity lectin allowing for its further *in vivo* evaluation as a hypoglycemic agent.

Acknowledgements

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ANEXO 2

Trabalhos Apresentados em Congressos Internacionais

IFCC-WorldLab Fortaleza 2008

20th International Congress of Clinical Chemistry and Laboratory Medicine
XXXV Brazilian Congress of Clinical Analysis
VIII Brazilian Congress of Clinical Citology

Fortaleza - Brasil
28 September - 2 October 2008

PIN: QS0GY
Last Name: ARAUJO
First Name: REGINA MS
Abstract title: Anticoagulant activity of Crataeva tapia bark lectin

ACCEPTED

Dear Colleague,

We are pleased to inform you that your abstract has been accepted for Poster Presentation at the IFCC-WORLDLAB FORTALEZA 2008 Congress!

A Poster Code has been assigned to your poster: **H144**.

This code will indicate the day and the poster panel you will have to hang your poster up (i.e. M001 = Monday, panel 001; T = Tuesday; W = Wednesday; H =Thursday).

XXXVI Annual Meeting of the SBBq and 10th IUBMB Conference "Infectious Diseases: Biochemistry of Parasites, Vectors and Hosts" - Resumo R-9007

PURIFICATION OF C*rataeva tapia* BARK LECTIN AND ITS USE AS BIOSPECIFIC ADSORBENT FOR GLYCOPROTEIN ISOLATION

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Lectins constitute a heterogeneous protein group that recognize and reversibly bind to saccharide moieties. Lectins have been isolated from vegetative tissues such as bark from legume trees. *Crataeva tapia* belongs to the Capparaceae family and its bark is popularly used at the Northeast of Brazil as hypoglycemic material. A lectin from *C. tapia* bark, CrataBL, was purified to electrophoretic homogeneity by ammonium sulphate fractionation and ion exchange chromatography. CrataBL hemagglutinating activity (HA) was obtained with human, chicken and rabbit erythrocytes, which was mainly inhibited by glycoproteins. CrataBL was thermostable and EDTA treatment or Ca^{2+}, Mg^{2+} and Mn^{2+} addition did not affect HA. CrataBL migrated as a single band after electrophoresis to native basic proteins and two polypeptide bands of molecular mass 21,000 Da (main band) and
40,000 Da after SDS-PAGE, with or without reducing agent; polypeptides stained using Schiff's reagent. Native CrataBL also interacted upon agarose gel with Cramoll 1,4, a glucose/mannose lectin, and showed molecular mass of 40,000 Da by gel filtration chromatography. CrataBL immobilized on Sepharose 4B bioselectively adsorbed casein, fetuin and ovalbumin. The immobilized homogeneous lectin can be used as affinity matrix for glycoprotein isolation.

Supported by: CNPq

Keywords: Bark lectin; Crataeva tapia; lectin immobilization; glycoprotein isolation.

LIQUID-LIQUID EXTRACTION OF A LECTIN FROM *CRATAEVA TAPIA* BARK WITH AOT REVERSED MICELLES

Nascimento, C.O[1,2]; Costa, R.M.P.B[1,2]; Araújo, R.M.S.[1]; Coelho, L.C.B.B. [1]; Paiva, P.M.G. [1]; Correia, M. T. S[1]; Carneiro-da-Cunha, M. G.[1,2]
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Lectins comprise a structurally versatile group of proteins and glycoproteins. Its binding specificity to carbohydrates can be used to isolate specific glycoproteins.The aim of the present work was to evaluate the extraction and back-extraction of a lectin from *Crataeva tapia* bark purified by ionic exchange chromatography (CrataBL) using the reversed micelles system of AOT / isooctane, looking for further application to crude extract (CE). On protein extraction, agitation contact time, ionic strength, temperature, aqueous phase pH and surfactant concentration were investigated. On back-extraction, pH and ionic strength of aqueous phase with 5 % of butanol were also evaluated. From extraction step was obtained 100% and 70% of protein content for CrataBL and CE, respectively, with 5 min of phase contact, 30 mM NaCl, citrate/phosphate buffer, pH 5.5, 27 °C and 5 mM AOT. From back-extraction step a protein recovery of 45.25% (CrataBL) and 80 % (CE). The optimal conditions for lectin purification from crude extract led to a protein and activity recovery yield of 56% and 80%, respectively. The obtention of 100% pure lectin preparation was confirmed by PAGE and gel permeation chromatography. These results clearly indicate the efficiency of reversed micellar systems in lectin purification.

