The International Journal of Biochemistry & Cell Biology 45 (2013) 807–815

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

# Crystal structure of *Dioclea violacea* lectin and a comparative study of vasorelaxant properties with *Dioclea rostrata* lectin

Maria Júlia Barbosa Bezerra[a], Natália Velloso Fontenelle Camelo Rodrigues[b], Alana de Freitas Pires[b], Gustavo Arruda Bezerra[a,c], Camila Bezerra Nobre[a], Kássia Lys de Lima Alencar[a], Pedro Marcos Gomes Soares[d], Kyria Santiago do Nascimento[a], Celso Shiniti Nagano[e], Jorge Luiz Martins[f], Karl Gruber[c], Alexandre Holanda Sampaio[e], Plinio Delatorre[g], Bruno Anderson Matias Rocha[a], Ana Maria Sampaio Assreuy[b,1], Benildo Sousa Cavada[a,*]

[a] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Av. Mister Hull s/n, Bloco 907, Box 6043, 60440-970, Fortaleza, Ceará, Brazil*
[b] *Instituto Superior de Ciências Biomédicas, Universidade Estadual do Ceará, Av. Paranjana 1700, 60740-000, Fortaleza, Ceará, Brazil*
[c] *Institute of Molecular Biosciences, University of Graz, Humboldtstrasse 50/3 A-8010, Graz, Austria*
[d] *Departamento de Morfologia, Universidade Federal do Ceará, Rua Delmiro de Farias s/n, 60430-170, Fortaleza, Ceará, Brazil*
[e] *Departamento de Engenharia de Pesca, Universidade Federal do Ceará, Av. Mister Hull s/n, Bloco 827, Fortaleza, Brazil*
[f] *Instituto de Química e Geociências, Universidade Federal de Pelotas, Rua Capão do Leão s/n, Box 354, 96010-900, Pelotas, Rio Grande do Sul, Brazil*
[g] *Departamento de Biologia Molecular, Universidade Federal da Paraíba, Cidade Universitária, 58059-900, João Pessoa, Brazil*

ARTICLE INFO

*Article history:*
Received 5 September 2012
Received in revised form
23 December 2012
Accepted 14 January 2013
Available online 23 January 2013

*Keywords:*
*Diocleinae* lectins
Isolated aorta
Vasorelaxant effect
Crystal structure

ABSTRACT

Lectins from *Diocleinae* subtribe belong to the family of legume lectins and are characterized by high identity between their amino acids sequences. It has been shown that punctual differences in amino acid sequences, such as one single amino acid or an alternative conformation, represent changes in biological activities caused by these lectins. Therefore, a more detailed understanding of three-dimensional structures of these proteins is essential for accurate analyzing the relationship between structure and function. In this study lectins purified from the seeds of *Dioclea violacea* (DVL) and *Dioclea rostrata* (DRL) were compared with regard to crystal structure and vasorelaxant properties. Differences in structure of lectins were found to be reflected in differences in vasorelaxant effects based on their high specificity and selectivity for cell glycans. Binding activity was related to the position of specific residues in the carbohydrate recognition domain (CRD). DVL complexed structure was solved by X-ray crystallography and was compared to native DVL and DRL. Therefore, DVL was co-crystallized with X-Man, and a molecular modeling with X-Man complexed with DVL was done to compare the complexed and native forms adjusted fit. The relatively narrow and deep CRD in DVL promotes little interaction with carbohydrates; in contrast, the wider and shallower CRD in DRL favors interaction. This seems to explain differences in the level of relaxation induced by DVL (43%) and DRL (96%) in rat aortic rings.



## 1. Introduction

Lectins are a structurally heterogeneous group of proteins with at least one non-catalytic domain binding reversibly to a specific mono- or oligosaccharide (Van Damme et al., 1998). Due to their ability to decipher membrane glycocodes, lectins participate in numerous cellular processes, such as cell communication, host defense, fertilization and development (Sharon and Lis, 2004).

*Diocleinae* lectins exhibit glucose/mannose-monosaccharide binding specificity. Studies on the chemical and physicochemical properties of *Diocleinae* lectins have revealed a high degree of identity in amino acid sequences and three-dimensional structure (Loris et al., 2004). However, small changes in amino acid sequence and conformation among the structures of these lectins can result in important differences in biological activity (Cavada et al., 2001; Nóbrega et al., 2012). In view of their multiple applications in science and technology, it is important to shed light on the relation between structure and function of *Diocleinae* lectins.

Many researches have shown that besides carbohydrate ligands, a number of lectins recognize small, predominantly hydrophobic ligands which bind at specific sites on the proteins (Komath et al.,

* Corresponding author at: BioMol-Lab, Departmento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Av. Mister Hull, 60440-970, Box 6043, Fortaleza, Ceará, Brazil. Tel.: +55 85 3366 9818; fax: +55 85 3366 9818.
*E-mail addresses:* anassreuy@gmail.com (A.M.S. Assreuy), bscavada@ufc.br (B.S. Cavada).
[1] Instituto Superior de Ciências Biomédicas, Universidade Estadual do Ceará, Av. Paranjana 1700, 60740-000, Fortaleza, Ceará, Brazil. Tel.: +55 8531019919; fax: +55 8531019810.

1357-2725 
http://dx.doi.org/10.1016/j.biocel.2013.01.012

2006). Inferences on the extent of the interaction between lectins and sugars may be made by comparing the distances between specific residues in the carbohydrate binding site and the way these are reflected in specific biological activities. The structural conformation of amino acids also alters the oligomerization of *Diocleinae* lectins (including His51, His131) contributing to these differences in activity. The pH-dependent dimer-tetramer equilibrium depends on the conformation of these residues and the presence of His or Asn in position 131, both are determinant to permit dimers interact in a tetrameric assembly (Nagano et al., 2008; Oliveira et al., 2008; Wah et al., 2001).In a previous research of Gadelha et al. (2005), the lectin of *Canavalia maritma* (ConM), which is very similar to the lectin of *Canavalia ensiformis* (ConA), was shown to relax endothelized rat aortic rings mediated by nitric oxide (NO). Likewise, in a comparative study on the vascular effects elicited *in vitro* by lectins of the *Canavalia* group, ConBr and CGL produced endothelium-dependent relaxant effects with the participation of different vasodilators in isolated rat aortic rings precontracted with phenylephrine (Assreuy et al., 2009). These studies support the notion that highly similar lectins can express different biological activities – or similar activities at varying levels of potency and efficacy.

The effects of the lectin of *Dioclea violacea* (DVL), purified by Moreira et al. (1996), and *Dioclea rostrata* (DRL), purified by Cavada et al. (1996), in the inflammatory process are well described (Assreuy et al., 1997, 1999; Barbosa et al., 2001; Figueiredo et al., 2009). All these activities were reversed by binding the lectin to glucose or mannose. However, no effect on the mechanical activity of vascular smooth muscles has been reported so far.

