Acta Botanica Brasilica, 2024, 38: e20230155
doi: https://doi.org/10.1590/1677-941X-ABB-2023-0155

Original Article

A new lectin from *Crotalaria incana* seeds and studies of toxicity in *Artemia salina* nauplii

Antônio Mateus Gomes Pereira[1,4#], Maria Gleiciane de Queiroz Martins[1#], Messias Vital de Oliveira[2], Claudia Figueiredo Lóssio[2], Elnatan Bezerra de Souza[3], Francisco Fernandes de Araújo[3], Benildo Sousa Cavada[2*], Kyria Santiago do Nascimento[2], João Batista Cajazeiras[1*]

Received: June 22, 2023
Accepted: November 20, 2023

ABSTRACT

Lectins are proteins that recognize and bind to carbohydrates in a reversible and specific manner. In this work, a lectin from *Crotalaria incana* L. seeds was purified by Sephadex G-50 affinity chromatography. The purified lectin was named CiL and presented affinity towards D-mannose, D-glucose, D-galactose, α-methyl-D-mannoside and derivatives. CiL was stable over a wide range of temperatures and pH values, and it was divalent cation-dependent. SDS-PAGE analysis indicated that CiL is composed of two subunits with apparent masses of 29 and 30 kDa. The amino acid sequence of five tryptic peptides was obtained through mass spectrometry. Partial primary structure data indicated the similarity between CiL and lectins from *Phaseolus vulgaris*, *Cladrastis kentukea*, *Lens culinaris*, *Pisum sativum*, *Crotalaria pallida* and *C. juncea*. CiL showed no toxicity to *Artemia salina* nauplii at the concentration of 2 mg/mL, thus reinforcing the potential of this protein for further studies in other biological models and elucidation of possible effects.

Keywords: Toxicity, Lectin, Leguminous, Biological Activity, Affinity Chromatography.

Introduction

Crotalaria incana Linnaeus is a plant species belonging to the Fabaceae family and the *Crotalaria* genus. It is widely distributed on the American, Asian and African continents. In Brazil, *Crotalaria incana* is popularly known as guizo-de-cascavel, xique-xique and crotalaria (Aremu *et al.* 2012; Silva-López & Pacheco 2010). It is an erect, branched subshrub with pubescent target stems capable of reaching up to 1.5 m

1 Núcleo de Bioprospecção e Experimentação Molecular Aplicada (NUBEM), Centro Universitário INTA - UNINTA, Sobral, 62050-100, Ceará, Brazil
2 Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza, Brazil
3 Centro de Ciências Agrárias e Biológicas, Herbário Professor José de Abreu Matos, Universidade Estadual Vale do Acaraú, Campus Betânia, 62040-370, Sobral, Ceará, Brazil
4 Programa de Pós-Graduação em Biotecnologia - PPGB- RENORBIO, Universidade Estadual do Ceará, Fortaleza, CE, Brazil

[#]These authors contributed equally to the article.
[*]Corresponding authors: bscavada@ufc.br; jcajazeiras@gmail.com

in height, but usually measuring between 50 and 120 cm. *C. incana* presents trifoliate compound alternate leaves, with pubescent petiole, and axillary inflorescences in racemes of light yellow flowers, typically measuring between 10 and 20 cm. Its fruit can be described as an oblong, inflated, tomentose-pubescent pod 35 to 40 mm in length. When shaken near dehiscence, it emits a sound similar that of a rattlesnake, and each plant pod has, on average, 25 reniform seeds (Santos *et al.* 2019).

Lectins are present in various organisms from viruses to mammals, but they are found in greater abundance in the Plant Kingdom, mainly in seeds of plants belonging to the Fabaceae family from which many of these proteins have been purified and tested, both *in vitro* and *in vivo*. Such testing has shown antibacterial, antifungal, antiviral, insecticidal, anthelmintic, anti-inflammatory and anti-glioma activity (Sano & Ogawa 2014; Osterne *et al.* 2017; Hasan *et al.* 2021). Lectins comprise a group of proteins of nonimmunological origin and have the ability to selectively and reversibly bind to carbohydrates or glycoproteins without altering their structures (Van Damme *et al.* 1998).

Plant lectins are a heterogeneous group of proteins that present diverse biochemical and physicochemical properties, evolutionary relationships, structural characterizations, carbohydrate specificities and biological activities (Cavada *et al.* 2018). Research involving lectins from the *Crotalaria* genus has aroused scientific interest because these proteins have not been extensively bioprospected. Only a few studies in the literature have reported the purification and characterization of lectins from this genus. Some of these lectins include those from *Crotalaria juncea* (Ersson 1977), *C. striata* (Khang *et al.* 1990), *C. pallida* (Rego *et al.* 2002) and *C. paulina* (Pando *et al.* 2004). Thus, the present work aimed to purify and characterize a new lectin from the seeds of *Crotalaria incana* L., potentially identifying novel biological activities of possible therapeutic interest.

Materials and methods

Botanical material

Crotalaria incana L. was collected in the city of Alcântaras, which is located in the northern part of Ceará State in northeastern Brazil. An exsicata was prepared and identified at the Herbarium Professor José de Abreu de Matos (HUVA) at the Vale do Acaraú State University (UVA) in Sobral, Ceará, Brazil, and incorporated into the HUVA collection under accession number 21639.

