In depth analysis on the carbohydrate-binding properties of a vasorelaxant lectin from *Dioclea lasiophylla* Mart Ex. Benth seeds

Benildo Sousa Cavada, Vanir Reis Pinto-Junior, Vinicius Jose Silva Osterne, Messias Vital Oliveira, Ivanice Bezerra Silva, Eva Pollyanna Peixe Laranjeira, Alana Freitas Pires, Jorge Luiz Coelho Domingos, Wandemberg Paiva Ferreira, Jeanlex Soares Sousa, Ana Maria Sampaio Assreuy, and Kyria Santiago Nascimento

1. Introduction

Lectins are proteins, or glycoproteins, with carbohydrate-binding properties able to interact with monosaccharides and oligosaccharides (Peumans & Van Damme, 1995; Van Holle & Van Damme, 2019). This property enables these molecules to perform different biological functions in the organisms in which they are present, such as bacteria, and even in complex organisms, such as animals, plants and humans. Among their functions, we can highlight roles in cellular communication, pathogen-host cell interaction, defense of plants against insects, and leukocyte recruitment in the immune system (Kumar et al., 2012; Mishra et al., 2019). Owing to their specific recognition of carbohydrates, these proteins have sparked interest in applications related to the agronomic, industrial, food and biomedical areas (Mishra et al., 2019; Tsaneva & Van Damme, 2020).

Lectins of the Diocleinae subtribe from the *Leguminosae* family are part of the most extensively studied class of lectins with several works demonstrating methods of purification, physicochemical and structural characterization, as well as biological activities (Cavada et al., 2018). Regarding structural properties, the monomers of these proteins are non-glycosylated and have molecular mass varying between 25-30 kDa, with a metal-binding site (MBS) and a carbohydrate recognition domain (CRD). Lectins in this subtribe show high similarity at primary structure level, with all amino acid residues from MBS and CRD being conserved. Despite this, these lectins have different types, or intensities, of effects in biological activity assays, suggesting that these small differences may influence the way that lectins interact with glycosylated receptors (Cavada et al., 2001, 2018). Another factor that can influence this interaction is the oligomerization of these proteins in solution that may be influenced by the environment in which they are found. Many lectins in this subtribe are present in dimeric or tetrameric form, depending on the pH of the medium (Nagano et al., 2008; Zamora-Caballero et al., 2015). Different oligomeric states can be important in establishing multivalent biological interactions, providing greater resistance to shear stress present in biological recognition systems, establishing interactions with greater kinetics than that of monovalent interaction systems, and providing different spatial arrangements from the same site recognition, favoring the stabilization of interactions (Cavada et al., 2018).
The genera *Canavalia*, *Dioclea*, *Cratyli*a and *Camptosema* are part of this subtribe, and the mannose-specific lectins purified from their seeds are known as ConA-like lectins, nomenclature used for reference lectins similar to ConA, the *Canavalia ensiformis* lectin, which was one of the first lectins to be studied, accumulating, to date, more than 100 years of published works involving this protein (Cavada et al., 2019). *Dioclea lasiophylla* lectin (DlyL), the protein of interest, is a ConA-like lectin purified from seeds through saline extraction, followed by affinity chromatography in Sephadex G-50 matrix (Pinto-Junior et al., 2013). Its primary and tertiary structures have already been determined by mass spectrometry and X-ray crystallography, respectively. Its CRD has also been investigated by molecular docking assays with N-glycans. It has been demonstrated that DlyL has proinflammatory and cytotoxic activity against C6 glioma cells (Leal et al., 2018; Pinto-Junior et al., 2017a).

With this previous accumulated data in mind, the present work aims to investigate the dynamic behavior of DlyL as well as the CRD properties and its relevant residues by analyzing the detailed interaction of the lectin with a mannose-derivative carbohydrate (Xman) and high mannose N-glycans (MAN3, MAN5 and MAN9) using molecular docking and dynamics. We evaluated the interactions of Xman (XMN), the ligand with which DlyL was co-crystallized, and high mannose N-glycans, important ligands at which the lectin can interact to elicit biological activities such as proinflammation and X-ray crystallography, respectively. Its CRD has also been investigated by molecular docking assays with N-glycans. It has been demonstrated that DlyL has proinflammatory and cytotoxic activity against C6 glioma cells (Leal et al., 2018; Pinto-Junior et al., 2017a).