Acknowledgements: CNPq/FACEPE/ALFA-VALNATURA

Keywords: *Crataeva tapia*, lectin, protein purification, reversed micelles system.

VIII Reunião Regional Nordestel da SBBq e 3rd International Symposium in Biochemistry of Macromolecules and Biotechnology - Resumo R0445-1

REVERSED MICELLAR SYSTEM A POTENTIAL METHOD TO OBTAIN *CRATAEVA TAPIA* BARK LECTIN

Nascimento, C.O[1,2]; Costa, R.M.P.B[1,2]; Araújo, R.M.S.[1]; Coelho, L.C.B.B. [1]; Paiva, P.M.G. [1]; Correia, M. T. S[1]; Carneiro-da-Cunha, M. G.[1,2]
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Lectins are ubiquitous proteins that specifically bind carbohydrate moieties including cells. The aim of this work was to evaluate the extraction and back-extraction of a *Crataeva tapia* bark lectin with the reversed micellar system of AOT in isooctane. Crude extract from powdered bark was obtained by agitation of 10 % (w/v) mixture in 150 mM NaCl overnight at 4 ^{o}C, followed by filtration and centrifugation at 4,000 g for 15 min. On protein extraction, agitation contact time, ionic strength, temperature, salt type and concentration, aqueous phase pH and surfactant concentration were investigated. On back-extraction, pH and ionic strength of aqueous phase with 5 % of butanol were also evaluated. Agitation speed and protein concentration maintained constant in all experiments. From extraction step was obtained 70% of protein content with 5 min of phase contact, 30 mM NaCl, citrate/phosphate buffer, pH 5.5, 27 °C and
5 mM surfactant. From back-extraction step a protein recovery of 80.65% (CE) with 50% of hemagglutinating activity was found under citrate/phosphate buffer, pH 5.5 added by 1000 mM KCl. PAGE for basic protein and SDS-PAGE revealed one single and two bands, respectively. These results showed the efficiency of reversed micellar system in lectin purification.

Supported by: PIBIC/CNPq/FACEPE/ALFA-VALNATURA

Key words: *Crataeva tapia*, lectins, protein purification, reversed micellar system.

ANEXO 3

Trabalhos Apresentados em Congressos Nacionais

XXXV Reunião Anual da SBBqResumoID:8567

Purification of a Lectin from *Crataeva Tapia* Bark Using Reversed Micelar System

Nascimento, C. O[1,2]; Costa, R. M. P. B[1,2]; Araújo, R. M. S.[1]; Correia, M. T. S[1]; Carneiro-da-Cunha, M. G.[1,2]

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The lectins are ubiquitous protein in the nature that reversibly bind to mono, oligo, polysaccharides and glycoconjugates. They do not present catalytic activity and unlike antibodies, are not products of immune reply. The aim of the present work was to evaluate the extraction and back-extraction of a lectin from *Crataeva tapia* bark using the reversed micellar system of sodium di(2-ethylhexyl)sulfosuccinate (AOT) in isooctane. The *Crataeva tapia* bark was collected in the region of Recife city (Pernambuco, Brazil) and the extract [10 % (w/v) in 150 mM NaCl] was obtained by trituration and agitation for 16 h at 4 ^{o}C, filtered on gaze and centrifuged (4.000 x g for 15 min). The supernantant obtained was called crude extract (CE). The factors that affect the protein extraction, such as: agitation contact time (5 - 20 min), ionic strength, salt type (NaCl, KCl e $CaCl_2$) and concentration (30 – 300 mM) included, pH of aqueous phase (pH 3.0 –12.0) and surfactant concentration (5 - 100 mM AOT), were investigated. For the back-extraction the following parameters were evaluated: pH of aqueous phase (pH 5.0 – 7.0) and ionic strength (50 - 1000 mM KCl) being added to the system 5 % of Butanol. The parameters, agitation speed (900 rpm), temperature (25^{o}C) and protein concentration (0.374 mg/ml), were maintained constant in all experiments. The best results for the extraction were obtained with 5 min of contact time between the two phases, 30 mM of NaCl, citrate/phosphate buffer, pH 5.5 and 5 mM of AOT, where it was possible to obtain 70% of protein extraction. For the back-extraction, the best conditions were; citrate/phosphate buffer, pH 5.5 added by 1000 mM of KCl, where it was possible to obtain a protein recovery of 80.65% (CE) with 50% of hemagglutinating activity. Samples from the best conditions of extraction and back-extraction revealed only one single PAGE band for basic protein and two SDS-PAGE bands. These results showed the efficiency of the reversed micellar system in the lectin purification.