Protein extraction

The lectin of *Crotalaria incana* L. (CiL) was extracted according to protocols performed by Benevides *et al.* (2012) and Araripe *et al.* (2017), but with modifications. *C. incana* seeds were peeled and ground in a coffee grinder (Cadence MDR301 Monovolt) to obtain a fine powder. To extract total soluble protein, 150 mM NaCl were added to the powder in the proportion of 1:10 (w/v). The suspension was kept under constant agitation for 4h at room temperature (25 °C), followed by centrifugation at 10,000 x g, 20 min, 4 °C. The supernatant was collected, filtered on Whatman™ filter paper and named crude extract. The residue was discarded.

Protein and carbohydrate content

Total soluble protein content was determined following (Bradford 1976). Bovine serum albumin (BSA) was used as standard, and absorbance was monitored at 595 nm (nanometers) in a visible light spectrophotometer (GE Healthcare Ultrospec™ 2100 pro UV/Visible). Carbohydrate content of the lectin was determined according to (DuBois *et al.* 1956), and lactose was used as standard.

Hemagglutinating activity and sugar inhibition assay

Both hemagglutinating activity and sugar inhibition assay were performed with a 3% rabbit erythrocyte suspension prepared in 150 mM NaCl, which was treated with trypsin and papain or kept in native condition. To determine hemagglutinating activity, 50 µL of samples were diluted in triplicate in a 96-well polystyrene plate containing 50 µL of 150 mM NaCl saline with 5 mM $CaCl_2$ and 5 mM $MnCl_2$. Afterwards, 50 µL of erythrocytes were added to the plate, which was subsequently incubated at 37 °C for 30 minutes, followed by incubating for another 30 minutes at room temperature. Hemagglutination titer was expressed as hemagglutination unit (H.U.), which is typically determined by the highest dilution that shows hemagglutination. After establishing the H.U., the specific activity [H.U./mg of protein (mgP)] of carbohydrate content for CiL was determined by the minimum inhibitory concentration (MIC) of the sugar or glycoprotein capable of inhibiting hemagglutinating activity. To accomplish this, solutions of sugars and glycoproteins were prepared at 100 mM and 2mg/mL, respectively: D-glucose, D-galactose, L-rhamnose, D-ribose, D-mannose, α-lactose, α-methyl-D-mannoside, N-acetylglucosamine, L-fucose, deoxyribose, inosine, mucin and ovalbumin. The results of H.U. titers in the physicochemical characterization of the lectin were represented in graphs by the average values of the replicates (Nascimento *et al.* 2012).

CiL purification

CiL purification was performed in a single-step affinity chromatography on a Sephadex G-50® matrix (15 x 50 mm Sigma-Aldrich, St. Louis, MO, USA). The crude extract applied to the matrix corresponded to one-third of the column volume (empty space), with an approximate concentration of 29 mg/mL. This crude extract was applied to the column previously equilibrated with 0.15M NaCl and kept in contact overnight. Non-binding proteins (PI) were removed with the same equilibration solution, while binding proteins (PIII) were eluted with 100 M glycine buffer, pH 9.0, containing

calcium and manganese at a concentration of 5 mM and 150 mM NaCl at a flow rate of 1 mL/min. Absorbance at 280 nm was used to monitor the chromatographic fractions using a spectrophotometer (Amersham Biosciences; Ultrospec 2100 Pro). PIII was accumulated, dialyzed against distilled water, and then lyophilized.

SDS-PAGE and size exclusion chromatography

Purity and apparent molecular weight of CiL were monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as adapted from the method described by Laemmli (1970). To accomplish this, 12 µL (4 mg/mL) of protein and 4 µL of BenchMark (ninex) commercial molecular marker (MM) were applied in discontinuous SDS-PAGE (12.5% separation gel and 4% stacking gel). Electrophoresis was conducted in a Mini-Protean II mini-gel system (Bio-Rad; Milan, Italy) under the following parameters: 25 mA, 50 V, and 10 W. After electrophoresis, the gel was stained with colloidal Coomassie Blue G-250 in order to observe bands corresponding to possible proteins.

To verify the purity profile of the purified lectin, size exclusion chromatography coupled to an ultra-performance system (UPLC) was performed. A 250 µL of the lectin at 2 mg/mL concentration was applied to the BioSuite ™ 250, 5 µm HR SEC column (7.8 × 300 mm) previously equilibrated with 100 mM sodium phosphate buffer pH 7.0. Lectin elution occurred in isocratic mode monitored at 280 nm with a flow rate of 250 µL/min.

Physicochemical characterization of CiL

To characterize physicochemical properties, a solution of CiL was prepared at 2mg/mL in 150 mM NaCl. To verify the influence of pH on CiL through the hemagglutinating activity test, the lectin was diluted in different buffers at 100 mM containing 150 mM NaCl: sodium acetate, pH 4.0 and pH 5.0; sodium citrate, pH 6.0; Tris-HCl, pH 7.0 and pH 8.0; and Glycine, pH 9.0 and pH 10.0. Thermostability was checked by incubating CiL at different temperatures from 30 to 100 °C (with 10 °C intervals) for 1 h. Divalent cation dependence was observed by dialyzing CiL for 24 h against 100 mM EDTA containing 150 mM NaCl, followed by dialyzing three times against 150 mM NaCl to remove excess EDTA. Afterwards, hemagglutinating activity was determined in the presence and absence of $CaCl_2$ and/or $MnCl_2$. Before performing the hemagglutination tests, all samples were centrifuged at 9000 x g for 5 min at 4 °C (Osterne *et al.* 2023).

