MONISE VIANA ABRANCHES

PURIFICAÇÃO E CARACTERIZAÇÃO PARCIAL DE LECTINA OBTIDA DE COUVE-FLOR (*Brassica oleracea* var. Botrytis*)* E POTENCIAIS AÇÕES BIOLÓGICAS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para a obtenção do título de *Doctor Scientiae*.

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LISTA DE ABREVIATURAS E SÍMBOLOS

CRD: carbohydrate recognition domain

DMSO: dimethyl sulfoxide

DRC: domínio de reconhecimento de carboidratos

Galβ1-3GlcNAc: Gal, galactose; GlcNAc, N-acetylglucosamine

H_2O_2: peróxido de hidrogênio

HepG2: linhagem celular de carcinoma hepatocelular humano

IFNγ: interferon-gamma

IL-12: interleukin 12

kDa: é uma unidade para massa molecular ou massa de uma banda particular em um gel de separação que equivale a 1.000 daltons (Da) ou 1.000 g/mol

MAPKs: mitogen-activated protein kinases

MCF-7: linhagem celular de carcinoma ductal invasivo de mama. Acrônimo de Michigan Cancer Foundation-7.

MDA-MB-231: linhagem celular de adenocarcinoma de mama

Mg: magnésio

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

NO^-: óxido nítrico

^oC: grau Celsius

PNA: peanut seed lectin

PNGase: peptídeo-N-glicosidase

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Eletrophoresis

TLR2: Toll-like receptor 2

TLR4: Toll-like receptor 4

TNFα: Tumor necrosis factor

Zym: Zymosan

RESUMO

ABRANCHES, Monise Viana, D.Sc., Universidade Federal de Viçosa, abril de 2013. **Purificação e caracterização parcial de lectina obtida de couve-flor (*Brassica oleracea* var. Botrytis) e potenciais ações biológicas**. Orientador: Leandro Licursi de Oliveira. Coorientadora: Silvia Almeida Cardoso.

As lectinas são proteínas capazes de interagir de forma seletiva e reversível com carboidratos. As interações proteína-carboidrato estão envolvidas em muitos processos biológicos, atuando como mediadoras de várias atividades celulares, que incluem: interação célula-célula, célula-matriz, controle do crescimento celular e a apoptose. Além disso, tais interações estão implicadas no desenvolvimento de doenças. Adicionalmente, as lectinas podem promover a eliminação de agentes não próprios pela opsonização e parecem ativar a resposta imune inata. Nesse estudo foi purificada e parcialmente caracterizada uma lectina de couve-flor (*Brassica oleracea* var. Botrytis). A atividade dessa proteína contra a viabilidade de células tumorais (MDA-MB-231, MCF-7 e HepG2) e sobre a ativação de macrófagos foi avaliada. O processo de purificação da lectina envolveu três etapas cromatográficas (por afinidade em coluna Hitrap Blue HP, por troca catiônica em coluna Hitrap CaptoS e exclusão por peso molecular em coluna Sephadex 200 HR 10/30). Sua massa molecular foi estimada por SDS-PAGE sob condições redutora e não redutora, bem como após tratamento com PNGase. A atividade da proteína sobre eritrócitos humanos e de carneiro, em diferentes temperaturas e valores de pH, na presença de diferentes cátions bivalentes, e sua capacidade de se ligar a diferentes carboidratos foi investigada. A sequência de aminoácidos da porção N-terminal da proteína foi analisada por degradação de Edman em sequenciador automático de proteína (modelo PPSQ-33A, Shimadzu). A viabilidade das células tumorais na presença da lectina pelo ensaio com MTT. Adicionalmente, foi realizado ensaio de fagocitose por macrófagos e estimada a produção de H_2O_2 e NO^- sob a influência da lectina. As etapas cromatográficas proporcionaram uma purificação de 139 vezes. A análise por SDS-PAGE em condições redutora e não redutora revelou uma única banda estimada em 34 kDa. Não foi observada diferença de massa quando a lectina foi previamente tratada com PNGase. Observou-se que a proteína foi capaz de

hemaglutinar eritrócitos de humanos (Grupos O e B) e de carneiro. A atividade máxima da proteína se manteve até 60 ^{o}C e entre os valores de pH 7 e 8. A lectina apresentou especificidade de ligação a carboidratos complexos (fetuína e asialofetuína) e teve sua atividade hemaglutinante melhorada pelo Mg. A sequência N-terminal da lectina encontrada foi ETRAFREERPSSKIVTIAG. A lectina reduziu a viabilidade das linhagens MDA-MB-231 e HepG2, mas o mesmo não foi constatado para a linhagem MCF-7. Na presença da lectina a fagocitose foi estimulada, bem como a produção de H_2O_2 e NO^-. É sugerido que a proteína estudada é uma nova lectina que pode inibir a proliferação das células MDA-MB-231 e HepG2. Além disso, essa lectina também pode ser explorada como agente imunoestimulador, capaz de auxiliar a imunidade inata, favorecendo a remoção de antígenos não próprios.

ABSTRACT

ABRANCHES, Monise Viana, D.Sc., Universidade Federal de Viçosa, April, 2013. **Purification and partial characterization of lectin obtained from cauliflower (*Brassica oleracea* var. Botrytis) and potential biological actions**. Adviser: Leandro Licursi de Oliveira. Co-adviser: Silvia Almeida Cardoso.

The lectins are proteins that interact selectively and reversibly with carbohydrates. Protein-carbohydrate interactions are involved in many biological processes, such as mediator of several cellular activities including cell-cell and cell-matrix recognition, control the cellular growing and apoptosis. Moreover, these interactions are implicated in the development of diseases. Additionally, the lectins can promote foreign agents elimination by opsonization and it seems activating the innate immune response. In this study, a lectin of cauliflower has been purified and partially characterized (*Brassica oleracea* var. Botrytis). In addition, the protein activity against the viability of tumor cells (MDA-MB-231, MCF-7 e HepG2) and on macrophages activation has been assessed too. The lectin purification involved three chromatographic steps (FPLC-affinity on a Hitrap Blue HP column, FPLC-cation exchange on a Hitrap CaptoS column and HPLC-molecular weight exclusion chromatography on a Sephadex 200 HR 10/30 column). The protein molecular mass was estimated by SDS-PAGE under reducing and non-reducing conditions and after PNGase treatment. The protein activity on human and sheep erythrocytes in different temperature and pH conditions, in the presence of divalent cations, and the protein ability to bind different carbohydrate were investigated. The N-terminal amino acid sequence was analyzed by Edman degradation method in the Shimadzu PPSQ-30 Series Protein Sequencer. The viability of tumor cells in the presence of lectin was investigated by MTT assay. Furthermore, it was performed the macrophage phagocytosis assay and it assessed H_2O_2 and NO^- production under influence of lectin. The purification steps have provided a 139-fold purification. The SDS-PAGE analysis in reducing and non-reducing conditions revealed a single band estimated at 34 kDa. It was not observed any difference in the molecular mass when the lectin was previously treated with PNGase. It was noted that the protein was able to cause hemagglutination of

human (O and B groups) and sheep erythrocytes. The protein optimal activity was maintained until 60 ^{O}C and between pH 7 and pH 8. The lectin presented a binding specificity to complex carbohydrates (fetuin and asialofetuin) and the Mg has improved the lectin hemagglutinating activity. The N-terminal sequence of the encountered lectin was ETRAFREERPSSKIVTIAG. The lectin reduced the viability of the MDA MB 231 and HepG2 bloodlines, but it did not reduce the viability of the MCF-7 cells. In the presence of lectin the phagocytosis was stimulated, as well as H_2O_2 and NO^- production. It is suggested that the studied protein is a new lectin that can inhibit MDA-MB-231 and HepG2 proliferation. In addition, this lectin can also be probed like immunostimulant agent, capable to help the innate immunity, favoring the clearance of non-self-agents.