The primary structure of DRL and DVL are 98% identical (NCBI database). Thus, in order to better understand the relation between three-dimensional structure and function in *Diocleinae* lectins, DRL and DVL were subjected to a comparative study of crystal structure and vasorelaxation in rat aortic rings. Therefore, DVL was co-crystallized with X-Man (5-bromo-6-chloro-3-indolyl-α-D-mannopyranoside), which was previously reported as an stabilizer of flexible loops in carbohydrate recognition domain (Nóbrega et al., 2012; Bezerra et al., 2011). It was also done a molecular docking of X-Man complexed with native DVL to compare the complexed and native forms adjusted fit, once mannose is one of the main components of glycans involved in biosignalization in smooth muscle (Carmignac and Durbeej, 2012). The differences in the level of relaxation induced by DVL (43%) and DRL (96%) in rat aortic rings are structurally explained by the relatively narrow and deep CRD in DVL which promotes little interaction with carbohydrates, and the wider and shallower CRD in DRL which favors this interaction.

## 2. Experimental

### 2.1. Lectins

Lectins extracted from the seeds of *D. violacea* (DVL) (Moreira et al., 1996) and *D. rostrata* (DRL) (Cavada et al., 1996) were purified by affinity chromatography on a Sephadex G-50 matrix, dialyzed against distilled water and freeze-dried. For the experiments, the lectins were dissolved in sterile saline solution (0.15 M NaCl).

### 2.2. Crystallization and data collection

The purified lectin (DVL) (Moreira et al., 1996) was solubilized in 20 mM Tris–HCl (pH 7.6) containing 5 mM $CaCl_2$ and $MnCl_2$ buffer and incubated during 1 h before the crystallization experiments with 3 mM X-Man (5-bromo-6-chloro-3-indolyl-α-D-mannopyranoside) for stabilization, in order to obtain well-diffracting crystals. The best condition of crystal growth was screened by the hanging-drop vapor diffusion method with drops containing 2 μL protein and 2 μL crystallization solution and equilibrated against 300 μL of the same solution (Hampton Research Screens I and II) (Jancarik and Kim, 1991).

DVL crystals complexed with X-Man (DVL_X-Man) belonging to the orthorhombic space group *I222* were grown in 4.0 M sodium formate (Crystal Screen I, condition #33). The X-ray diffraction data were collected at 2.6 Å of resolution at a beamline MX1 station (Brazilian Synchrotron Light Laboratory – LNLS, Campinas, Brazil) at 100 K, using 10% PEG 400 as cryoprotectant to avoid ice formation. Following adjustment of dose and beam line intensity, 120 images were collected during 60 s of exposure each with a CCD detector (Marresearch GmbH, Germany) placed at 130 mm from the crystal, with 1° oscillation.

The collected data were indexed and processed with XDS (Kabsch, 2010) and scaled using Scala (Evans, 2006). Based on a molecular weight of 25.5 kDa, Matthews coefficient was calculated as 2.12 $Å^3\ Da^{-1}$ and indicated the presence of a monomer in the asymmetric unit and a solvent content of 42% (Matthews, 1968).

### 2.3. Molecular replacement and refinement

The amino acid sequence of DVL was aligned with all amino acid sequences in our *Diocleinae* database using BLAST (Altschul et al., 1990) in order to identify the best match. The corresponding model was used as template for molecular replacement. The structure of DVL_X-man at 2.6 Å resolution was obtained by molecular replacement using the coordinates of DRL (PDB ID: 2ZBJ) and both belongs to the same space group (Oliveira et al., 2008). This template was chosen based on sequence alignment with other members of the genus *Dioclea*. The structure of DVL_X-Man was determined by molecular replacement using PHASER (Mccoy et al., 2007) implemented in CCP4 (CCP4, 1994) with a maximum likelihood gain of 848,883, an RFZ of 13.1 and a TFZ of 30.0 for the best solution. Several rounds of iterative refinement were performed with the PHENIX suite (Adams et al., 2010), adding water molecules until achieving the optimal X-ray weighting factor.

Manual adjustments in COOT were made after each refinement step and an X-Man molecule was finally added to the model in order to explain the residual density at the ligand binding site. After the last refinement step, $R_{factor}$ was 0.23 and $R_{free}$ was 0.27. The stereochemistry of the structure was assessed with a Ramachandran plot analysis with MolProbity (Chen et al., 2010), analyzing the $\varphi$ and $\psi$ angles and the root mean square deviation of the bonds. The graph confirmed the absence of residues in disallowed regions, and deviations were within the normal range. Molecular replacement and refinement data are shown in Table 1. The structure factors and coordinates were deposited in the Protein Data Bank under accession code number 3AX4.

### 2.4. Molecular docking of X-Man with native structure of DVL

Native structure obtained from PDB of DVL (PDB ID: 2GDF) was submitted to molecular docking with the X-Man ligand. Molecular docking analysis was performed with MEDock (Maximum-Entropy based Docking) web server, which is aimed at providing an efficient utility for prediction of ligand binding site. A major distinction in the design of MEDock is that its global search mechanism is based on a novel optimization algorithm that exploits the maximum entropy property of the Gaussian distribution (Chang et al., 2005).

### 2.5. Biological assays

#### 2.5.1. Drugs and reagents

Indomethacin, D-glucose, 5-bromo-4-chloro-3-indolyl-α-D-mannose (X-Man), $N^{\omega}$-nitro-L-arginine-methyl-ester (L-NAME),

**Table 1**
Statistics of data collection, refinement and structure quality.

| Parameters | Values |
|---|---|
| Data collection | |
| Beamline wavelength | 1.42 Å |
| Space group | *I222* |
| Exposure time per frame (s) | 60 |
| Mosaicity | 0.67° |
| Unit cell parameters (Å) | |
| A | 61.34 |
| B | 66.11 |
| C | 106.69 |
| Total reflections | 22900 (2438) |
| Number of unique reflections | 6354 (810) |
| Molecules per asymmetric unit | Monomer |
| Resolution limits (Å) | 34.3–2.6 (2.7–2.6) |
| $R_{merge}$ [a] (%) | 9.7 (22.6)[d] |
| Completeness (%) | 92.7(83.1)[d] |
| Multiplicity | 3.6 (3.0) |
| $I/\sigma$ (average) | 7.0 (3.3)[d] |
| Molecular replacement | |
| RFZ | 13.1 |
| TFZ | 30.0 |
| Refinement | |
| Resolution range (Å) | 34.3–2.6 (3.3–2.6) |
| $R_{factor}$ [b] (%) | 23.0 (25.5) |
| $R_{free}$ [c] (%) | 27.5 (30.1) |
| B factor (Å$^2$) | 25.9 |
| Wilson B (Å$^2$) | 19.8 |
| Number of residues in asymmetric unit | 237 |
| Number of water molecules | 60 |
| RMS deviations | |
| Bond length (Å) | 0.014 |
| Bond angle (°) | 0.848 |
| Ramachandran plot | |
| Residues in most favored regions (%) | 92.5% |
| Residues in additional allowed regions (%) | 6.61% |

[a] The $R_{merge} = \frac{\sum_{hkl}\sum_i \left| I(hkl) - \langle I(hkl)i \rangle \right|}{\sum_{hkl}\sum_i \langle I(hkl)i \rangle}$ where $I(hkl)i$ is the intensity of i$^{th}$ measurement of the reflection $h$ and $I(hkl)$ is the mean value of the $I(hkl)i$ for all I measurements.
[b] $R_{factor} = \frac{|F_{obs}| - |F_{calc}|}{|F_{obs}|}$..
[c] Calculated with 5% of the reflections omitted from refinement.
[d] Values in parenthesis represent the high resolution shell.

phenylephrine (Phe), acetylcholine (ACh) and tetraethylammonium (TEA) were purchased from Sigma (St. Louis, MO, USA). All substances were solubilized directly in sterile saline (0.15 M NaCl), except for indomethacin, which was initially dissolved in dimethyl sulfoxide up to 10% of the total volume, then in saline.