Partial primary structure of CiL by ESI-MS

To determine the amino acid sequence of CiL, bands corresponding to this lectin were excised from the SDS-PAGE. The excised bands were bleached in 100 mM ammonium bicarbonate solution with acetonitrile (1:1 v/v) and dried with acetonitrile. Proteolytic cleavage was performed with a trypsin solution (Promega, Madison, WI, USA) with 50 mM ammonium bicarbonate. The obtained peptides were extracted on formic acid/acetonitrile solution [(5% / 100%) 1: 2 v/v] and dried in SpeedVac (Shevchenko *et al.* 2006). Peptides were then resuspended in 0.1% trifluoroacetic acid (TFA), separated by liquid chromatography (LC) on a BEH300 C18 column (100 µm x 100 mm) using the nanoAcquity™ system (Waters) and eluted at 600 µL/min with acetonitrile concentration gradient (10-85%) containing 0.1% formic acid. The LC system was coupled to the nano-electrospray mass spectrometry (SYNAPT HDMS System). The mass spectrometer was run in positive mode, using a temperature source of 80 °C and capillary voltage of 3.5 kV. Double protonated [Glu1]-fibrinopeptide B (Sigma-Aldrich) ion fragments (m/z 785.84) were used for instrument calibration. For LC-MS/MS analyses, MS/MS was selected from the doubly or triply charged precursor ions, which were fragmented by collision-induced dissociation (CID) with argon gas. Data collected from the ionized peptides were interpreted using Mass Lynx 4.1 software (Waters). Peptides were manually sequenced (*de novo* sequencing) using the PepSeq tool, and their theoretical mass was calculated using the Compute pI/Mw tool from ExPASy (Expert Protein Analysis System). Sequences obtained from the peptides were submitted to similarity analysis against the protein database present in the platform of the National Center of Biotechnology Information (NCBI). For this the program BLAST (Basic Local Alignment Search Tool) (Altschul *et al.* 1997) and the tool Protein BLAST (BlastP) were used. The peptides sequenced from CiL were compared with the protein database using Blastp and the alignment was performed in the program ESPript (Gouet *et al.* 2003).

Analysis of CiL toxicity on artemia nauplii

The toxicity of CiL against artemia was analyzed by preparing a 2 mg/mL lectin solution in seawater collected from Camocim Beach in Ceará-Brazil. For this purpose, 1 g of *Artemia salina* cysts was incubated in one liter of seawater at 28 °C under constant aeration. After 48 h *Artemia salina* that presented good motility when migrating towards the light were collected.

Lectin and seawater solution were added to a 24-well Limbro plate until concentrations of 2, 1, 0.5, 0.250 and 0.125 mg/mL were obtained for a final volume of 1 mL in each well. Then, 10 *Artemia salina* nauplii were added to each well. The experiment was conducted in triplicate. The negative control contained only seawater and 10 nauplii. The positive control differed from the negative control only by the presence of 1% SDS. After 24 and 48 h, the number of dead nauplii was calculated according to (Arruda *et al.* 2013).

Statistical analysis

Results were presented as mean ± standard error of the mean (SEM) in GraphPad Prism. Subsequent results were analyzed by ANOVA, followed by Bonferroni's test. Values of $p < 0.05$ were considered significant.

Results and Discussion

Extraction and purification of CiL

The protein extract was prepared using a 1/10 (w/v) ratio of ground powder from *Crotalaria incana* seeds in 150 mM NaCl. The best hemagglutinating activity was observed when papain-treated erythrocytes were used. The specific hemagglutinating activity of CiL was higher than that found by Pando *et al.* (2004), who reported on *C. paulina* lectin, or that found by Khang *et al.* (1990) for *C. juncea* and *C. pallida* lectins, both of which showed lower specific hemagglutinating activity than that provided by the results of Pando *et al.* (2004).

The lectin was inhibited in the presence of carbohydrates D-glucose, D-galactose, L-rhamnose, D-mannose, α-lactose, α-methyl-D-mannoside, N-acetyl-D-glucosamine and the glycoproteins mucin and ovalbumin. It was not inhibited by any of the other tests (Table 1). Other species of this genus are also specific for several carbohydrates, such as *C. pallida*, which shows affinity for D-galactose and D-raffinose; *C. striata*, which binds to D-galactosamine, D-galactose and N-Acetyl-D-Galactosamine; *C. paulina*, which has affinity for D-galactose and N-Acetyl-D-Galactosamine and *C. juncea*, which binds to D-galactose (Silva Filho *et al.* 2014), thus reaffirming our findings.