INTRODUÇÃO GERAL

As lectinas são proteínas que possuem a característica de interagir com carboidratos de forma específica e reversível sem, contudo, causar modificações catalíticas sobre as glicanas com as quais interagem, diferente do que acontece em detrimento da ação de glicosidases e glicosiltransferases (Cummings, 2009). O termo lectina é originário da palavra em latim *legere*, que significa selecionar. Ele foi inicialmente utilizado em referência à capacidade de algumas proteínas aglutinarem eritrócitos humanos de um grupo particular do sistema ABO. Entretanto, o termo lectina não é utilizado por alguns autores devido ao fato de algumas proteínas aglutinar diferentes tipos celulares, sendo sugerido o uso do termo aglutinina (Peumans e Van Damme, 1995).

Os carboidratos constituem uma das mais abundantes classes de biomoléculas da Terra (Aurnaut *et al.* 2013). Combinados, os monômeros de carboidratos podem originar uma ampla variedade de glicanas (Zeng *et al.*, 2012). Ao conjunto de glicanas de um organismo, sejam elas mono ou polissacarídeos, livres ou ligados a proteínas ou lipídeos, atribui-se o termo glicoma (Cummings, 2009). Tem sido estimado que aproximadamente 70% das proteínas celulares totais humanas e, mais especificamente, 80% das proteínas presentes na superfície celular são glicosiladas. Portanto, a glicosilação é considerada uma das mais comuns e relevantes modificações pós-traducionais já descritas (Aurnaut *et al.* 2013), responsável pela diversidade de glicanas existentes.

O reconhecimento das glicanas pelas lectinas permite a “interpretação” do “glico código” e consequente sinalização celular por meio dos glicoconjugados (Feizi e Mulloy, 2003; Mariño *et al.*, 2010). A região da proteína onde ocorre o reconhecimento dos carboidratos é chamada domínio de reconhecimento de carboidratos (DRC) e, após essa interação, diversas atividades biológicas vitais são desencadeadas tais como: fertilização, defesa imune; diferenciação, crescimento e sinalização celular; interação célula-matriz extracelular, reconhecimento célula-célula, tráfego celular e sinalização transmembrana (Paulson *et al.,* 2006; Aurnaut *et al.*, 2013; Castanheira *et al.,* 2013). Ainda, as interações lectinas-carboidratos são responsáveis por uma ampla variedade de processos patológicos como: interações patógeno-

hospedeiro, crescimento e metástase tumoral e desenvolvimento de doenças neurológicas (Ghazarian *et al.*, 2011; Clark, 2013; Shin, 2013).

A região da molécula de carboidrato que é alvo de reconhecimento pela lectina é denominada determinante glicana. Evidências atuais indicam que os DRC podem acomodar determinantes glicana constituídos por 2 a 6 monossacarídeos lineares, juntamente com suas cadeias laterais contendo outros açúcares e, ou modificações como sulfatação, fosforilação e acetilação. Isso se deve ao fato de proteína e carboidrato necessitarem de múltiplas interações para alcançar o efeito biológico (Paulson *et al.*, 2006). A importância das pesquisas voltadas à descoberta de determinantes glicana é justificada pela necessidade de compreender os mecanismos fisiológicos e patológicos, bem como obter potenciais alvos para ação de novas drogas (Cummings, 2009).

Com base em sua estrutura, as lectinas são classificadas em: 1) merolectinas (possuem um único DRC, sendo, portanto, incapazes de precipitar glicoconjugados ou aglutinar células); 2) hololectinas (possuem exclusivamente DRC, apresentando dois ou mais domínios idênticos ou homólogos, sendo capazes de aglutinar células e precipitar glicoconjugados); 3) quimerolectinas (possuem um DRC e outro com atividade catalítica ou outra atividade biológica que atua de forma independente ao DRC) e 4) superlectinas, possuem dois ou mais DRC, com especificidade para açúcares diferentes. (Peumans e Van Damme, 1995; Oliveira 2002).

As lectinas estão presentes em toda parte na natureza, podendo ser encontradas desde em vírus até em seres humanos (Sharon, 2008). Nas plantas, elas compõem parte do sistema de defesa contra diferentes tipos de agressores como insetos e micro-organismos, além de reconhecerem sítios de ligação específicos para o estabelecimento da simbiose, principalmente na raiz (Peumans e Van Damme, 1995; Coelho *et al*., 2007).

Técnicas bioquímicas clássicas permitem obter lectinas de plantas purificadas dada a afinidade das mesmas por carboidratos imobilizados em colunas cromatográficas (Sharon, 2008). Atualmente, além do princípio da afinidade, é comum o emprego dos princípios da troca iônica e da exclusão molecular para o isolamento de novas proteínas (Wong e Ng, 2006; Lam *et al.*, 2009). Muitos estudos que visam o isolamento e caracterização de lectinas de

origem vegetal são desenvolvidos a partir da extração das proteínas das sementes, dada a maior concentração dessas proteínas nessas estruturas (Leung *et al.*, 2008; Chen *et al.*, 2009). Não somente a estrutura, mas também a especificidade de ligação das lectinas vem sendo estudadas e identificadas. No Quadro 1 são citados alguns exemplos (Huang *et al.,* 2008; Wu *et al.*, 2009; Swanson *et al.*, 2010).

Quadro 1. Lectinas de plantas e respectivos determinantes glicana.

Lectina	Origem	Determinante glicana
Lectinas que interagem com galactose / N-acetilgalactosamina		
SBA	soja	galactose 3-α-1-N-acetilgalactosamina
Jacalina	jaca	galactose β-1-N-acetilgalactosamina
Lectinas que interagem com Manose		
Con A	feijão	manose, glicose e oligossacarideos N-ligados
LCH	lentilha	manose, glicose e oligossacarideos N-ligados
BanLec	banana	estruturas ricas em manose
Lectina que interage com ácido siálico		
PLC	feijão-da-espanha	fetuína, ácido siálico
Lectina que interage com galactose		
MCL	Melão-de-são-caetano	galatose

A toxicidade das lectinas de vegetais é observada quando alimentos com alta concentração destas proteínas são ingeridos. As lectinas que não são eficientemente degradadas pelas enzimas digestivas e que tem afinidade pela superfície das células epiteliais do intestino podem ser prejudiciais ao organismo, causando náuseas, vômito e diarreia. Em modelos animais, podem provocar alta taxa de renovação celular, hiperplasia intestinal e perda de peso (Miyake *et al.,* 2007).