#### 2.5.2. Animals

Male Wistar rats (250–300 g) were kept in cages in a controlled environment (circadian cycle, 25 °C, food and water *ad libitum*). The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Universidade Estadual do Ceará State University of Ceara (UECE, Fortaleza, Ceará, Brazil) under (CEUA-UECE No., 10130208-8/40) following the recommendations of the Guide for the Care and Use of Laboratory Animals of the US Department of Health and Human Services (NIH publication No. 85–23, revised 1985).

#### 2.5.3. Lectin-induced vasorelaxation

The animals were euthanized by stunning, followed by prompt excision of the thoracic aorta and removal of adhering fat and connective tissue. Ring segments (3–5 mm) were mounted for tension recording (2 g) in 10-mL organ baths filled with modified Tyrode solution (in mM: 136 NaCl, 5 KCl, 0.98 $MgCl_2$, 2 $CaCl_2$, 0.36 $NaH_2PO_4$, 11.9 $NaHCO_3$ and 5.5 glucose). The rings were kept at 310 K, gassed with 95% $O_2$/5% $CO_2$ (pH 7.4) and allowed to equilibrate for 45 min. The contractile response (isometric tension/g) was measured using a force transducer linked to a pre-amplifier and a computerized data acquisition system (PowerLab, Chart 4.2, ADInstruments). Following equilibration, rings were challenged with 60 mM KCl to ensure tissue viability.

Cumulative concentration curves were plotted for DVL and DRL (1–100 μg/mL) at the contraction plateau induced by 0.1 μM Phe or at aortic basal tone in both endothelized and de-endothelized rings. De-endothelization was assessed by mechanical rubbing of the internal aortic surface. The endothelium was considered intact when the relaxation response to 1 μM acetylcholine was above 75% of the Phe-induced tone (Furchgott and Zawadzki, 1980). The animals in the control group received the same amount of Tyrode. To investigate the mechanism of lectin-induced vasorelaxation, 100 μM L-NAME (a nitric oxide synthase inhibitor), 10 μM indomethacin (a nonselective cyclooxygenase inhibitor) or 5 mM tetraethylammonium (a potassium channel blocker) were added to tissues with intact endothelium 30 min prior to treatment with Phe.

DVL and DRL at their most active concentration were incubated with 0.1 M D-glucose for 1 h at 310 K to allow for lectin–sugar interaction prior to the experiments. Lectin and sugar controls were prepared and incubated individually under similar conditions.

### 2.6. Statistical analysis

Results were expressed as mean values ± SEM for each group of 5–7 animals. Differences were analyzed with ANOVA or Student's *t* test, as appropriate. The level of statistical significance was set at $p < 0.05$.

## 3. Results and discussion

### 3.1. Overall structure of DVL

The structure of DVL_X-Man at 2.6 Å resolution (Table 1) was obtained using DRL (PDB ID: 2ZBJ) as search model, which is 98% identical with DVL (Fig. 1). The primary structures of DVL and DRL differ only in the position of nine amino acids. The refined structure of DVL_X-Man contained 60 water molecules, one calcium ion and one manganese ion and a molecule of X-Man. The biological assembly is a tetramer composed by two perpendicular canonical dimers similar to ConA. The tetramer of DVL_X-Man is formed by the superposition of two perpendicular symmetry axes and consists of two dimers tied in a canonical β-sheet structure. This quaternary arrangement, which is often referred to as the "jelly roll", is observed in all ConA-like lectins (Bouckaert et al., 2000).

As with other *Diocleinae* lectins, the electron density map is unreliable in the loop regions corresponding to residues 117–125 and 149–151. The first loop is completely inconclusive in DVL_X-Man structure. Therefore, the residues in this loop were excluded.

The structure of DVL_X-Man includes a metal-binding site (Fig. 2) containing the conserved residues Asn14 and Try12 which interact with calcium, Glu8 and His24 which interact with manganese and Asp10 and Asp19 which interact with both. Four water molecules interact indirectly with residues Val32, Ser34, Asp208 and Arg228 and with the metal ion. The orientation of the residues is similar to that of other ConA-like lectins. In addition, the peptide bond of Ala207 and Asn208 in the *cis* configuration is isomerized. This fact is very important for the stability of the carbohydrate binding site through the interaction with Asn14 and Arg228 (Bouckaert et al., 2000; Loris et al., 1998). The substitution of Glu (DVL and DVL_X-Man) for Asp (DRL) in position 205 may also indicate a significant repositioning of the amino acids involved in carbohydrate recognition. The mannose moiety of X-Man binds to the carbohydrate binding site according to electron

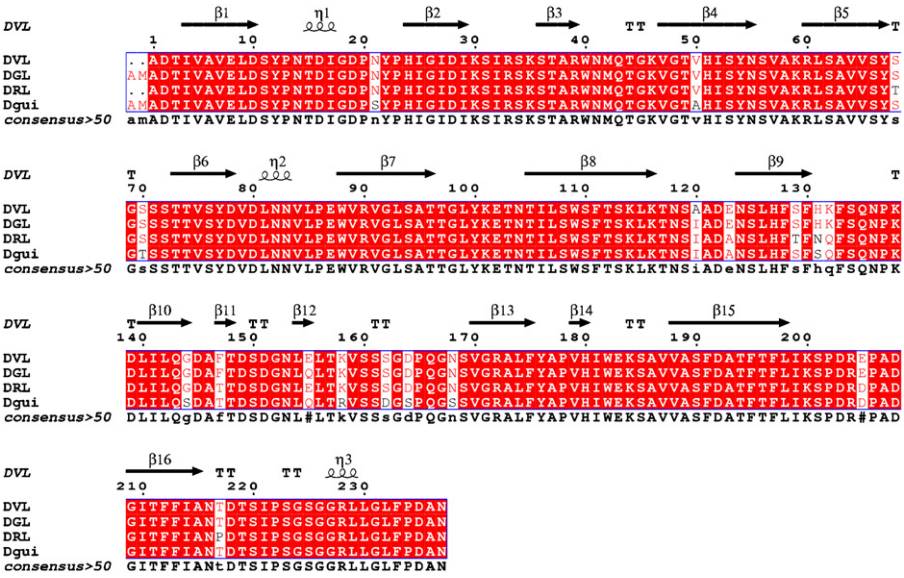


**Fig. 1.** Alignment of lectins of the genus *Dioclea*. DVL (*D. violacea* lectin), DGL (*D. grandiflora* lectin; Uniprot accession number: P08902), DRL (*D. rostrata* lectin) and Dgui (*D.guianensis* lectin; Uniprot accession number: P81637).

density (Fig. 3), stabilizing the protein. This produces a more ordered crystal lattice and improves the quality of the crystals formed.