The lectin was purified on Sephadex G-50 affinity chromatography. During chromatography, PI and PIII fractions, corresponding to non-retained and retained fractions, respectively, were obtained, both accompanied by SDS-PAGE (Fig. 1A, B). PIII demonstrated hemagglutinating activity corresponding to the pure lectin, which was termed as CiL (Table 2).

CiL purification was verified by a single peak in the molecular exclusion chromatography. SDS-PAGE showed two subunits with approximate weight of 29 and 30 kDa (Fig. 1C). This electrophoresis pattern is similar to that observed in other species of *Crotalaria* with weight of 30 kDa, including *C. paulina*, *C. pallida*, *C. striata* and *C. juncea*. These findings are well within data from other Fabaceae family lectins that present two to four subunits with an average weight of 25 to 30 kDa (Sharon & Lis 1990).

Apparent molecular mass and N-terminal sequence of CiL

The purified lectin was confirmed by 1) molecular exclusion chromatography showing a single peak and 2) SDS-PAGE with two subunits and approximate weight of 29 and 30 kDa (Fig. 1B). For sequence determination by MALDI-TOF-TOF, the SDS-PAGE bands were digested by trypsin, and the sequence was analyzed in ESI-MS, according to the intensity of the spectrum (Fig. 2A). Using the BLAST tool, the peptides obtained from CiL were compared in the database with peptides from other lectins and showed similarity to *Phaseolus vulgaris*, *Cladrastis kentukea*, *Lens culinaris*, *Pisum sativum*, *Crotalaria pallida* and *C. juncea*.

Primary sequence determination was done by MALDI-TOF. The SDS-PAGE band was digested by trypsin. Each peptide was sequenced by manual interpretation, and five peptide sequences, corresponding, on average, to 30% of the total sequence, were obtained. The lectin from *Vatairea macrocarpa* (VML) was selected as the template for assembling the partial primary structure of CiL, corresponding to 24% coverage.

Table 1. Inhibition of hemagglutinating activity of lectin of *Crotalaria incana* L. seeds.

Carbohydrates and glycoproteins	MIC (mM)
D-glucose	12.5
D-galactose	0.39
L-ramnose	50
D-ribose	NI*
D-mannose	1.56
α-lactose	6.25
α-methyl-D-mannoside	1.56
L-fucose	NI*
Deoxyribose	NI*
Inosine	NI*
N-acetyl-D-glycosamine	12.5
Mucine	12.5 mg/mL
Ovalbumin	50 mg/mL

*NI, non-inhibitory sugar; MIC = (minimum inhibitory concentration).