Embora sejam consideradas um tipo de fator antinutricional, as lectinas de plantas são utilizadas no campo da biologia e da medicina como ferramentas de estudos dos carboidratos simples e complexos, em solução e sobre as superfícies celulares, além de servirem para a caracterização celular. Exemplo disso, é que lectinas isoladas de gérmen de trigo (Valentiner *et al.*, 2003), feijão (Leung *et al.*, 2008; Chen *et al.*, 2009), soja (Valentiner *et al.*, 2003) e banana (Wong e Ng, 2006) podem atuar de forma específica contra as

linhagens L1210 (leucemia), MCF-7 (câncer de mama) e HepG2 (hepatoma). Isso se deve, possivelmente, às alterações dos padrões de glicosilação das superfícies celulares dos diferentes tipos de câncer, que ocorre concomitante às modificações genéticas (Aub *et al.* 1965; Filho *et al.,* 2006; Sharon, 2008). Nos tumores, as alterações nas estruturas das glicanas (como aumento do número de ramificações e adição de ácido siálico) acontecem devido aos distúrbios na expressão e atividade de glicosiltransferases e glicosidases, que atuam nas vias secretórias do retículo endoplasmático granular e do complexo de Golgi (Kuzmanov *et al.*, 2013). Essas evidências tem levado ao uso das lectinas em pesquisas sobre o câncer.

Outra aplicação das lectinas é como ativadoras do sistema imune. Conforme proposto por Coltri *et al.* (2008) essa ação possivelmente ocorre via receptores Toll-like, especialmente Toll-like 2 (TLR2), presentes na superfície de macrófagos e células dendríticas, evidenciado por meio do estímulo com ArtinM, lectina extraída de sementes de jaca. Coltri *et al.* (2008) sugerem que a imunomodulação desencadeada por esta lectina é dependente de carboidratos, em que a lectina interage com N-glicanas ligadas ao sítio de reconhecimento de padrões moleculares associados a patógenos de TLR2. Corroborando com esses achados, Park *et al.* (2010) observaram que quando macrófagos peritoneais de camundongos foram tratados com KML-C (lectina de visco branco coreano - *Viscum album*), ambos os processos de transcrição e tradução de TLR4 foram estimulados, bem como observou-se ativação de macrófagos e produção de Fator de Necrose Tumoral alfa (TNF-α). A interação entre KML-C e TRL4 foi confirmada pela coprecipitação dessas moléculas, indicando que as mesmas podem ser associadas.

Além disso, as lectinas podem ser utilizadas no fracionamento de células em subpopulações biologicamente distintas, como ocorre com o uso de PNA (lectina de amendoim) como marcadores de linfócitos maduros. A aplicação das lectinas vegetais também permite decifrar vias de glicosilação de proteínas, bem como foi adotada pela indústria biotecnológica para a produção de drogas constituídas por glicoproteínas. As aplicações mais recentes das lectinas são seu emprego em *microarrays*, para análise do rendimento da glicosilação de proteínas e do perfil de carboidratos presentes na superfície de diferentes tipos celulares (Sharon, 2008).

Diante do exposto, o isolamento de novas lectinas e o estudo das interações entre estas e os carboidratos tem se tornado foco de pesquisas no campo da proteoglicômica, tendo em vista a necessidade de avaliar a sua aplicabilidade. Todavia, ainda não são descritas na literatura lectinas originárias de vegetais do gênero *Brassica*, um dos gêneros mais cultivados no Brasil, e que tem a couve-flor (*Brassica oleracea* var. Botrytis) como um de seus representantes (Embrapa Hortaliças e Sebrae, 2010).

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ARTIGO

Title: Purification and partial characterization of lectin obtained from cauliflower (*Brassica oleracea* var. Botrytis) and potential biological actions

Short title: Isolation and characterization of a cauliflower lectin

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Author Contributions

Conceived and designed the experiments: MVA, SAC and LLO. Performed the experiments: MVA, NCSC, LNM and GRP. Analyzed the data: MVA, NCSC, LNM, GRP, SOP and LLO. Contributed reagents/materials/analysis tools: SOP and LLO. Wrote the paper: MVA, SAC and LLO.

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Abstract

Lectins are proteins that interact selectively and reversibly with carbohydrates. Protein-carbohydrate interactions is involved in a range of biological mechanisms, i.e., they mediate diverse cellular activities, including cell recognition, growth control, apoptosis and pathologies such as tumor spread. In the field of immunology, lectins favor the elimination of non-self-agents by opsonization mechanism and appear to activate the innate immune response. In this study, Bol lectin was purified from cauliflower (*Brassica oleracea* var. Botrytis). The purification involved three chromatographic steps: affinity chromatography on Blue-sepharose, ion-exchange chromatography on CaptoS and size exclusion chromatography on Sephadex 200 HR 10/30. Bol was a complex carbohydrates-specific lectin, as evidenced by inhibit only with fetuin and asialofetuin. The purified lectin lacks glycosylation and presented as a single 34 kDa band both in reducing and non-reducing conditions on SDS-PAGE. The N-terminal amino acid sequence of the purified lectin was ETRAFREERPSSKIVTIAG. Bol displayed moderate thermo stability, retaining full hemagglutinating activity after heating up to 60^{o}C. It manifested optimal activity at pH 7 and 8. The lectin exhibits antiproliferative activity against some tumor lineages including hepatoma HepG2 cells and breast cancer MDA-MB-231 cells. Additionally, the lectin stimulated phagocytosis and induced the production of H_2O_2 and NO by macrophages. The data presented indicate that the methodology used was efficient to lectin from cauliflower purification. The lectin isolated can activate innate immunity and it potentially can act against cancer.

Keywords: Plant Lectin, Chromatography, Agglutinin, *Brassica oleracea*,

Introduction

Lectins are proteins that bind to carbohydrates in a reversible and selective manner, and the region of protein responsible for this binding is known as carbohydrate recognition domain (CRD) (Castanheira *et al.,* 2013). The recognition of carbohydrates present on the cell surface by lectins produces many biological information that include: differentiation growth and signaling cellular, cell-cell and cell-matrix recognition, immunostimulation, glycoprotein correct folding, apoptosis and diseases, for example, cancer (Feizi and Mulloy, 2003; Mariño *et al.*, 2010; Ghazarian *et al.*, 2011; Zeng, *et al.*, 2012, Arnaud *et al.*, 2013; Clark, 2013).

Although chromatography is a method relatively simple and inexpensive, it allows the isolation of proteins, especially lectins in its biologically active form (Wong and Ng, 2006; Singha *et al.*, 2007; Leung *et al.*, 2008,). Nevertheless, the efficiency of the method varies with the type of biological sample (for example, plants and fungi), columns and solutions employed, among other factors (Konozy *et al.*, 2002; Li *et al.*, 2008).

Lectins from plants have been found especially in seeds (Leung *et al.*, 2008; Chen *et al.*, 2009). In these structures there is a high concentration of proteins that probably defend seeds against aggressors (Peumans and Van, 1995). The structure and ligation specificity with carbohydrates of lectins also has been studied, for example SBA (soybean) interacts with α-1-3 N-acetilgalactosamine-galactose; jacalin (jackfruit) with β-1-galactose-N-acetilgalactosamine; Con A (jack bean) and LCH (lentil) with mannose, glucose and oligosaccharide N-linked (Wu *et al.*, 2009); *Ficus benghalensis* (fig) with galactose β-1, 3-N-acetilgalactosamine β-1, 4-galactose β-1, 4- glucose β (Singha *et al.,* 2007). However, lectins are presents in other plants structures as leaves (Konozy *et al.*, 2002).