### 2. Materials and methods

#### 2.1. In silico studies

##### 2.1.1. Molecular docking

To generate the coordinates of DlyL in complex with N-glycans, a molecular docking was performed using the same methodology as that used by Leal et al. (Leal et al. 2018). Before docking trials, the coordinates of the high mannose N-glycans (MAN3, MAN5 and MAN9; Supplementary Figure 1) were generated through the Carbohydrate Builder server (Woods Group, Glycan Web, University of Georgia at Athens, GA, http://glycam.org) and then minimized using GLYCAM_06 force field (Kirschner et al., 2008). The initial structures included the lectins, Ca$^{2+}$ and Mn$^{2+}$ ions, and with/without carbohydrate ligands. The system was solvated with the TIP3P water model and was neutralized by adding counter ions (Na$^+$). To create the solvated systems, 22,667, 22,669, 24,449, 25,107 and 27,005 water molecules were added to the DlyL, DlyL-XMN, DlyL-MAN3, DlyL-MAN5 and DlyL-MAN9 systems respectively, as well as a sodium ion in each system for neutralization.

Energy minimization was performed using the steepest descent and conjugate gradient methods with 10 kJ/mol as convergence criteria. The minimization was followed by 600,000 equilibration steps (500 ps) of NVT followed by 600,000 steps in the NPT ensemble. A temperature of 300 K was maintained using a Langevin thermostat with a friction coefficient of 1 ps$^{-1}$. In addition, an isotropic Monte-Carlo barostat was used for pressure control (Berendsen et al., 1984) to 1 bar. The simulation time step was set to 2 fs in conjunction with the SHAKE algorithm (Ryckaert et al., 1977) to constrain the covalent bonds involving hydrogen atoms. Long-distance electrostatic interactions were calculated by the Particle Mesh Ewald (PME) method with a threshold of 10 Å (Essmann et al., 1995). MD trajectory analysis consisted of root mean square deviation (RMSD), root mean square fluctuations (RMSF), radius of gyration (RoG), system energy, intermolecular hydrogen bonds (HB), contact frequency (CF) and solvent accessible surface area (SASA) to determine data quality and compare systems. Cpptraj (Roe and Cheatham III, 2013), Xmgrace and VMD (Humphrey et al., 1996) were used to analyze the MD simulation results of all systems.

##### 2.1.2. Molecular dynamics simulations

MD simulations were performed for the unbound and bound forms of DlyL to observe the molecular behavior of the lectin in its native form and when interacting with different ligands, as well as to evaluate the stability and compaction of the biomolecule. For the bound forms, the PDB ID 6CJ9 model was used to evaluate the interaction of DlyL with Xman, and in the case of MAN3, MAN5 and MAN9, coordinates from the molecular docking have been used. In this study, 100 ns (20,000 frames) MD simulations were prepared using the Web-based CHARMM-GUI (Jo et al., 2008; Lee et al., 2020) and performed in the AMBER20 suite (Case et al., 2020) with parameters of ff19SB force field (Tian et al., 2020) for the system and GLYCAM_06j for the carbohydrates (Kirschner et al., 2008). The initial structures included the lectins, Ca$^{2+}$ and Mn$^{2+}$ ions, and with/without carbohydrate ligands. The system was solvated with the TIP3P water model and was neutralized by adding counter ions (Na$^+$). To create the solvated systems, 22,667, 22,669, 24,449, 25,107 and 27,005 water molecules were added to the DlyL, DlyL-XMN, DlyL-MAN3, DlyL-MAN5 and DlyL-MAN9 systems respectively, as well as a sodium ion in each system for neutralization.

Energy minimization was performed using the steepest descent and conjugate gradient methods with 10 kJ/mol as convergence criteria. The minimization was followed by 600,000 equilibration steps (500 ps) of NVT followed by 600,000 steps in the NPT ensemble. A temperature of 300 K was maintained using a Langevin thermostat with a friction coefficient of 1 ps$^{-1}$. In addition, an isotropic Monte-Carlo barostat was used for pressure control (Berendsen et al., 1984) to 1 bar. The simulation time step was set to 2 fs in conjunction with the SHAKE algorithm (Ryckaert et al., 1977) to constrain the covalent bonds involving hydrogen atoms. Long-distance electrostatic interactions were calculated by the Particle Mesh Ewald (PME) method with a threshold of 10 Å (Essmann et al., 1995). MD trajectory analysis consisted of root mean square deviation (RMSD), root mean square fluctuations (RMSF), radius of gyration (RoG), system energy, intermolecular hydrogen bonds (HB), contact frequency (CF) and solvent accessible surface area (SASA) to determine data quality and compare systems. Cpptraj (Roe and Cheatham III, 2013), Xmgrace and VMD (Humphrey et al., 1996) were used to analyze the MD simulation results of all systems.