The ligand forms hydrophilic interactions (H-bonds) and Van der Waals contacts with DVL_X-Man. The polar interactions involve seven H-bonds (Fig. 3) with oxygens of mannose: $O_3$ (Arg228N – 3.1 Å), $O_4$ (Asn14ND2 – 3.0 Å and Asp208OD1 – 2.6 Å), $O_5$ (Leu99N – 3.0 Å), and $O_6$ (Tyr100 N – 2.9 Å and Asp208OD2 – 2.9 Å); and also involves the nitrogen of indolyl group of X-Man with Tyr12OH at 3.0 Å. The amino acids of carbohydrate binding site (Tyr12, Leu99 and Tyr100) interact with X-Man through Van der Waals contacts and hydrophobic interactions.

### 3.2. Dimer-tetramer equilibrium

The dimer-tetramer equilibrium is known to have an important influence on biological activity in lectins. pH-dependent changes in dimer and tetramer structure and the orientation of the residues in the carbohydrate binding site are responsible for variations in the potency of the biological effects induced by lectins (Calvete et al., 1999; Nagano et al., 2008).

DVL and DVL_X-Man, pH-independent tetramers (Nóbrega et al., 2012; Delatorre et al., 2011), were compared to DGL (*Dioclea grandiflora*), a predominantly pH-independent tetramer, and to Dgui (*Dioclea guianensis*), a pH-independent dimer (Wah et al.,

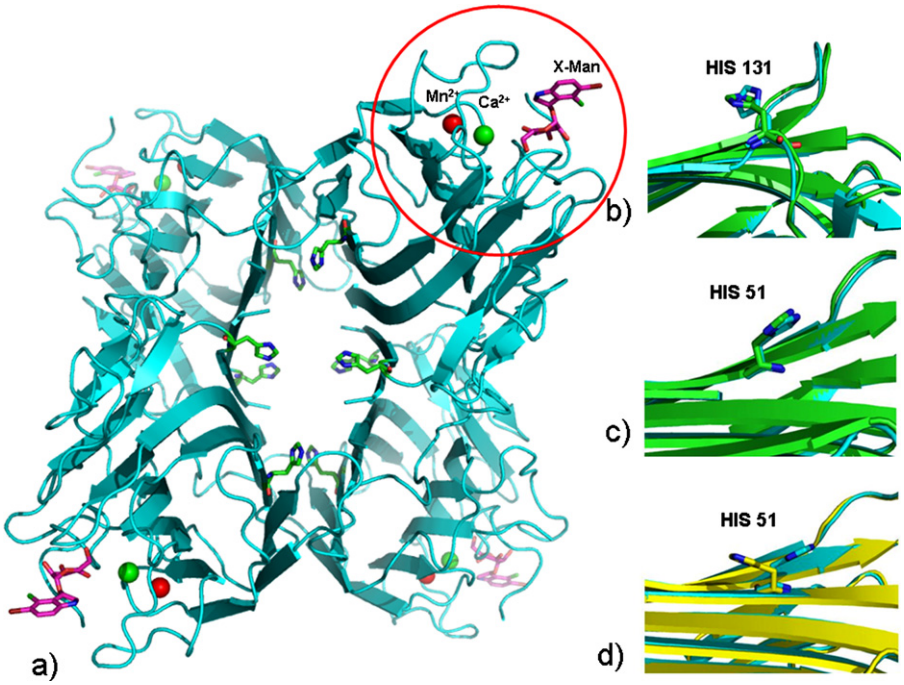


**Fig. 2.** Overall structure of DVL. (a) Tetrameric structure of DVL with the presence of $Mn^{2+}$ and $Ca^{2+}$ (red and green spheres, respectively) and the ligand X-Man (pink). The residues His51 and His131 are involved in oligomeric stablization. (b) Superposition of His131 in DVL_X-Man (blue) and DGL (green). (c) Superposition of DVL_X-Man (blue) and DGL (green); (d) Dgui (yellow) showing differences in the orientation of His51.

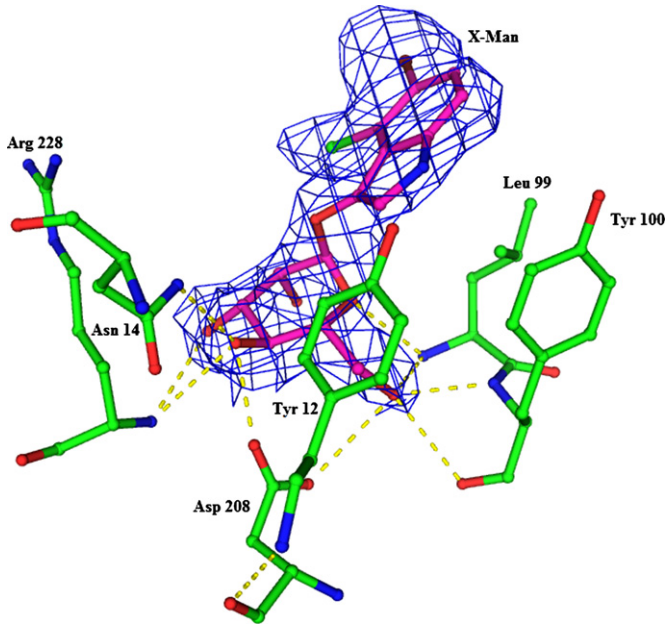


**Fig. 3.** Carbohydrate binding site of DVL interacting with X-Man by polar contacts. Omit map representation of the electron density contoured at 1$\sigma$ of X-Man. Yellow dashes represent polar contacts.

2001). Differences in oligomerization behavior are mainly dependent on residues His51 and His131 (Fig. 2(b)–(d)). His131 enhances interactions between dimers participating in the formation of the tetramer. This residue is present in DGL but is replaced by an Asn in Dgui, resulting in a lower level of dimer interaction. His51 induces interdimeric interactions depending on its position in the structure. Changes in these key residues affect the three-dimensional conformation of the crystal and, consequently, its biological properties. Based on the comparison of specific residues, it was confirmed that DVL is a pH-independent tetramer (Fig. 2).

Both DVL and DGL contain the residue His131 in their structure. This residue enhances dimer interaction, favoring tetramer formation (Fig. 2(b)). Structural comparisons show that His51 is in the same position in DVL and DGL (Fig. 2(c)), but in a different position in Dgui (Fig. 2(d)). The former conformation allows many series have hydrophobic and Van der Waals interactions with other residues in the same chain (Thr49, Val64 and Thr194) and with residues of the dimer (Lys166, Val187 and Val188), producing a zipper-like appearance (Fig. 2(a)).

The pH interference on quaternary assembly was analytically and structurally determined for DRL by Calvete et al. (1999) and Oliveira et al. (2008), respectively. It was suggested that this protein represents an intermediate case between Dgui and DGL regarding pH-dependent oligomerization profile. Superposition of the dimer-dimer interface of DRL, Dgui and DGL crystal structures indicated two structural reasons for this intermediate behavior; the position of Asn131 of DRL is similar to that of Dgui and does not allow for the establishment of interdimer interactions because Asn131 is not able to interact with the 117-123 loop, and His51, which is conserved in all three lectins, has a similar orientation in the crystal structures of DRL and DGL (this permits many Van der Waals and hydrophobic interactions between the monomers), contrasting with a different orientation in the crystal structure of Dgui (Wah et al., 2001).