Traditionally, plant lectins are considered antinutritional factors, but they exert positive actions as tools in immunology and glycobiology. Their carbohydrate specificity has allowed them to be used in applications as purification and characterization of complex carbohydrates and glycoconjugates, activation of innate immunity and recognition of tumor cells in

biopsies, for example (Loris *et al.,* 1998; Wong and Ng, 2006; Solórzano *et al.*, 2012). In Brazil, vegetables of specie *Brassica oleracea* are most cultivated and the cauliflower (*Brassica oleracea* var. Botrytis) is one kind representative this specie (Embrapa Hortaliças e Sebrae, 2010), whose lectin are not described in the literature. In this study we report protocol to isolation and characterization of a lectin of cauliflower and evaluate its biological effects against tumor cells (MDA-MB-231, MCF-7 and HepG2) and on macrophage activation.

Materials and Methods

Biological material

The cauliflower (*Brassica oleracea* var. Botrytis) was purchased from different suppliers in Viçosa, Brazil. Fresh human blood was obtained from Health Division of the Federal University of Viçosa - UFV. Sheep erythrocytes and BALB/c mice were provided by Department of Veterinary of UFV, Brazil. This study was carried out in strict accordance with Ethical Principles, the protocols were approved by the Committee on the Ethics of Animal Experiments of the Federal University of Viçosa (Permit Number: 21/2012) and by the Committee on the Ethics of Humans of the Federal University of Viçosa (Permit Number:108/2012/CEPH/wmt). The tumor lineages MDA-MB-231 (ATCC HTB-26) - human breast adenocarcinoma cell line; MCF-7 (ATCC HTB 22) - human breast adenocarcinoma cell line and HepG2 (ATCC HB 8065) - human hepatocellular carcinoma were kindly provided by Dr. Fábio Trindade Maranhão Costa (UNICAMP, Brazil).

Preparation of cauliflower extract

The cauliflower were ground and homogenized using a crusher, in phosphate-buffered saline (PBS) (NaH_2PO_4 1.8 mM, Na_2HPO_4 8.1 mM, NaCl 154.0 mM) at pH 7.4 in a ratio of 1:1 (w/v), and then it was kept at 4°C for 8 hours. The mixture was clarified using a cotton filter and subsequently the mixture was centrifuged at 15,560 x *g* (Centrifuge 5804 R eppendorf) for 40 minutes at 4°C. The supernatant was filtered using a filter paper (14 µm pore size, J. Prolab, Brazil), and then was filtered again using a cellulose acetate membrane (0.45 µm pore size, Schleicher & Schull, German) to obtain the cauliflower crude extract. This extract was used in the subsequent purification processes.

Hemagglutinating activity assay

Hemagglutinating activity was assayed in a 96-well microtiter U plate (NUNC, Denmark) according to a two-fold serial dilution using 2% (w/v) untreated erythrocyte suspension in PBS (pH 7.4). The lectin solution (50 µl)

was mixed with 50 µl of erythrocyte suspension and incubated at room temperature for 1 h. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, is reckoned as one hemagglutination unit (Wang *et al.*, 2000). The haemagglutinating activity was tested on human and sheep erythrocytes.

Lectin purification

The cauliflower crude extract were loaded on a Hitrap Blue HP (GE Healthcare, China) column (0.7 cm x 2.5 cm) which had previously been equilibrated with 10 mM Tris-HCl buffer (pH 7.4) at a flow rate of 1,0 mL/minute. After the unbound proteins had been washed with the same buffer, bound proteins with hemagglutinating activity were eluted with 1M NaCl in 10 mM Tris-HCl buffer (pH 7.4), dialyzed and then subjected to ion exchange chromatography on a Hitrap Capto S (GE Healthcare, China) column (0.7 cm x 2.5 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4) at a flow rate of 1mL/minute. After the unbound proteins had been washed, bound proteins with hemagglutinating activity were eluted with 100 mM NaCl in 10 mM Tris-HCl buffer (pH 7.4). The lectin fraction was further purified by gel filtration on a Sephadex 200 HR 10/30 (Waters, Ireland) column (7.8 mm x 300 mm) equilibrated with 0.85% (w/v) NaCl at a flow rate of 0.7 mL/minute for the final polishing step. The absorbance in all chromatographic steps was monitored at 280 nm.

Protein concentration determination

The concentration of proteins in the cauliflower crude extract and in the fractions obtained in the purification processes was determined using BCA *Protein Assay* kit (Thermo Scientific, USA) according to manufacturer's instructions.

Molecular mass estimation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by HPLC gel filtration

To determine molecular weight under denaturing conditions the SDS-PAGE was carried out in accordance with the procedure of Laemmli (1970),

using a 15% resolving gel and a 5% stacking gel. Samples were treated under reducing and non-reducing condition, with or without PNGase (Sigma-Aldrich, USA) and boiled at 100°C for 10 min. The gel was stained with 2% (w/v) Coomassie Brilliant Blue G-250. The molecular weight has been determined by migration patterns of PageRuler Prestained Protein Ladder (Pierce Biotechnology, USA). The molecular mass of the native lectin was estimated by gel filtration, using a Protein Pak 300 SW (Waters, Ireland) column (7.8 mm x 300 mm) equilibrated with 0.85% (w/v) NaCl at a flow rate of 0.7 mL/minute that had been calibrated with molecular mass standards (Sigma, USA) to estimate the molecular mass of the purified lectin.

N-terminal Sequencing

The N-terminal amino acid sequence was analyzed by Edman degradation method. Briefly, a band of lectin on the 15% SDS-polyacrylamide gel was electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) for 2 h at 350 V, 100 mA and 4 °C. The electroblotted protein on the PVDF membrane was then stained with Coomassie Blue Brilliant R-250. The N-terminal amino acid sequence of the lectin on the PVDF membrane was analyzed using a Shimadzu PPSQ-30 Series Protein Sequencer, and the phenylthiohydantoin (PTH) derivatives were identified by comparing their retention times to that of the 20 PTH amino acid standards. The N-terminal sequence of the lectin was compared with sequences available in the BLAST database.

Inhibition of lectin-induced hemagglutination by carbohydrates

The hemagglutinating inhibition tests were performed in a same manner to the hemagglutination test. A serial twofold dilution of each sugar samples was prepared in PBS. All of the dilutions were mixed with an equal volume (25 µL) of a solution with 2 hemagglutination units of lectin. The mixture was allowed to stand for 30 minutes at room temperature and then mixed with 25 µL of a 2% sheep erythrocyte suspension. It was calculated the minimum concentration of the sugar which completely inhibited 1 hemagglutination units of the lectin (Wang *et al.*, 2000). Carbohydrates tested were D-arabinose, D-

cellobiose, D-fructose, D-galactose, D-glucose, D-lactose, D-mannose, D-raffinose, D-ribose, D-trehalose, D-xylose, maltose, melibiose, N-acetylglucosamine, saccharose, and glycoproteins asialofetuin, fetuin and casein

Thermal, pH and metal ions stability

Aliquots of lectin in PBS were incubated at different temperatures (4°C – 100°C) for 30 minutes and cooled in ice. The hemagglutinating activity of the aliquots was tested. The pH stability of lectin was measured by dialyzing the aliquots of lectin against the following buffers for 6 h at 4°C: 100 mM glycine buffer (pH 2.0 and 3.0), 20 mM acetate buffer (pH 4.0 and 5.0), 100 mM phosphate buffer (pH 6.0 and 7.0), and 100 mM glycine–NaOH buffer (pH 10.0, 11.0 and 12.0). The pH of the lectin solution was adjusted to 7.0 by the addition of 0.1N HCl or 0.1N NaOH before hemagglutination activity was determined. To determine the metal ion dependence, the protein was dialyzed against 100 mM Tris-HCl, 10 mM EDTA at pH 7.4 for 12 hours. After this period, the lectin was dialyzed again, but now against 100 mM Tris-HCl at pH 7.4, followed by the hemagglutination assay. Additionally, fractions of the dialyzed protein were dialyzed again 50 mM $CaCl_2$, 50 mM $MgCl_2$, 50 mM $MnCl_2$ or 50 mM $ZnCl_2$, followed by the hemagglutination assay.