##### 2.1.3. Binding free energy calculation

The post-processing trajectory analysis technique, binding free energy estimation, was carried out on all the simulated DlyL-carbohydrate complexes using the MMPBSA.py (Case et al., 2005; Gohlke & Case, 2004; Miller et al., 2012) script available in AMBERTOOLS20 (Case et al., 2020). The GB model (igb = 2, saltcon = 0.15) was used to compute binding free energy for all four DlyL-carbohydrate complexes using the molecular mechanics generalized Born (GB) model representation of the ligands are shown in Supplementary Figure 2.
The binding free energy was calculated as:

$$
\Delta G_{\text{binding}} = G_{\text{complex}} - G_{\text{protein}} - G_{\text{ligand}}
$$

where $$\Delta G_{\text{binding}}$$ is the binding free energy, $$G_{\text{complex}}$$ is the free energy of the complex, $$G_{\text{protein}}$$ is the free energy of protein, and $$G_{\text{ligand}}$$ is the free energy of ligand. Further, the binding free energy was calculated using the following equation:

$$
\Delta G_{\text{total}} = \Delta E_{\text{MM}} + \Delta G_{\text{GB}} + \Delta G_{\text{NP}} - T\Delta S
$$

where $$\Delta E_{\text{MM}}$$ is the interaction energy of the gas phase estimated by the SANDER program for the DlyL complexes with carbohydrates, and $$\Delta G_{\text{GB}}$$ and $$\Delta G_{\text{NP}}$$ correspond to the energetic contribution of polar and nonpolar components, respectively. The term $$- T\Delta S$$ corresponds to conformational entropy variation from carbohydrate binding. The main objective of the present investigation was to obtain comparisons relative to binding free energy and not to calculate the absolute value; therefore, entropy has not been calculated. In addition, entropy calculation procedures require high processing and can still overestimate entropy values. The $$\Delta G_{\text{GB}}$$ corresponds to the polar solvation-free energy calculated using GB equations present in AMBERTOOLS20, while $$\Delta G_{\text{NP}}$$ corresponds to the nonpolar contribution. In addition, the nonpolar component was calculated using the following equation:

$$
\Delta G_{\text{NP}} = \gamma \text{SASA} + \beta,
$$

where $$\gamma$$ represents the surface tension and is set to 0.0072 kcal/(molÅ²), $$\beta$$ is set to 0.92 kcal/mol, and SASA is the solvent accessible surface area. The default dielectric parameters were set to 1 and 80 for interior solute and exterior solvent, respectively.

From the MM/GBSA data, the free energy decomposition (FED) of the DlyL-carbohydrate complexes was also performed. Thus, it was possible to evaluate which residues contribute energetically to binding free energy for the formation of the complex (Case et al., 2005; Gohlke & Case, 2004; Miller et al., 2012). The comp module present in AMBER20 was used for this purpose. It was also possible to determine the contribution of four important energy components for each residue, namely electrostatic ($$\Delta G_{\text{ELE}}$$), van der Waals ($$\Delta G_{\text{VDW}}$$), polar solvation ($$\Delta G_{\text{P}}$$) and nonpolar solvation ($$\Delta G_{\text{NP}}$$). Potential energy-contributing residues were selected based on an energy value greater than $$-1$$ kcal/mol.

$$
\Delta G_{\text{carbohydrate–residue}} = \Delta G_{\text{ELE}} + \Delta G_{\text{VDW}} + \Delta G_{\text{P}} + \Delta G_{\text{NP}}
$$

### 2.2. In vitro assays

#### 2.2.1. DlyL purification

DlyL was purified from powdered *Dioclea lasiophylla* seeds as described previously (Pinto-Junior et al., 2013). Peeled seeds were ground in a coffee grinder (Cadence ™ MDR301 Monovolt, Cadence Design Systems, San Jose, CA, USA) until a fine powder was obtained. Then, this material was subjected to saline extraction (150 mM NaCl; 1:10 w/v ratio) under continuous agitation (4 h, 25 °C) to soluble proteins. The insoluble material was separated by centrifugation at 10,000 × g at 4 °C for 20 min (Eppendorf Centrifuge 5810 R). The resulting supernatant (crude extract) was applied to a Sephadex® G-50 affinity column (6.5 × 1.8 cm, GE Healthcare, Little Chalfont, UK) equilibrated with the same extraction solution containing CaCl₂ and MnCl₂, both at 5 mM. Unbound material was eluted with the equilibration solution, and the lectin was eluted with 100 mM D-glucose added to the equilibration solution. The purified lectin was dialyzed against ultrapure water and lyophilized for further analysis.