### 3.3. Molecular docking of X-Man in native structure of DVL

Molecular docking was developed with the aim of verifying the X-Man binding capacity to native DVL (PDB ID: 2GDF) and compare it with the crystallographic data. Docking was processed to both structures (DRL and DVL), although the binding of DVL with X-Man revealed a favorable energy (Lowest Docked Energy = −6.88 kcal/mol) to a solution which positioned X-Man correctly in the carbohydrate binding site. Very similar interactions can be observed between native DVL and X-Man complex obtained by molecular docking and crystallographic structure DVL_X-Man.

DVL and DVL_X-man present Arg228 in distal position differing of DRL which has the same amino acid in a proximal position related to X-Man (Fig. 4). The structural alignment among native DRL and DVL, and DVL_X-man did not show a significant adjusted fit induced by the ligand regarding the amino acids that compose the carbohydrate binding site in both structures; native and complexed DVL. In comparison with the different positions of these same amino acids in DRL, it can be seen some conformational changes in the side chains that became important to the binding of X-Man, such as Arg228, Tyr100 and Tyr12 (Fig. 4). Molecular docking of native DVL with X-Man showed that small conformational changes in the carbohydrate binding site imply different affinities for glycidic derivative ligands.

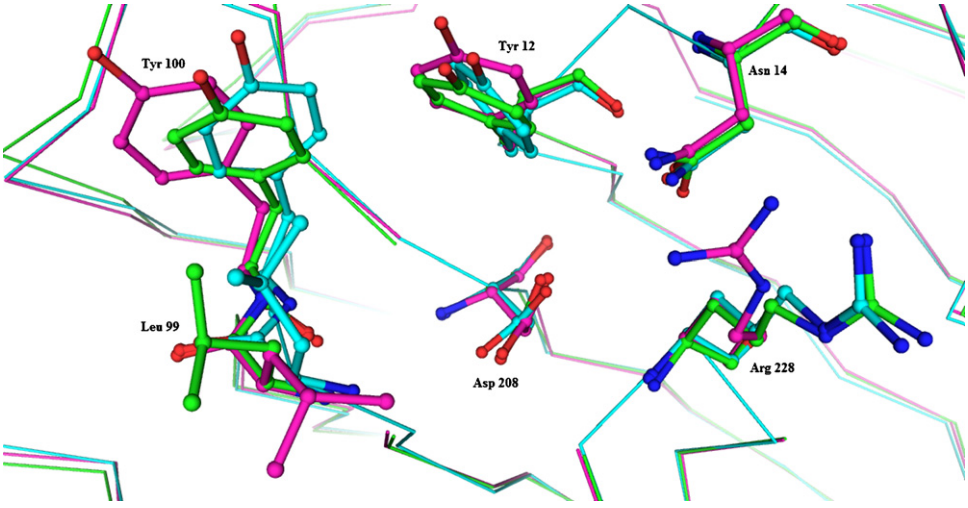


**Fig. 4.** Structural alignemnet of native DVL (2GDF; green) and complexed DVL (3AX4; blue) and native DRL (2ZBJ; magenta). Arg228 presents a distal position in native and complexed DVL and a proximal position in DRL. The ligand was removed to enhance the visualization of amino acids.

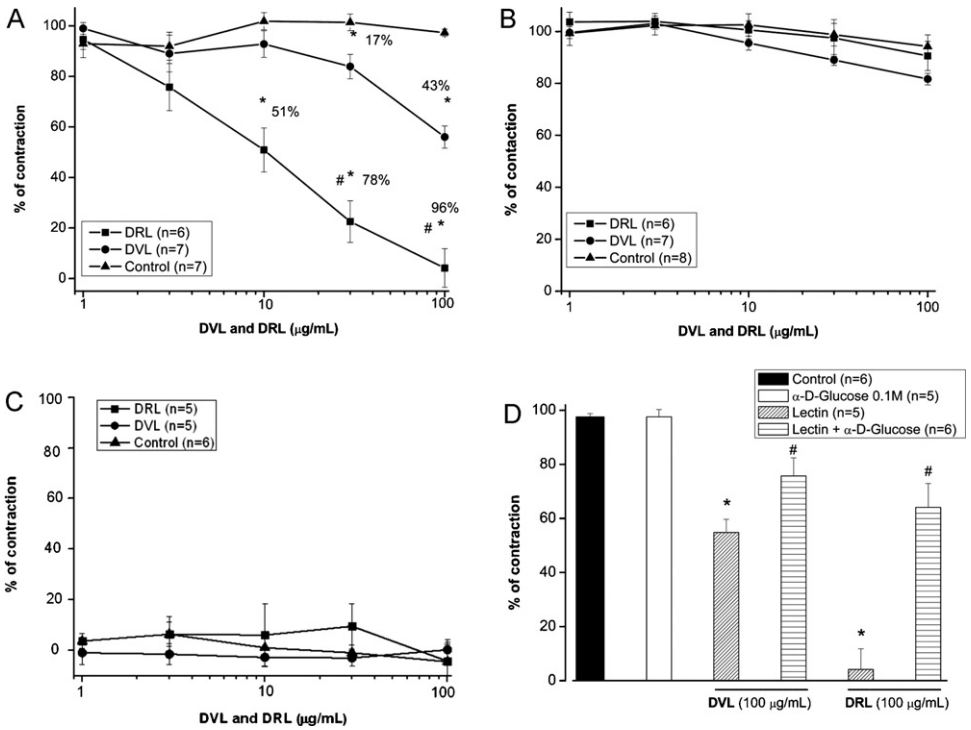


**Fig. 5.** DVL and DRL inducing endothelium-dependent relaxation in isolated rat aorta via the lectin domain. Data comparing the responses of DVL (●) and DRL (■) (1–100 μg/mL) to control (▲) in tissues with endothelium (A) and without endothelium (B) pre-contracted with phenylephrine or at basal tonus (C). The data show reversion of relaxation response after 60 min incubation of lectins (100 μg/mL) with D-glucose (0.1 M) (D). Mean values ± SEM; $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus lectins.

### 3.4. Vasorelaxant effect of DRL and DVL

Phenylephrine (Phe) induced tonic contractions in endothelized and de-endothelized aortic rings with amplitude of 0.445 ± 0.06 g ($n = 15$) and 0.67± 0.085 g ($n = 11$), respectively. DRL relaxed precontracted endothelialized rings at concentrations between 10 μg/mL (initial response) and 100 μg/mL (peak response: 95.7 ± 7.64%). DVL was effective at concentrations between 30 and 100 μg/mL (42.5 ± 4.37%) (Fig. 5(A)). However, no relaxation was observed in de-endothelized rings (Fig. 5(B)). DVL and DRL did not affect tissue responsiveness in any of the protocols (the contractile response to KCl was similar to baseline). In addition, the basal tonus was not significantly affected by DVL or DRL at 1–100 μg/mL (Fig. 5(C)).

The vasorelaxant effect induced by 100 μg/mL lectin (DVL: 42.5 ± 4.37%, $n = 6$ vs. DRL: 95.7 ± 7.64%, $n = 5$) was partially inhibited by D-glucose (DVL: 22.41 ± 6.70% vs. DRL: 34.28 ± 6.70%). However, D-glucose had no significant effect on Phe-induced contraction (Fig. 5(D)). The glucose/mannose binding lectins are equally inhibited by both monosaccharides. However, the methylation of these sugars (e.g. methyl-mannosides) became them more potent than the unmodified ones (Cavada et al., 2001).