Growth inhibition assay

Lineages tumor cells (MDA-MB-231, MCF-7 and HepG2) were resuspended in D-MEM (Dubelco`s, USA) culture medium supplemented with 10% fetal bovine serum, 500 units penicillin/mL and 0.1 mg streptomycin/mL, adjusted to a cell density of 10^5 cells/mL and 100 µL these suspension were seeded in 96-well culture plates. Plates were incubated for 24 hours at 37°C in humidified atmosphere with 5% CO_2. The supernatant was removed and different known concentrations of the lectin (10, 100 and 1,000 µg/mL) diluted in 100 µL complete D-MEM medium were added to the wells followed by incubation for 24 hours. After this period, 10 µL of a 5 mg/mL solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in phosphate buffered saline was added into each well, and the plates were incubated for 4

hours. The supernatant was carefully removed, and 100 µL of dimethyl sulfoxide (DMSO) was added in each well. The absorbance at 550 nm was then measured with a microplate reader (BioRad, USA) within 10 minutes (Mosmann, 1983).

Mitogenic activity

Spleens from BALB/c mice were aseptically removed and spleen cells were isolated and resuspended with RPMI 1640 culture medium (Gibco, USA), supplemented with 10% fetal bovine serum, 500 units penicillin/mL and 0.1 mg streptomycin/mL. A 100 µL aliquot of this cell suspension (10^6 cells/mL) was seeded to a well of a 96-well plate, followed by incubation for 24 hours. A range of different concentrations of lectin or Con A (positive control) in 100 µL complete RPMI 1640 medium were then added to the wells followed by incubation for 48 hours. After this period the MTT assay was performed (Mosmann, 1983).

Phagocytic activity of peritoneal macrophages

Macrophages from the BALB/c mice peritoneal cavity were resuspended with RPMI 1640 culture medium (Gibco, USA), supplemented with 10% fetal bovine serum, 500 units penicillin/mL and 0.1 mg streptomycin/mL. A aliquot (200 µL) of this cell suspension (10^6 cells/mL) was seeded to a well of a 6-well plate covered with coverslip, followed by incubation for 2 hours at 37^{o}C in humidified atmosphere of 5% CO_2. A range of different concentrations of lectin in 200 µL complete RPMI 1640 medium were then added to the wells followed by incubation for 30 minutes. After that, a *Pichia pastoris* suspension (1 x 10^6 cells/well) was added and the plates were incubated for 2 hours. The supernatant was removed and 400 µL of 10% formaldehyde in PBS was added. The coverslip were stained with HEMA 3 panoptic dye (Renylab, Brazil) and analyzed by light field optical microscopy (Olympus, Japan).

NO production by peritoneal macrophages assay

Macrophages from the BALB/c mice peritoneal cavity were washed and resuspended with RPMI 1640 culture medium (Gibco, USA) supplemented with

10% fetal bovine serum, 500 units penicillin/mL and 0.1 mg streptomycin/mL. The cells were seeded in a 96-well culture plate (2 x 10^5 cells/well) and incubated at 37^{o}C in humidified atmosphere with 5% CO_2 for 2 hours. The cells were stimulated with a range of different concentrations of lectin or zymozan (2.5 mg/mL) (positive control), followed by incubation for 48 hours. The supernatant was collected and the amount of nitric oxide in the culture medium was determined as describe by Wong and Ng (2006). Briefly, 100 µL aliquot of the supernatant was allowed to react with 50 µL of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthalene-ethylenediamine dihydrochloride) for 10 minutes. A calibration curve was constructed by using sodium nitrite (Dinâmica, Brazil). The absorbance was read a 540 nm with a microplate reader (BioRad, USA). The results were expressed in NO_2^- micromolar.

H_2O_2 production by peritoneal macrophages assay

The assay was conducted as described by Pick and Keisari (1980). Macrophages from the BALB/c mice peritoneal cavity were washed with PBS and resuspended in phenol red buffer (140 mM NaCl, 10 mM potassium phosphate, 5.5 mM dextrose, 0.56 mM phenol red and 0.01 mg/mL peroxidase type II, pH 7.0). Cell aliquots (2 x 10^5 cells/well) were seeded in a 96-well culture plate and they were incubated with a range of different concentrations of lectin or zymozan (2.5 mg/mL) for 1 hour at 37°C in humidified atmosphere with 5% CO_2. The reaction was stopped by the addition of 10 µL/well of 1 M NaOH. The H_2O_2 present in the medium was determined by a colorimetric method, in which the absorbance was read at 620 nm. The results were expressed in H_2O_2 micromolar, based on a calibration curve used H_2O_2.

Statistical analysis

Data analyses were performed using Graph Pad Prim software version 5.0. The ANOVA test was used to test for differences between the groups. Dunnett's test was used to analyze differences between mean values of groups and control and differences were considered statistically significant at $P < 0.05$.

Results

Protein Purification

Purification of the cauliflower lectin involved initial extraction in PBS (pH 7.4) and three-step chromatography including affinity chromatography, ion-exchange chromatography, and gel filtration. Fractionation of the crude extract using Hitrap Blue HP revealed the presence of a slightly smaller adsorbed fraction designated as FI (Figure 1A). This fraction presented hemagglutinating activity and it was subsequently applied on Mono S column, by means of which a fraction designated as FII (Figure 1B) was obtained. The adsorbed fraction with hemagglutinating activity was resolved into a large peak (FIII) by gel filtration (Figure 1C). The purified lectin, represented by FIII, appeared as a single peak of 28 kDa in gel filtration on Protein Pak column (Figure 1D) and a single band with a molecular mass of 34 kDa in SDS-PAGE (Figure 1E). It was not observed any difference in the molecular mass when the protein was previously treated with PNGase (data not shown). The lectin was obtained with a specific activity of 2,370 HA/mg of protein, a 139-fold purification and a recovery of 12% (Table 1).

Biochemical characterization

The N-terminal sequence of the lectin founded was ETRAFREERPSSKIVTIAG. The novel protein has named Bol, “Bol” represents the specie, *Brassica oleracea*. The hemagglutinating activity of Bol (2.7 µg/mL) could not be inhibited by any of the sugars tested (D-glucose, D-galactose, D-arabinose, D-xylose, N-acetyl-glicosamine, D-fructose, D-mannose, D-ribose, melibiose, maltose, D-lactose, D-cellobiose, D-trehalose, sacarose, and D-raffinose), but it was inhibited by complex carbohydrates asialofetuin and fetuin at 7,8 µg/mL and 15,6 µg/mL, respectively (Table 2). The lectin hemagglutinating activity at 350 µg/mL was completely stable between 4^{o}C and 60^{o}C, but considerable loss in activity occurred starting at 70^{o}C to 100^{o}C (Figure 2A). Bol (at 350 µg/mL) exhibited remarkable stability over the entire range of pH 7–8 (Figure 2B) and in the presence of Mg^{+2}, but the activity was affected by Mn^{+2} (Figure 2C). The protein showed hemagglutinating activity

against human erythrocytes of blood groups B and O, as well as sheep erythrocytes at 175.0 µg/mL, 10.9 µg/mL and 2.7 µg/mL, respectively (Figure 2D).