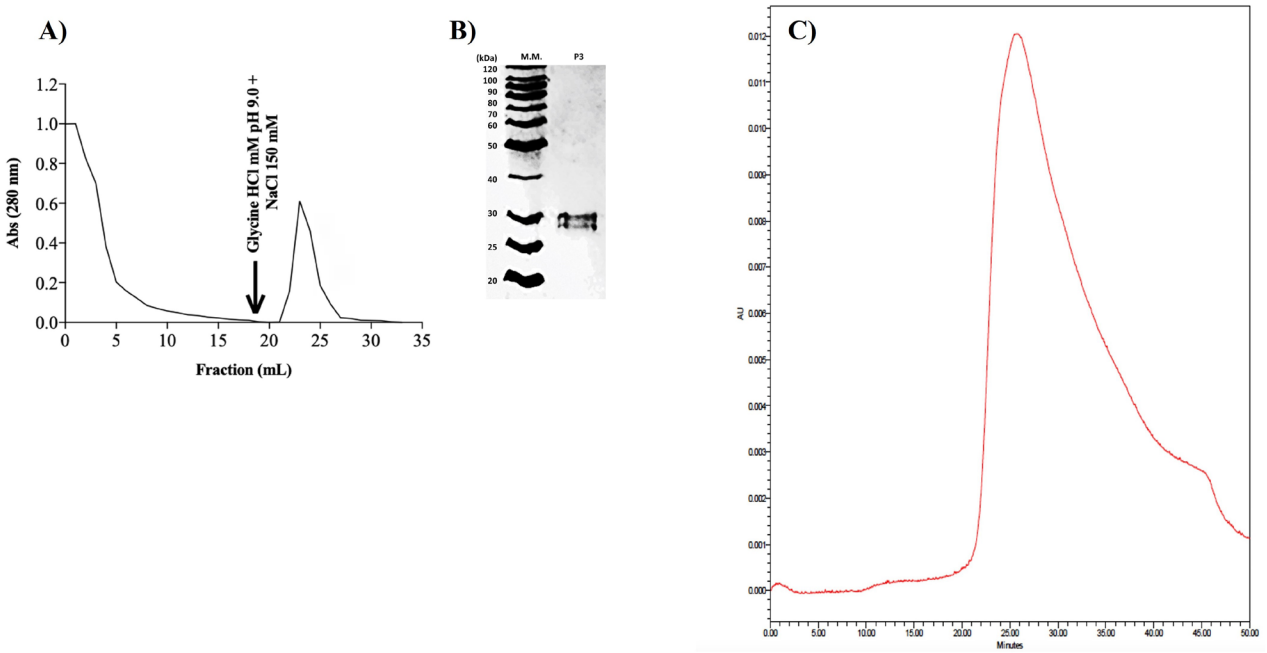

Figure 1. Chromatogram in Sephadex G-50 and electrophoretic profile of CiL. **(A)** Purification of CiL in Sephadex G-50 affinity chromatography. Fractions of 3 mL were collected and monitored at 280 nm. Lectin was eluted with 100 mM Gly solution pH 9.0 with 150 mM NaCl. **(B)** Lane M.M: Promega Broad Range molecular marker (Reference: V849A). P3 corresponds to the lectin fraction (CiL) obtained from affinity chromatography. **(C)** Molecular exclusion chromatography of CiL. Lyophilized lectin was dissolved in 100 mM sodium phosphate buffer pH 7.0 at a concentration of 2 mg/mL and loaded onto the Waters® silica-based BioSuiteTM 250 column. The chromatography was monitored at 280 nm. AU: Absorbance Unit.

Table 2. Purification of lectin from *Crotalaria incana* L seeds.

Fractions	[a]Proteins mg/mL	[b]Total H.A. U.H / mL	[c] Specific activity (U.H./mg)	Purification fold
Crude extract	29.79	64	2.14	1
G-50 (PIII)	1.46	16	10.95	5

[a]Protein content determined by the Bradford method
[b]Hemagglutination titer (H.U.)
[c]Specific activity as a ratio of hemagglutination activity to protein content

Alignment of the partial primary structure of CiL with other plant seed lectin sequences by the Protein BLAST tool showed CiL as having 93.10% of sequence similarity with both *V. macrocarpa* lectin (UniProtKB/Swiss-Prot: P81371) and *V. guianensis* lectin (UniProtKB/Swiss-Prot: P86893) (Fig. 2B).

When compared with the database, the peptide sequences of CiL's primary structure showed homology with lectins of the Crotalarie tribe, Dioclenae tribe and other tribes. Despite their structural similarities, these lectins still show different binding properties and specificities to carbohydrates, as well as differences in conserved residues in certain regions of the sequence compared to other lectins of the same tribe (Foriers *et al.* 1981; Gatehouse *et al.* 1987; Anthony *et al.* 1991; Peumans & Van Damme 1995). However, total sequence information is still needed to make further comparisons.

Figure 2C shows the N-terminal sequence of CiL and the alignment of its amino acid sequence with other proteins. Therefore, it was now possible to analyze the homology of CiL with the *Crotalaria* and legume clades using Protein BLAST. This analysis resulted in finding considerable similarity among legume lectins from *Cladrastis kentukea* (Q39529), *Crotalaria pallida* (P16351), *C. juncea* (P16352), *Cymbosema roseum* (P86795), *Cytisus scoparius* (P29257), *Lablab purpureus* (P38662), *Lens culinaris* (P02870), *Phaseolus vulgaris* (Q43629), *Pisum sativum* (Q40987), *Robinia pseudoacacia* (Q42372), *Spatholobus parviflorus* (P86353), *Ulex europaeus* (P22973), *Vatairea guianensis* (P86893), *V. macrocarpa* (P81371) and *Vicia villosa* (P56625). The highest amino acid sequence similarity of CiL was observed between VML and VGL, both 93.1%, and the lowest similarity was observed between CiL and C. juncea (52.94%).