This study demonstrates the ability of DVL and DRL to induce vasorelaxant effects in rat aortic rings. Relaxation was found to be strictly dependent on the presence of intact endothelium and on carbohydrate binding site conformation.

Other lectins of the *Diocleinae* subtribe, such as ConM, ConA (Gadelha et al., 2005), ConBr and CGL (Assreuy et al., 2009), were shown to induce relaxation in pre-contracted rat aortic rings using an *in vitro* model of contractility. In all the lectins studied so far, the mechanism of relaxation is endothelium-dependent and reversible by addition of the respective binding sugar. However, even lectins belonging to the same genus display important differences in efficacy and mechanisms of action. This was confirmed in the present study, in which two *Dioclea* lectins, DRL and DVL, produced relaxation at different levels of potency despite structural homology.

Since DVL and DRL-induced relaxation was strictly dependent on the presence of endothelium, the involvement of the key endothelial relaxing factors NO, EDHF and prostacyclin was investigated.

In aortis rings pre-incubated with L-NAME (a non-selective nitric oxide synthase inhibitor), Phe-induced contractions presented an amplitude of 0.80 ± 0.025 g. L-NAME inhibited relaxation induced by 30 and 100 μg/mL DVL (Fig. 6(A)) and blocked relaxation induced by 10, 30 and 100 μg/mL DRL (Fig. 6(B)). The effect of DVL and DRL was completely inhibited by L-NAME. In fact, NO is the main mediator of endothelium-dependent relaxation in vascular smooth muscle (Mollace et al., 2005) and of vasorelaxation in other Diocleinae lectins (Gadelha et al., 2005; Assreuy et al., 2009).

In aorta incubated with indomethacin (a non-selective cyclooxygenase inhibitor), Phe-induced contractions presented amplitude of 0.44 ± 0.075 g. Indomethacin did not reverse the effect of DVL (Fig. 6(A)), but partially recovered relaxation induced by 10, 30 and 100 μg/mL DRL (60.68 ± 6.58%) (Fig. 6(B)). Indomethacin partially reversed the dose-dependent vasorelaxation induced by DRL, but not by DVL, suggesting the involvement of prostacyclin in the former, but not in the latter. In a previous study, prostacyclin was found to be involved in relaxation induced by CGL and ConBr (Assreuy et al., 2009). Many substances stimulate NO synthesis and release, which in turn can elicit prostacyclin production – a major product of the cyclooxygenase pathway in vascular endothelial cells (Mollace et al., 2005). The frequently observed inhibitory effect of indomethacin and L-NAME supports the increasing evidence in the literature of a significant level of cross-talk between NO and the prostaglandin biosynthetic pathways (Edwards et al., 1998; Salvemini, 1997).

This study also evaluated the effect of endothelium-derived hyperpolarizing factor (EDHF) on lectin-induced relaxation. Thus, in the presence of TEA, Phe-induced tonic contraction presented

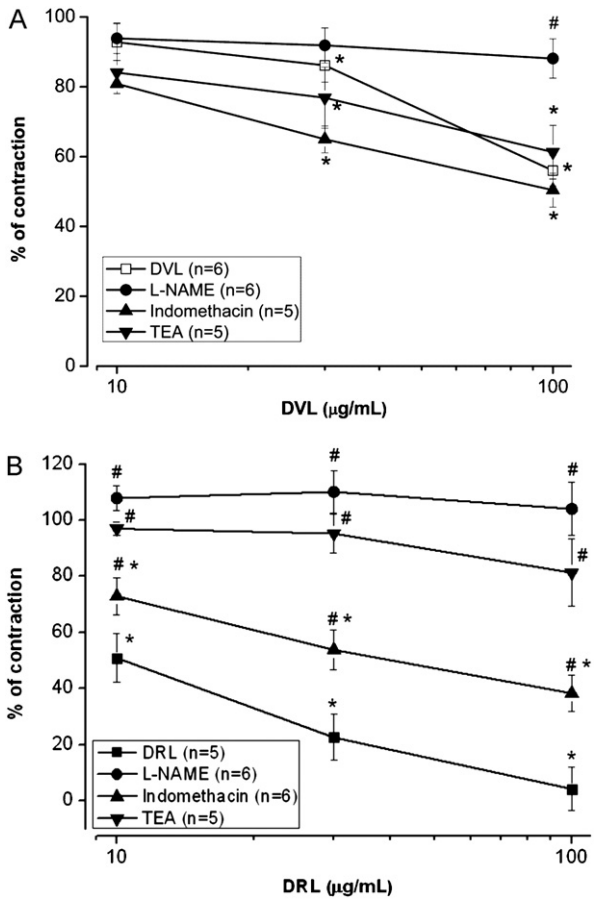


**Fig. 6.** Role of endothelium-derived relaxant factors in the effect of DVL and DRL. Comparative effects of 10–100 μg/mL DVL (A; □) and DRL (B; ■) on phenylephrine (0.1 μM)-induced contraction of endothelialized aorta in the absence or presence of 100 μM L-NAME (●), 10 μM indomethacin (▲) or 500 μM TEA (▼); Mean values ± SEM; $^{*}p < 0.05$ versus control; $\#p < 0.05$ versus lectins.

amplitude of 0.55 ± 0.10 g. TEA blocked the relaxation induced by 10, 30 and 100 μg/mL DRL (Fig. 6(B)), but the effect of DVL remained unchanged (Fig. 6(A)). Activation of potassium channels may lead to EDHF release in vascular smooth muscle, which can be blocked by TEA (Barral-Netto et al., 1992). In our experiments, TEA did not affect DVL-induced vasodilatation, but significantly inhibited the effects of DRL. In a previous study, CGL was shown to produce a greater effect (involving the three endothelium-derived relaxant factors) than ConBr (Assreuy et al., 2009). Likewise, in the present study DRL was more efficacious than DVL.

**Table 2**
Distances among residues of the carbohydrate-binding domain of lectins from genus Dioclea.

| Amino acids | Distances (Å) | |
|---|---|---|
| | DVL_X-Man | DRL |
| ARG 228 N–TRY 12 OH* | 9.55 | 9.00 |
| ARG 228 N–ASN 14 ND2* | 4.86 | 3.79 |
| TYR 100 N–TYR 12 OH | 6.91 | 7.30 |
| TYR 100 N–LEU 99 N | 2.65 | 2.78 |
| TYR 12 OH–ASN 14 ND2 | 5.36 | 5.77 |
| ARG 228 N–LEU 99 N | 10.25 | 9.80 |
| ARG 228 N–TYR 100N* | 11.82 | 11.07 |

* Significant distances for comparison are those with DPI higher than 0.5 Å.

Different levels of biological activity have previously been reported for *Diocleinae* lectins (Cavada et al., 2001; Gadelha et al., 2005; Andrade et al., 1999; Bento et al., 1993). In this study, DVL and DRL dose-dependently induced reversible vasorelaxation in pre-contracted aorta with the participation of different vasodilators, provided the rings were endothelialized. Several factors may contribute to these differences, such as pH-dependent oligomerization, observed in some lectins, the relative amino acids position in the carbohydrate binding site (Wah et al., 2001; Brinda et al., 2004) and small changes in amino acid residues in key positions in the structure (Sanz-Aparicio et al., 1997).