Biological Assays

The activity of BoI against HepG2, MDA-MB-231 and MCF-7 cells is shown in Figure 3. The lectin displayed inhibition of proliferation toward HepG2 cells at 100 µg/mL, while its concentration toward MDA-MB-231 was 1,000 µg/mL, but the protein did not inhibit the proliferation of MCF-7 cells at concentrations tested.

The lectin did not stimulate the mitogenic response of mouse splenocytes (Figure 4). However, it stimulated the phagocytosis of yeast by peritoneal macrophages on 50 µg/mL of lectin (Figures 5A and 5B), as well as increased nitric oxide production (Figure 5C) and hydrogen peroxide (Figure 5D) by mouse macrophages.

Discussion

The isolation, purification, characterization and biological applications of plant lectins have been the focal point of many studies in the last years (Oliveira *et al*, 2008; Sharma *et al.,* 2009; Swanson *et al.*, 2010). Some therapeutic applications have been proposed for them, including cancer therapy (Castanheira *et al.,* 2013). In the present work, we describe an efficient and simple method for extraction and isolation Bol, a highly purified lectin from *Brassica oleracea* var. Botrytis. The protocol used in the purification was divided in three successive chromatography stages. Similar to Anasazi bean lectin (Sharma *et al.,* 2009), Bol lectin was adsorbed on Affi-gel blue gel as well on Capto S. The chromatographic process allowed the recovery of 12% of the total hemagglutinating activity present in the crude extract and 139-fold purification. Lam *et al.*, (2009) used ion-exchange chromatography and gel filtration to purify *Capparis spinosa* lectin seeds also obtained high purification fold (100-fold) this protein. Wong e Ng (2006) purified emperor banana lectin using the same chromatographic steps of the present study. The autors reported recovery of 20% of the hemagglutinating activity.

The homogeneity of the Bol lectin preparation was evidenced by the presence of a single band in SDS-PAGE and a single pick on the filtration gel. The results of SDS-PAGE and gel filtration chromatography together revealed that the lectin exists as a single unit. The molecular weight of Bol was estimated at 34 kDa by SDS-PAGE and 28 kDa by filtration gel. These results suggest that Bol might be a compact form due to its disulfide bonds. The similar divergences between data of molecular weight determined were also found for Castanheira *et al.* (2013), who studied lectin from *Bothrops pauloensis* snake venom and Konozy *et al.* (2002), who studied lectin from *Erythrina indica* leaf. It was observed that lectins from diverse sources lacked primary sequence similarity, but shared similarities in their tertiary structures (Ghazarian *et al.* 2011). The N-terminal sequence of the lectin (ETRAFREERPSSKIVTIAG) was distinct from sequences available in the BLAST database.

The lectin remained moderately stable when submitted at different temperatures or pH, the same as occurs with other plant species lectins, for

example soybean - *Glycine max* (Lin *et al.*, 2008) e feijão - *Phaseolus coccineus* (Chen *et al.*, 2009). These characteristics increase the applications of the lectin in studies. Furthermore bivalent cation Mn^{+2} decreases Bol hemagglutinating activity, this activity was not inhibited by mono-, di- or tri-saccharides like other lectins (Oliveira *et al.*, 2008; Leung *et al.*, 2008), but complexes carbohydrates structures can inhibit the hemagglutination. The inhibition of the hemagglutinating activity by polysaccharides was also observed by Li *et al.* (2008), who found that inulin (a fructose polymer) inhibits the action of the lectin obtained from *Pleurotus citrinopileatus,* a mushroom. These data suggest that Mn^{+2} affect the ligation of the lectin with the glyconjugates.

Additionally, Bol showed specificity for agglutinating native erythrocytes from sheep and blood groups O and B human. Otherwise, human erythrocytes from blood group A were not agglutinated by our lectin at dose up to 350 µg/mL. Similarly, Konozy *et al.* (2002) also observed that *Erythrina indica* leaf lectin has a slight preference for the O blood group. This hemagglutinating property of lectins until now has been described as a result of lectin binding to carbohydrates to the erythrocyte surface. Carbohydrates are of great importance for preventing natural adhesion between these cells due to the repulsion resultant from negative charges exerted by the sugars. When negative charges are neutralized by the binding of lectins, hemagglutination occurs. Probably, Bol was not capable of agglutinating human erythrocytes from blood group A because of the different composition of surface. The literature describes that disaccharide N-acetyllactosamine (Galβ1-3GlcNAc: Gal, galactose; GlcNAc, N-acetylglucosamine) participate of glycoconjugate structure on human erythrocytes surfaces and on human erythrocytes of blood group O, they are the outer portion of complex carbohydrates (Cummings, 2009). Additionally, these sugars also are present in end portions of asialofetuin and fetuin structures, as well as this disaccharide is recognized preferably by of Jacalin (Kabir, 1998), peanut agglutinin (PNA) (Rogerieux, 1993), *Erythrina cristagalli* and *Ricinus communis* (Iskratsch *et al.,* 2009). Singha *et al.* (2007) reported that lectin from the seeds of *Ficus benghalensis* fruits can isolated by affinity chromatography on fetuin-agarose column and that this lectin could accommodate the tetrasaccharide Galβ1,3GalNAcβ1,4Galβ1,4Glcβ. This data

suggest a potential determinant glycan to Bol, but this hypothesis should be confirmed by further studies.

It is well established that a common characteristic of oncologic malignancies is glycosylation pattern modified (Rêgo *et al.,* 2013). These patterns have been observed in almost all types of experimental and human tumors (Kuzmanov *et al.*, 2013). It's relevant to highlight that the glycosylation is one of the most frequent and important post-translational modifications of proteins (Nunes *et al.*, 2012) and that increase of branching N-glycans and sialylation are changes associated with cancer (Aarnoudse *et al.,* 2006). In Brazil, the breast cancer is the type of cancer most incident in woman (Brasil, 2009) and the first cause of women cancer related deaths worldwide (Rêgo *et al.*, 2013) The hepatocellular carcinoma is a leading cause of cancer-related death worldwide, and the burden of this devastating cancer is expected to increase further in coming years (Venook *et al.* 2010). These data were considered to choose the lineage cells used in this work.

The lectin isolated from the cauliflower was characterized by its potential anti-proliferative effect on HepG2 and MDA-MB-231 lineages, but not on MCF-7 cells. These differences observed in this study, maybe is due to observed changes in the glycosylation pattern of each cell type (Peiris *et al.*, 2012). Peanut seed lectin, for example, is widely used to identify tumor-specific antigens on the eukaryotic cell surface (Ortíz *et al.,* 2000), which may also be evaluated for lectin studied. Similar to that found in the present study, Lin and Ng (2008) and Lam *et al.* (2009) demonstrated that lectin purified from *Bauhinia variegate* seeds and *Capparis spinosa* seeds inhibited the HepG2 proliferation, respectively.