Supported by: PIBIC/CNPq/FACEPE

II Reunião Regional da FeSBE

04.033 - Produtos Naturais

PURIFICAÇÃO DA LECTINA DE ENTRECASCA DE CRATAEVA TAPIA E SEU USO COMO ADSORVENTE BIOESPECÍFICO

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Objetivo:

Lectinas constituem um grupo heterogêneo de proteínas ou glicoproteínas que reconhecem e se ligam reversivelmente a mono ou oligossacarídeos específicos. Amplamente distribuídas na natureza, lectinas têm sido isoladas de tecidos vegetativos tais como entrecasca de árvores. Crataeva tapia pertence à família Capparaceae e sua entrecasca é popularmente utilizada no Nordeste do Brasil como hipoglicemiante. Este trabalho teve como objetivo purificar a lectina da entrecasca de C. tapia**,** caracterizá-la parcialmente e imobilizá-la em Sepharose CL-4B.

Métodos e Resultados:

A lectina da entrecasca de C. tapia**,** CrataBL, foi purificada após fracionamento salino com sulfato de amônio e cromatografia de troca iônica. Atividade hemaglutinante (AH) de CrataBL aglutinou eritrócitos de humanos, coelho e galinha, os quais foram principalmente inibidos com glicoproteínas. CrataBL foi termoestável e o tratamento com EDTA ou a adição de íons Ca^{2+}, Mg^{2+} e Mn^{2+} não afetou a AH. CrataBL migrou como uma única banda após eletroforese para proteínas nativas básicas e duas bandas polipeptídicas de massa molecular 21.000 Da (banda principal) e 40.000 Da após SDS-PAGE, com ou sem agente redutor; polipeptídeos foram corados usando reagente de Schiff. CrataBL também interagiu sobre um gel de agarose com Cramoll 1,4, uma lectina glicose/manose, e apresentou massa molecular de 40.000 Da por cromatografia de filtração a gel. CrataBL imobilizada em Sepharose CL-4B adsorveu bioseletivamente caseína, fetuína e ovoalbumina.

Conclusões:

A lectina da entrecasca de C. tapia é uma glicoproteína termoresistente que quando imobilizada em Sepharose CL-4B pode ser utilizada como matriz de afinidade para isolamento de glicoproteínas.

Apoio Financeiro: CNPq

Data de Apresentação: 01/06/2007

ANEXO 4

Guia para Autores

BIORESOURCE TECHNOLOGY

Guide for Authors

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Varon D., Schulman S., Dardick R., Barzilal A., Bashart D. & Martinowitz U. (1994) High versus ultra-high purity Factor VIII for the treatment of previously untreated patients with haemophilia A. *New England Journal of Medicine* **328,** 453-459. References in the text should use the Harvard system and be in full on first mention, e.g. (Brown, Smith & Williams 1978), but subsequently abbreviated to (Brown *et al.* 1978), unless there are more than three authors to a paper, in which case *et al.* should be used throughout. Authors are responsible for the accuracy of their references.
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5. General Instructions

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References to books should include the author's name, title of article, editors, title of book, edition, city where the publisher is located, publisher, year of publication, and initial and final pages of the cited article or section.

e.g., Margineanu DG, Klitgaard H. Levetiracetam: mechanisms of action. In: Levy RH, Mattson RH, Meldrum BS, Perucca E, editors. Antiepileptic drugs. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2002. p. 419 – 427.

For formats of other published materials, please refer to the Vancouver style (see the "References" section in the *"Uniform Requirements for Manuscripts Submitted to Biomedical Journals"* http://www.icmje.org/index.html).

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