### 3.5. Three-dimensional CRD conformation and vasorelaxant properties

The three-dimensional structure of DVL_X-Man was carefully compared to that of DRL and differences in distance between the residues in the carbohydrate binding sites of DVL_X-Man and DRL were found to be associated with differences in the ability to induce vasorelaxation. Table 2 shows the distances between the residues Tyr12, Asn14, Leu99, Try100, Asp208 and Arg228 in each lectin.

The conformation of the CRD appears to be changed by the substitution of an amino acid positioned next to carbohydrate binding site: Glu205 in DVL is replaced by Asp205 in DRL. The

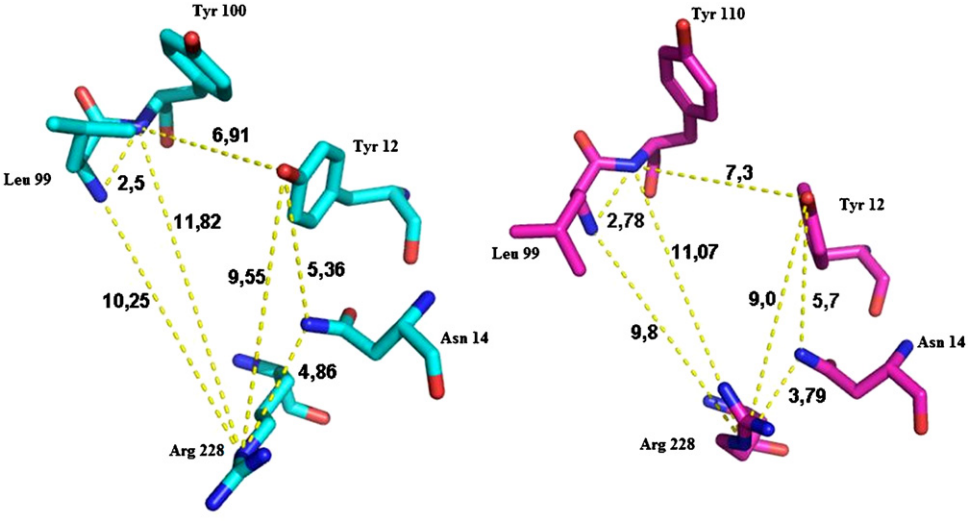


**Fig. 7.** CRD conformation in DVL_X-Man and DRL. Distances between carbohydrate-binding residues in DVL_X-Man (blue) and DRL (pink).

position has been identified as important to carbohydrate binding orientation and affinity in ConA-like lectins (Gadelha et al., 2005). As shown in Fig. 1, DGL and DRL have the same residue in this position, suggesting that the position is important for CRD conformation.

The geometry of the carbohydrate binding site may be expressed in terms of the distances between the residues it contains. This geometry is conserved among *Diocleinae* lectins, but small differences with confirmed significance (more than 0.5 DPI) (Blow, 2002) in the distances between residues in the binding site are reflected in different carbohydrate specificities (Dam et al., 1998) and in different levels of biological activity (Fig. 7; Table 2).

Differences in distance between Arg228 and Asn14, and between Arg228 and Try100, play an important role in the depth of the carbohydrate binding site in DRL and DVL. Arg228 drive the interaction and can favor the binding with glycans in proximal position (such as DRL) or reduce the interaction in distal position (such as DVL). This was previously described to *Dioclea virgata* lectin (DvirL) and some other *Diocleinae* lectins (Nóbrega et al., 2012). Likewise, the width of the site depends on the distance between Tyr100 and Tyr12. These distances determine the geometry of the carbohydrate binding pocket. The overall geometry (and consequently the biological properties) is the result of the distances between all the residues in the CRD. The relatively narrow and deep CRD in DVL promotes low lectin–carbohydrate interaction; in contrast, the wide and shallow CRD in DRL favors interaction.

Our comparison of the three-dimensional structures of DVL and DRL helps to shed light on the relation between structure and function in *Diocleinae* lectins. CRD conformation is probably the main structural aspect determining lectin–glycan interactions in smooth muscle cells mediated by NO and prostaglandin biosynthetic pathways.

## 4. Conclusion

Lectins isolated from the seeds of *D. violacea* and *D. rostrata* were shown to induce different levels of relaxation in isolated endothelized rat aorta. Relaxation induced by DRL involved NO, prostacyclin and EDHF. Relaxation induced by DVL involved NO only. The biological properties of *Diocleinae* lectins depend on several factors, including pH-dependent oligomerization, the position of residues in the carbohydrate binding site and small conformational changes in key amino acids (Tyr12, Asn14, Tyr 100 and Arg228). Differences in the distance between these key residues are reflected in the level of vasorelaxation induced by DVL and DRL in vascular smooth muscle. These variations in distances are caused by side chain conformations which leads to changes in the site volume. Arg228 drive the interaction and can favor the binding with glycans in proximal position or reduce the interaction in distal position. Based on the site volume, it was confirmed that the interaction with glycans, that stimulates the activity of nitric oxide syntase and promotes NO production, is more effective in DRL that presents the wide and shallow CRD.

## Acknowledgments

This study was partly funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We would also like to thank Laboratório Nacional de Luz Síncrotron (LNLS), Campinas, Brasil. KSN, CSN, AHS, PD, BAMR, AMSA and BSC are senior investigators of CNPq. Additional funding was provided by the Doktoratskolleg "Molecular Enzymology" Grant W901-B05 from the Austrian Science Fund (to KG and GAB).