Biological activities of lectins include cellular agglutination and cellular growing inhibition. When interacting with cell surface glycoconjugates, the lectins can promote the cross-linking of adjacent cells, triggering the agglutination process (Pereira, 2005). Some lectins has been demonstrating to induce envenoming, disorganization of actin filaments and apoptosis in different cell types (Pereira, 2005; Liu *et al.*, 2009; Faheina-Martins, 2009; Castanheira *et al.* 2013), however the mechanisms by which they act is not elucidated. It has been suggested that cellular receptors (for example, tumor necrosis factor 1

receptor and CD95/Fas), that modulate the signalization pathway mitogen-activated protein kinases (MAPKs), are glycoproteins, so they can be target by lectins and lead to apoptosis (Kagan *et al.*, 2000; Pereira, 2005; Liu *et al.*, 2009; Faheina-Martins, 2009; Hanahan e Weinberg, 2011; Nunes *et al.,* 2012). Besides this, some lectins may act by its internalization and afterward lysosomal proteases liberation (Faheina-Martins, 2009; Hamshou *et al.*, 2012). These reports suggest the potential effect caused in the MDA-MB231 and HepG2 cells by Bol.

In the immune system, the lectins are important components of the host innate defense since they direct the foreign agent elimination, by act in opsonization process and facilitate the phagocytosis by dendritic cells and macrophages, which activates the adaptive immune response (Ghazarian *et al.*, 2011). The studies led by Lin and Ng (2008) and Li *et al.* (2008) showed that lectins from *Bauhinia variegata* seeds and *Pleurotus citrinopileatus*, respectively favored leucocytes mitosis, different of what was found in the present study. However, the immunostimulatory effect triggered by Bol was evidenced by its capacity of stimulate the phagocytosis and the NO and H_2O_2 production by peritoneal macrophages. Wong and Ng (2006) reported that the banana lectin increased the NO production by macrophages, in a dose-dependent manner, which is similar to the cauliflower lectin results. According to the demonstrated by Coltri *et al.* (2008), a possible mechanism of inflammatory compounds synthesis stimulation by macrophages can be triggered by the interactions of the lectin with TLR2 receptors carbohydrates, which culminates in a larger secretion of IL-12, IFNγ, TNFα and NO^-, due to action of the NFκB nuclear transcriptional factor. Similar results were observed by Park *et al.* (2010) that evaluated the action of Korean mistletoe lectin on macrophage activation via TLR4.

The data presented indicate that methodology used allowed lectin purification in efficient and simple manner. The novel lectin isolated from cauliflower can act against lineage of cancer cell lines (HepG2 and MDA-MB-231), as well as act as it can also be probed like immunostimulator agent, capable to help the innate immunity, favoring the clearance of non-self-agents. All these data indicate potential activities to be explored using this lectin. The

future of this search will include elucidating the activation mechanism of macrophages and of reduction process of cancer cells viability.

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Figures and legends

Figure 1

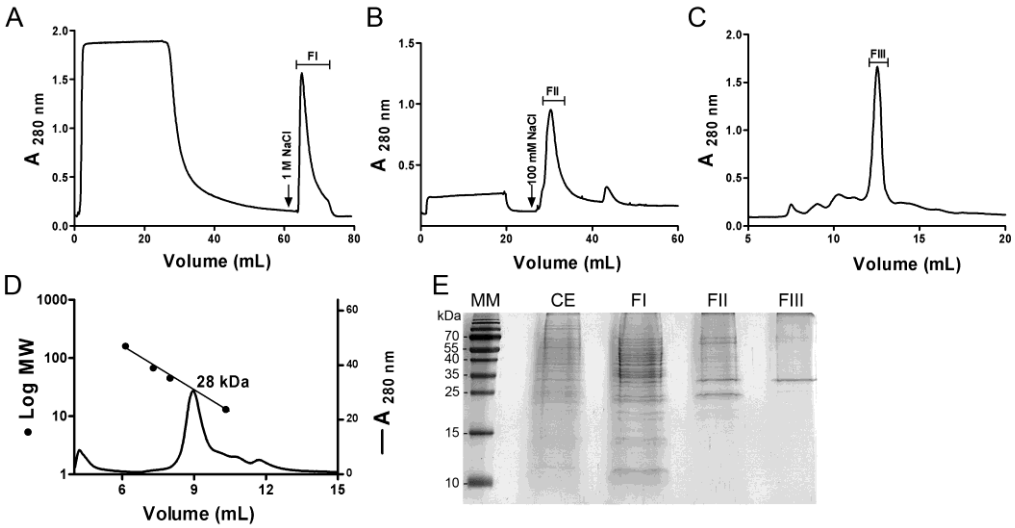

Fig. 1. Chromatography profile of cauliflower lectin purification. (A) Affinity chromatography on a Hitrap Blue HP column. **(B)** Ion exchange chromatography on a Hitrap CaptoS column. **(C)** Molecular weight exclusion chromatography on a Sephadex 200 HR 10/30 column. All elutions were monitored at 280 nm. The bar indicates the fractions which had hemagglutination activity. **(D)** Estimation of molecular weight by gel filtration. **(E)** SDS-PAGE of the fractions obtained in the chromatography steps. MM: molecular weight marker, CE: crude extract, FI: fraction obtained by affinity chromatography, FII: fraction obtained by ion exchange chromatography, FIII: fraction obtained by filtration gel.

Figure 2

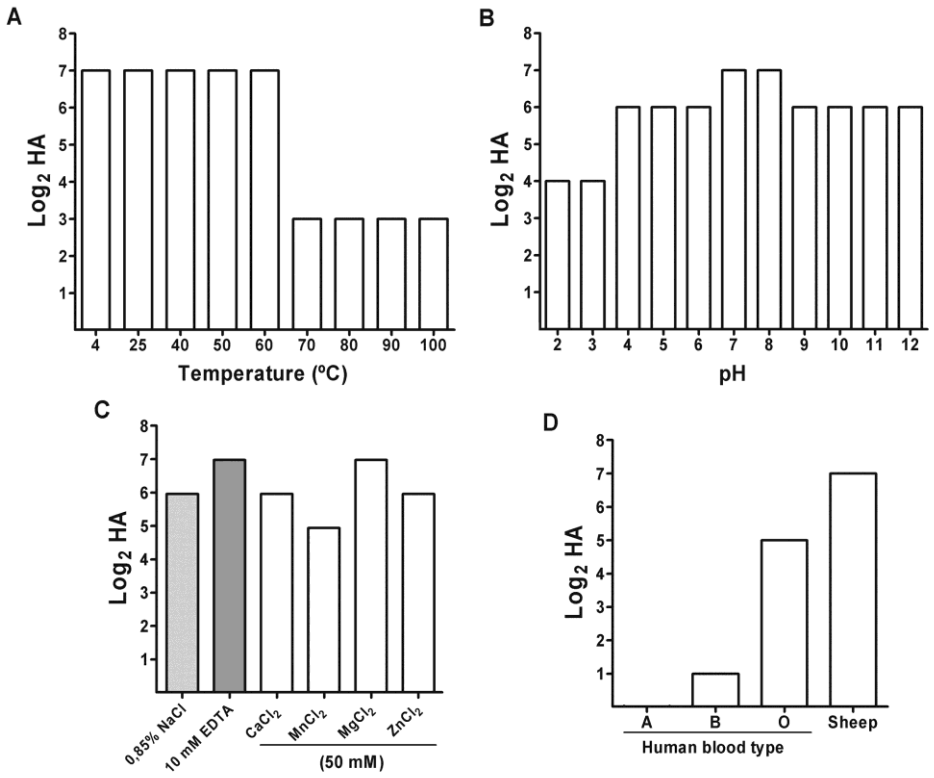

Fig. 2. Physicochemical characterization of the cauliflower lectin. (A) Lectin stability at different temperatures. **(B)** Lectin stability at different pH. **(C)** Metal ion dependence of lectin. **(D)** Specificity of binding to human and sheep erythrocytes.