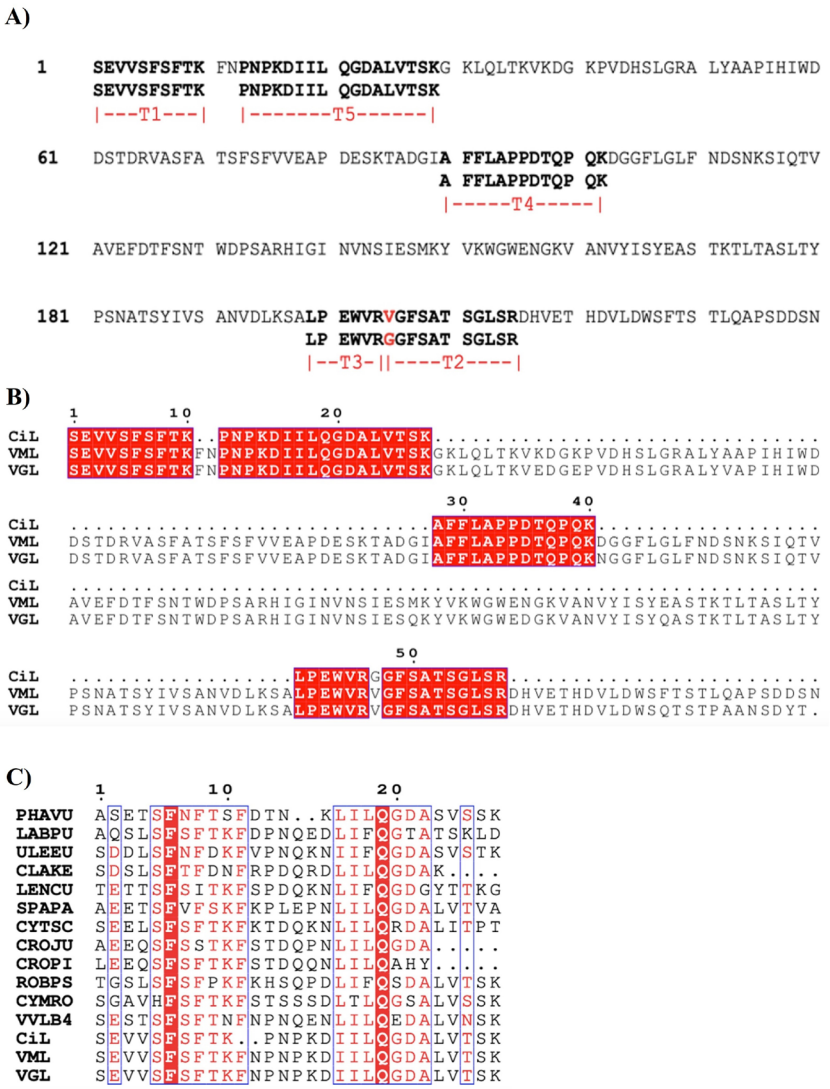

Figure 2. Partial primary structure of CiL obtained by superposition of the amino acid sequence of VML. **(A)** The first and second row correspond to the primary structure of lectin (VML) from *Vatairea macrocarpa* seed (UniProt: P81371) and to CiL peptides obtained by trypsin digestion (T). The amino acids in bold from CiL identical to VML are in black and those that differed are in red. **(B)** Partial sequence alignment of CiL with *V. macrocarpa* and *V. guianensis* (VGL) lectins. The alignment was done using ESPript 3.0 software. In red box are regions that showed homology between CiL, VML and VGL. **(C)** Alignment of the N-terminal sequence of CiL with that of other legume lectins: *Crotalaria incana* (CiL), *C. kentukea* (CLAKE), *C. pallida* (CROPI), *C. juncea* (CROJU), *C. roseum* (CYMRO), *C. scoparius* (CYTSC), *Lablab purpureus* (LABPU), *Lens culinaris* (LENCU), *Phaseolus vulgaris* (PHAVU), *Robinia pseudoacacia* (ROBPS), *Spatholobus parviflorus* (SPAPA), *Ulex europaeus* (ULEEU), *Vatairea guianensis* (VGL), *V. macrocarpa* (VML), and *Vicia villosa* (VVLB4). Alignment was done using ESPript 3.0 software.

Physicochemical characterization

CiL maintained its hemagglutinating activity, even after being incubated in a wide range of pH, having its optimum activity between pH 5 and 7, but presenting lower, but still considerable, activity of 12% in acidic pH (3.0 and 4.0) and in basic medium (8 and 9). CiL lost 100% of its hemagglutinating activity when it reached pH 10.0 (Fig. 3A). CiL exhibited thermostability with maximum activity at temperatures of 30, 40 and 50 °C, decreasing to 25% activity at temperatures of 60, 70, 80 and 90 °C, but still showing low activity at about 12% at 100 °C (Fig. 3B). CiL was shown to be dependent on divalent manganese (Mn^{2+}) and calcium (Ca^{2+}) ions. When tested in the presence of EDTA, it showed no hemagglutinating activity, but in the presence of manganese, it had about 50% of recovery. When treated with calcium, it also showed about 50% activity. Finally, when tested for hemagglutinating activity in the presence of calcium and manganese, CiL recovered 100% activity, thus showing its total dependence (Fig. 3C).

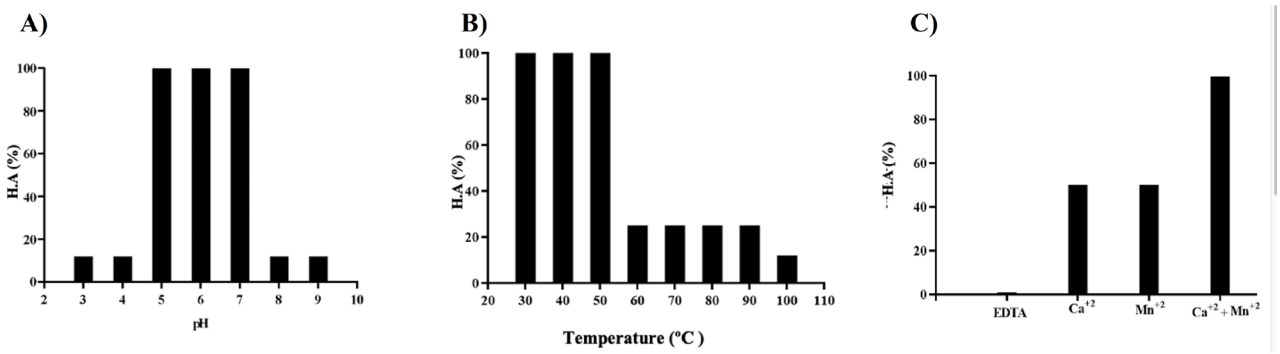

Figure 3. Average percentage of lectin hemagglutinating activity titers (CiL) tested under different conditions: pH **(A)**, temperatures **(B)**, and bivalent cations **(C)**.