## References

Adams PD, Afonine PV, Bunkóczi G, Chen VB, Davis IW, Echols N, et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallographica Section D 2010;66:213–21.
Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of Molecular Biology 1990;215:403–10.
Andrade JL, Arruda S, Barbosa T, Paim L, Ramos MV, Cavada BS, et al. Lectin-induced nitric oxide production. Cell Immunology 1999;194:98–102.
Assreuy AMS, Fontenele SR, Pires ADF, Fernandes DC, Rodrigues NVFC, Bezerra EHS, et al. Vasodilator effects of *Diocleinae* lectins from the *Canavalia* genus. Naunyn Schmiedebergs Archives of Pharmacology 2009;380:509–21.
Assreuy AM, Martins GJ, Moreira ME, Brito GA, Cavada BS, Ribeiro RA, et al. Prevention of cyclophosphamide-induced hemorrhagic cystitis by glucose-mannose binding plant lectins. Journal of Urology 1999;161:1988–93.
Assreuy AM, Shibuya MD, Martins GJ, Souza ML, Cavada BS, Moreira RA, et al. Anti-inflammatory effect of glucose-mannose binding lectins isolated from Brazilian beans. Mediators of Inflammation 1997;6:201–10.
Barbosa T, Arruda S, Cavada BS, Grangeiro T, Freitas LAR, Barral-Neto M. In vivo lymphocyte activation and apoptosis by lectins of the *Diocleinae* subtribe. Memorias do Instituto Oswaldo Cruz 2001;96:673–8.
Barral-Netto M, Santos SB, Barral A, Moreira LIM, Santos CF, Moreira RA, et al. Human lymphocyte stimulation by legume lectins from the *Diocleae* tribe. Immunological Investigations 1992;21:297–303.
Bento CA, Cavada BS, Oliveira JT, Moreira RA, Barja-Fidalgo C. Rat paw edema and leukocyte immigration induced by plant lectins. Agents Actions 1993;38:48–54.
Bezerra EHS, Rocha BAM, Nagano CS, Bezerra GAA, Moura TR, Bezerra MJB, et al. Structural analysis of ConBr reveals molecular correlation between the carbohydrate recognition domain and endothelial NO synthase activation. Biochemical and Biophysical Research Communications 2011;408:566–70.
Blow DM. Rearrangement of Cruickshank's formulae for the diffraction-component precision index. Acta Crystallographica Section D 2002;58:792–7.
Bouckaert J, Dewallef Y, Poortmans F, Wyns L, Loris R. The structural features of Concanavalin A governing non-proline peptide isomerization. Journal of Biological Chemistry 2000;275:19778–87.
Brinda KV, Mitra N, Surolia A, Vishveshwara S. Determinants of quaternary association in legume lectins. Protein Science 2004;13:1735–49.
Calvete JJ, Thole HH, Raida M, Urbanke C, Romero A, Grangeiro TB, et al. Molecular characterization and crystallization of *Diocleinae* lectins. Biochimica et Biophysica Acta 1999;1430:367–75.
Carmignac V, Durbeej M. Cell–matrix interactions in muscle disease. Journal of Pathology 2012;226:200–18.
Cavada BS, Barbosa T, Arruda S, Grangeiro TB, Barral-Netto M. Revisiting Proteus: do minor changes in lectin structure matter in biological activity lessons from and potential biotechnological uses of the *Diocleinae* subtribe lectins. Current Protein and Peptide Science 2001;2:123–35.
Cavada BS, Grangeiro TB, Ramos MV, Cordeiro EF, Oliveira JTA, Moreira RA. Isolation and partial characterization of a lectin from *Dioclea rostrata* Benth seeds. Revista Brasileira de Fisiologia Vegetal 1996;8:31–6.
Chang DT, Oyang Y, Lin J. MEDock: a web server for efficient prediction of ligand binding sites based on a novel optimization algorithm. Nucleic Acids Research 2005;33:233–8.
Chen VB, Arendall WB, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, et al. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallographica Section D 2010;66:12–21.
Collaborative Computational Project Number 4. The CCP4 suite: programs for protein crystallography. Acta Crystallographica Section D 1994;50:760–3.
Dam TK, Cavada BS, Grangeiro TB, Santos CF, Sousa FAM, Oscarsoni S, et al. Diocleinae Lectins are a group of proteins with conserved binding sites for the core trimannoside of asparagine-linked oligosaccharides and differential specificities for complex carbohydrates. Journal of Biological Chemistry 1998;273:12082–8.
Delatorre P, Rocha BAM, Simões RC, Pereira-Júnior FN, Silva HC, Bezerra EHS, et al. X-ray diffraction analysis of two crystal types of *Dioclea virgata* lectin: an antinociceptive protein candidate to structure/function analysis. Applied Biochemistry and Biotechnology 2011;164:741–54.
Edwards G, Dora KA, Gardener MJ, Garland CJ, Weston AH. $K^+$ is an endothelium-derived hyperpolarizing factor in rat arteries. Nature 1998;396:269–72.
Evans PR. Scaling and assessment of data quality. Acta Crystallographica Section D 2006;62:72–82.
Figueiredo JG, Bitencourt FS, Mota MRL, Silvestre PP, Aguiar CN, Benevides RG, et al. Pharmacological analysis of the neutrophil migration induced by *D. rostrata* lectin: involvement of cytokines and nitric oxide. Toxicon 2009;54:736–44.
Furchgott RT, Zawadzki ZV. The obligatory role of the endothelial cells in relaxation of arterial smooth muscle by acetycholine. Nature 1980;288:373–6.
Gadelha CAA, Moreno FBMB, Santi-Gadelha T, Cajazeiras JB, Rocha BAM, Assreuy AMS, et al. Native crystal structure of a nitric oxide-releasing lectin from the seeds of *Canavalia maritime*. Journal of Structural Biology 2005;152:185–94.
Jancarik J, Kim SH. Sparse matrix sampling: a screening method for crystallization of proteins. Journal of Applied Crystallography 1991;24:409–11.
Kabsch W. XDS. Acta Crystallographica Section D 2010;66:125–32.
Komath SS, Kavithab M, Swamy MJ. Beyond carbohydrate binding: new directions in plant lectin research. Organic and Biomolecular Chemistry 2006;4:973–88.

Loris R, Van Walle I, De Greve H, Beeckmans S, Deboeck F, Wyns L, et al. Structural basis of oligomannose recognition by the *Pterocarpus angolensis* seed lectin. Journal of Molecular Biology 2004;335:1227–40.

Loris R, Hamelryck T, Bouckaert J, Wyns L. Legume lectin structure. Biochemica et Biophysica Acta 1998;1383:9–36.

Matthews BW. The solvent content of protein crystals. Journal of Molecular Biology 1968;33:491–587.

Mccoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. Journal of Applied Crystallography 2007;40:658–74.

Mollace V, Muscoli C, Masini E, Cuzzocrea S, Salvemini D. Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors. Pharmacological Reviews 2005;57:17–52.

Moreira RA, Cordeiro EF, Ramos MV, Grangeiro TB, Martins JL, Oliveira JTA, et al. Isolation and partial characterization of a lectin from seeds of *Dioclea violacea*. Revista Brasileira de Fisiologia Vegetal 1996;8:23–9.

Nagano CS, Calvete JJ, Barenttino D, Pérez A, Cavada BS, Sanz L. Insights into the structural basis of the pH-dependent dimer–tetramer equilibrium through crystallographic analysis of recombinant Diocleinae lectins. Biochemistry Journal 2008;409:417–28.

Nóbrega RB, Rocha BAM, Gadelha CAA, Santi-Gadelha T, Pires AF, Assreuy AMS, et al. Structure of *Dioclea virgata* lectin: Relations between carbohydrate binding site and nitric oxide production. Biochimie 2012;94:900–6.

Oliveira TM, Delatorre P, Rocha BAM, Souza EP, Nascimento KS, Bezerra GA, et al. Crystal structure of *Dioclea rostrata* lectin: Insights into understanding the pH-dependent dimer-tetramer equilibrium and the structural basis for carbohydrate recognition in *Diocleinae* lectins. Journal of Structure Biology 2008;164:177–82.

Salvemini D. Regulation of ciclooxygenase enzymes by nitric oxide. Cellular and Molecular Life Science 1997;53:576–82.

Sanz-Aparicio J, Hermoso J, Grangeiro TB, Calvete JJ, Cavada BS. The crystal structure of *Canavalia brasiliensis* lectin suggests a correlation between its quaternary conformation and its distinct biological properties from Concanavalin A. FEBS Letters 1997;405:114–8.

Sharon N, Lis H. History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology 2004;14:53–62.

Van Damme EJM, Peumans WJ, Barral A, Rougè P. Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Critical Review in Plant Sciences 1998;17:575–692.

Wah DA, Romero A, Gallego del Sol F, Cavada BS, Ramos MV, Romero A, et al. Crystal structure of native and Cd/Cd substituted *Dioclea guianensis* seed lectin. A novel manganese-binding site and structural basis of dimer-tetramer association. Journal of Molecular Biology 2001;310:885–94.