Figure 3

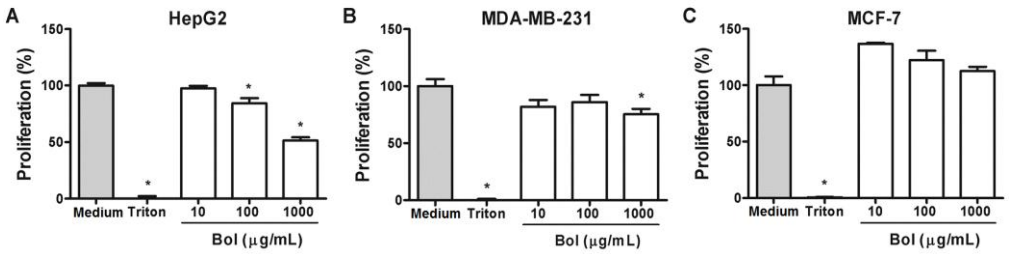

Fig. 3. Antitumor activity. Viability of the cancer cells lineages submitted to treatment with Bol. **(A)** Human HepG2 lineage. **(B)** Human MDA-MB-231 lineage. **(C)** Human MCF-7 lineage. Values normalized according to the medium. Each data position of is an average of three experiments and error bars. *$P < 0.05$.

Figure 4

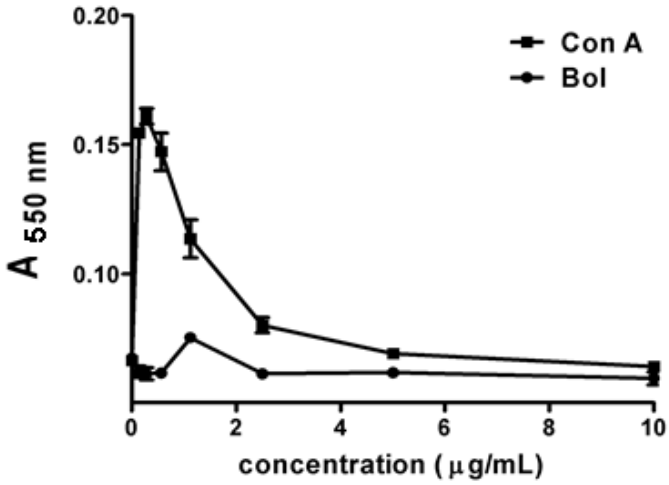

Fig. 4. Mitogenic Activity. Leucocytes obtained from BALB/c mice were treated with Bol. Values normalized according to the medium. Each data position of is an average of three experiments and error bars.

Figure 5

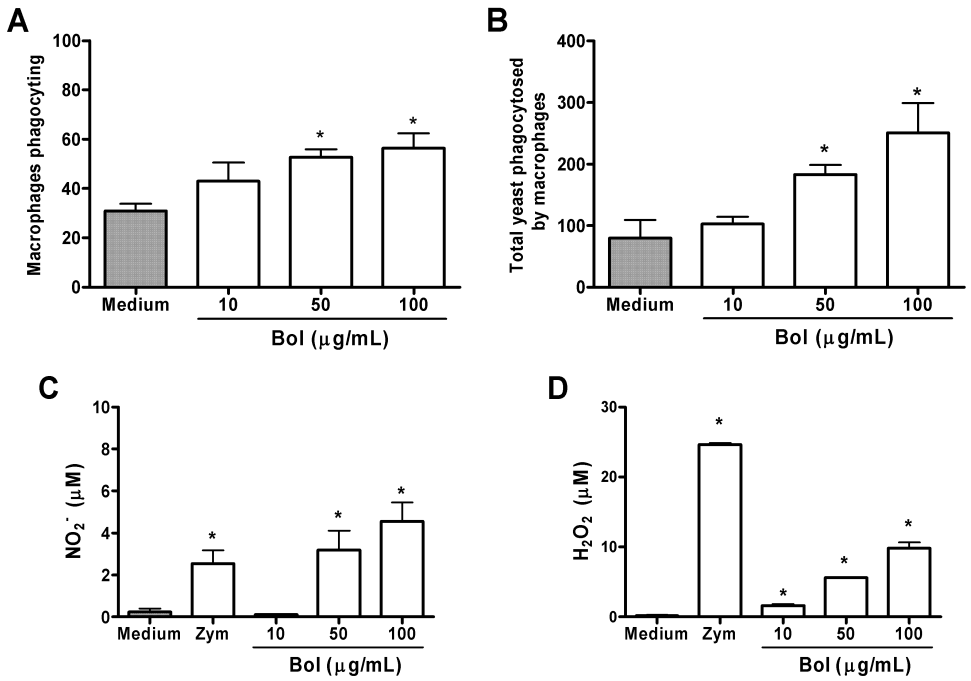

Fig. 5. Macrophages Activation. Peritoneal macrophages of BALB/c mice were treated with the cauliflower lectin. **(A)** Number of macrophages that made phagocytosis in each 200 analyzed macrophages. **(B)** Phagocytosed yeast number in each 200 analyzed macrophages. **(C)** Nitric oxide production by macrophages. **(D)** Hydrogen peroxide production by macrophages. Zym: Zymosan. Each data position of is an average of three experiments and error bars. *$P < 0.05$.

Tables

Table 1. Purification scheme of cauliflower lectin.

Purification steps	Total Protein (mg)*	Total Activity** (HA)†	Specific Activity (HA/mg)	Purification fold	Recovery (%)
Crude extract	10,642.50	180,000	17	1.0	100
Hitrap Blue HP	395.28	43,200	109	6.4	24
Hitrap Capto S	80.28	28,800	359	21.1	16
Sephadex 200 HR10/30	8.91	21,120	2,370	139.4	12

* Protein was measured by the method of BCA

** Measured with sheep erythrocyte

† HA: Haemagglutinating Activity was defined as the reciprocal of the end point dilution exhibiting the hemagglutination.

Table 2. Carbohydrates and glycoprotein effects on the cauliflower lectin.

Inhibitor	mM*	Inhibitor	mM
Lactose	–	Maltose	–
Galactose	–	Mannose	–
Arabinose	–	Saccharose	–
Melibiose	–	Ribose	–
Xylose	–	Trehalose	–
Cellobiose	–	Raffinose	–
N-Acetylglucosamine	–	Casein	–
Fructose	–	Asialofetuin**	7.8
Glucose	–	Fetuin**	15.6

–, There was not hemagglutination activity inhibition.

* Minimal carbohydrate concentration required to inhibit lectin at 2.7 µg/mL.

** µg/ml