Based on the activity of CiL at different pH, it was possible to verify optimum activity at acidic and neutral pH, results similar to those of other reports on lectins of the Fabaceae family, such as the lectins of *Dioclea reflexa* (Pinto-Junior *et al.* 2016), *Canavalia villosa* (Lossio *et al.* 2017) and *Parkia panurensis* (Cavada *et al.* 2019). Other studies report lectins from other botanical families with maximum activity in more acidic and basic regions, *e.g.*, *Clerodendrum infortunatum* from the Lamiaceae family with optimum activity at pH 2.0 (Surya & Haridas 2018) and *Litchi chinensis* from the Sapindaceae family with optimum activity at pH 8 and 9 (Bose *et al.* 2016).

The lectin proved to be thermostable with maximum activity at temperatures of 30 °C, 40 °C and 50 °C, but its activity was reduced when 100 °C was reached. Other lectins of the same botanical family show maximum activity at similar temperatures, such as the *Collaea speciosa* lectin isolated by Oliveira *et al.* (2021) and the lectin isolated by Alves *et al.* (2015) from seeds of *Clathrotropis nitida*, which showed low hemagglutinating activity at 100 °C. Total dependence of CiL on divalent ions, as noted above, closely resembled the findings of Pinto-Junior *et al.* (2016) who isolated a metal-dependent lectin from the seeds of *Dioclea reflexa*. These data diverge from those of other lectins, such as *Dioclea lasiophylla* (lectin II - specific lactose) (Cavada *et al.* 2020) and *Canavalia virosa* (Osterne *et al.* 2014).

Toxicity against artemia nauplii

CiL showed no toxicity against *Artemia salina* nauplii for 24 and 48 hours at the tested concentrations of 2, 1, 0.5, 0.25 and 0.125 mg/mL, and it was not possible to determine the lethal dose (LD_{50}) of the lectin. These findings are similar to those of the lectin from *Canavalia virosa* (ConV). They also differed from other lectins of the Fabaceae family, such as the *Parkia panurensis* (PpaL) lectin which showed toxicity with LC50 at a concentration of 20 µg/mL (Cavada *et al.* 2019) when tested against artemia nauplii for 24 and 48 hours.

Conclusions

This work demonstrated the purification, physicochemical properties and biological activity of a new lectin (CiL) isolated from *Crotalaria incana* seeds. CiL showed excellent thermostability, hemagglutinating activity within a wide range of pH, and dependence on divalent cations. No toxicity was observed at a dose of 2 mg/mL when the lectin was tested against *Artemia salina*. At the moment, due to the few studies of lectins of the genus *Crotalaria*, new research is needed to elucidate other biological and structural activities of this group of proteins.

Acknowledgements

This research was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP). Nascimento, K.S. and Cavada, B.S. are senior investigators of CNPq/Brazil. David Martin helped with the English editing of the manuscript. National Biosciences Laboratory (LNBio) of the National Center for Research, Energy and Materials (CNPEM) realized mass spectrometry analyses.

References

Altschul SF, Madden TL, Schäffer AA *et al.* 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research 25: 3389-3402.

Alves AC, Vasconcelos MA, Santiago MQ *et al.* 2015. A novel vasorelaxant lectin purified from seeds of *Clathrotropis nitida*: partial characterization and immobilization in chitosan beads. Archives of Biochemistry and Biophysics 588: 33-40.

Anthony JL, Haar RA, Hall TC. 1991. Nucleotide sequence of a genomic clone encoding arcelin, a lectin-like seed protein from *Phaseolus vulgaris*. Plant Physiology 97: 839-840.

Araripe DA, Pinto-Junior VR, Neco AHB *et al.* 2017. Partial characterization and immobilization in CNBr-activated Sepharose of a native lectin from *Platypodium elegans* seeds (PELa) and comparative study of edematogenic effect with the recombinant form. International Journal of Biological Macromolecules 102: 323-330.

Aremu MO, Bamidele TO, Amokaha JA. 2012. Compositional studies of rattle box (*Crotalaria retusa* L.) seeds found in Nasarawa State, Nigeria. Pakistan Journal of Nutrition 11: 880-885.

Arruda FVS, Melo AA, Vasconcelos MA *et al.* 2013. Toxicity and binding profile of lectins from the Genus *Canavalia* on brine shrimp. BioMed Research International 2013: 154542.

Benevides RG, Ganne G, Simões R da C *et al.* 2012. A lectin from *Platypodium elegans* with unusual specificity and affinity for asymmetric complex N-glycans. The Journal of Biological Chemistry 287: 26352-26364.

Bose PP, Bhattacharjee S, Singha S *et al.* 2016. A glucose/mannose binding lectin from litchi (Litchi chinensis) seeds: Biochemical and biophysical characterizations. Biochemistry and Biophysics Reports 6: 242-252.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Cavada BS, Araripe DA, Silva IB *et al.* 2018. Structural studies and nociceptive activity of a native lectin from *Platypodium elegans* seeds (nPELa). International Journal of Biological Macromolecules 107: 236-246.

Cavada BS, Bari AU, Pinto-Junior VR *et al.* 2019. Purification and partial characterization of a new lectin from *Parkia panurensis* Benth. ex H.C. Hopkins seeds (Leguminosae family; Mimosoideae subfamily) and evaluation of its biological effects. International Journal of Biological Macromolecules 145: 845-855.

Cavada BS, Pinto-Junior VR, Osterne VJS *et al.* 2020. A Diocleinae type II lectin from *Dioclea lasiophylla* Mart. Ex Benth seeds specific to α-lactose/GalNAc. Process Biochemistry 93: 104-114.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28: 350-356.

Ersson B. 1977. A phytohemagglutinin from Sunn hemp seeds (Crotalaria juncea). II. Purification by a high capacity biospecific affinity adsorbent and its physicochemical properties. Biochimica et Biophysica Acta 494: 51-60.

Foriers A, Lebrun E, Van Rapenbusch R, De Neve R, Strosberg AD. 1981. The structure of the lentil (Lens culinaris) lectin. Amino acid sequence determination and prediction of the secondary structure. The Journal of Biological Chemistry 256: 5550-5560.

Gatehouse JA, Bownd D, Evans IM *et al.* 1987. Sequence of the seed lectin gene from pea (*Pisum sativum* L.). Nucleic Acids Research 15: 7642.

Gouet P, Robert X, Courcelle E. 2003. ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. Nucleic Acids Research 31: 3320-3323.

Hasan I, Rahman SN, Islam MM *et al.* 2021. A N-acetyl-D-galactosamine-binding lectin from *Amaranthus gangeticus* seeds inhibits biofilm formation and Ehrlich ascites carcinoma cell growth in vivo in mice. International Journal of Biological Macromolecules 181: 928-936.

Khang NQ, Jean-Luc G, Hoebeke J. 1990. A blood group A specific lectin from the seeds of *Crotalaria striata*. Biochimica et Biophysica Acta 1033: 210-213.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lossio CF, Moreira CG, Amorim RMF *et al.* 2017. Lectin from *Canavalia villosa* seeds: A glucose/mannose-specific protein and a new tool for inflammation studies. International Journal of Biological Macromolecules 105: 272-280.

Nascimento KS, Cunha AI, Nascimento KS, Cavada BS, Azevedo AM, Aires-Barros MR. 2012. An overview of lectins purification strategies. Journal of Molecular Recognition 25: 527-541.

Oliveira MV, Osterne VJS, Lossio CF *et al.* 2021. Purification and characterization of a highly thermostable GlcNAc-binding lectin from *Collaea speciosa* seeds. International Journal of Biological Macromolecules 193: 1562-1571.

Osterne VJS, Oliveira MV, De Schutter K *et al.* 2023. A galactoside-specific Dalbergieae legume lectin from seeds of *Vataireopsis araroba* (Aguiar) Ducke. Glycoconjugate Journal 40: 85-95.

Osterne VJS, Santiago MQ, Pinto-Junior VR *et al.* 2014. Purification, partial characterization, and CNBr-sepharose immobilization of a vasorelaxant glucose/mannose lectin from *Canavalia virosa* seeds. Applied Biochemistry and Biotechnology 172: 3342-3353.

Osterne VJS, Silva-Filho JC, Santiago MQ *et al.* 2017. Structural characterization of a lectin from *Canavalia virosa* seeds with inflammatory and cytotoxic activities. International Journal of Biological Macromolecules 94: 271-282.

Pando LA, De Carvalho DD, Toyama MH *et al.* 2004. Purification and characterization of a lectin from *Crotalaria paulina* seeds. The Protein Journal 23: 437-444.

Peumans WJ, Van Damme EJM. 1995. Lectins as plant defense proteins. Plant Physiology 109: 347-352.

Pinto-Junior VR, Correia JLA, Pereira RI *et al.* 2016. Purification and molecular characterization of a novel mannose-specific lectin from *Dioclea reflexa* hook seeds with inflammatory activity. Journal of Molecular Recognition 29: 134-141.

Rego EJL, De Carvalho DD, Marangoni S, De Oliveira B, Novello JC. 2002. Lectins from seeds of *Crotalaria pallida* (smooth rattlebox). Phytochemistry 60: 441-446.

Sano K, Ogawa H. 2014. Hemagglutination (inhibition) assay. Methods in Molecular Biology (Clifton, N.J.) 1200: 47-52.

Santos RT da S, Oliveira MF de, Moura VB, Costa YKS da, Viana RG. 2019. Phenology and phytomass of *Crotalaria incana* L. in the Eastern Amazon, Brazil. Communications in Plant Sciences 9: 108-114.

Sharon N, Lis H. 1990. Legume lectins--a large family of homologous proteins. FASEB Journal: official publication of the Federation of American Societies for Experimental Biology 4: 3198-3208.

Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nature Protocols 1: 2856-2860.

Silva Filho V, Pereira W, Fernandes KF, Batista K de A. 2014. Extração, purificação parcial e caracterização de lectinas de sementes de *Crotalaria juncea*. Revista de Biotecnologia & Ciência 2: 12-24.

Silva-López RES, Pacheco JS. 2010. Genus *Crotalaria* L. (Leguminoseae). Revista Fitos 5: 43-52.

Surya S, Haridas M. 2018. A new galactose-specific lectin from *Clerodendrum infortunatum*. Iranian Journal of Biotechnology 16: e1449.

Van Damme EJM, Peumans WJ, Barre A, Rougé P. 1998. Plant lectins: A composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Critical Reviews in Plant Sciences 17: 575-692.