#### 2.2.2. Isolation of aorta

Male Wistar rats (250–300 g), treated in accordance with the principles established by the Ethics Committee of UECE (CEUA N° 10130208-8/40), were sacrificed by stunning, and the thoracic aorta was removed. Aortic 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 MgCl₂, 2 CaCl₂, 0.36 NaH₂PO₄, 11.9 NaHCO₃, and 5.5 glucose) at pH = 7.4, 37 °C, 95% O₂, and 5% CO₂. After an equilibrium period of 40 min, rings were challenged with 60 mM KCl to ensure tissue viability. The contractile response was measured using a force transducer connected to a pre-amplifier and computerized data acquisition system (Chart 4.1; PowerLab AD Instruments, Inc., Australia).

#### 2.2.3. Evaluation of relaxant effects

DlyL was added in cumulative concentrations (1, 3, 10, 30 and 100 μg/ml) at 10 min intervals to tissues precontracted with phenylephrine (0.1 μM) in either endothelium intact or denuded aorta. The endothelium removal was performed by mechanical rubbing of the internal aortic surface and intact endothelium considered for relaxant responses to acetylcholine (1 μM) greater than 75% (Furchgott & Zawadzki, 1980).

Effect of endothelium-derived relaxant factor (EDRF) nitric oxide on DlyL vasorelaxation was assessed by incubation of endothelized aorta with atropine (1 μM) antagonist at muscarinic receptor, N-(G)-nitro-L-arginine methyl ester (L-NAME; 100 μM), inhibitor of nitric oxide synthase, and 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ; 10 μM) inhibitor of guanylate cyclase, 30 min before addition of lectin on the plateau of phenylephrine-induced contraction. Involvement of the CRD was evaluated by the lectin (30 μg/ml) incubation with its binding sugar glucose (0.1 M) before addition into tissues.

### 3. Results and discussion

#### 3.1. In silico results

##### 3.1.1. Molecular docking

Representations of DlyL with high mannose N-glycans were the same as those obtained in the work of Leal et al. (Leal et al. 2018). The docking scores obtained for MAN3, MAN5 and MAN9 were −44.57, −49.73 and −46.35, respectively. All

---

**References:**

- Case et al., 2005
- Gohlke & Case, 2004
- Miller et al., 2012
- Pinto-Junior et al., 2013
- Furchgott & Zawadzki, 1980
- Leal et al. 2018
of them were close to the Xman docking score (-46.03), as obtained from redocking (Table 1). In addition, the pose adopted by Xman at the redocking was quite similar to that observed in the crystallographic structure (PDB ID: 6CJ9; Leal et al., 2018), which validates the applied docking settings (Supplementary Figure 3A). The torsions suffered by MAN3 (greater penalty for torsion than Xman) resulted in a conformation with a smaller contribution from non-polar interactions in the formation of the complex with DlyL, and these two factors negatively contributed to the final docking score resulting in a lower value than that of Xman (Table 1). For MAN5, although the torsion penalty is higher than MAN3, the position of the ligand favored buried interactions that resulted in the largest contribution when compared to all tested ligands, with a value more favorable than the others. This resulted in a high docking score for the DlyL-MAN5 complex, being higher than the docking score of MAN9, which had a higher contribution of hydrogen and non-polar interactions.

DlyL contains a highly conserved metal binding site (MBS) located in the vicinity of the CRD (Figure 1A). DlyL’ CRD is formed by four discontinuous segments: Tyr12-Phe13-Asn14-Thr15-Asp16, Gly98-Leu99-Tyr100, Ala207-Asp208 and Gly227-Arg228-Leu229. Redocking data demonstrates that a network of hydrogen bonds connecting Tyr12, Asn14, Leu99, Tyr100, Asp208 and Arg228 to the atoms N1, O3, O4, O5 and O6 of the Xman molecule stabilize the complex, along with van der Waals and hydrophobic interactions involving residues Gly98, Ala207 and Gly227 (Supplementary Figure 3B). The interactions are shown in Supplementary Figure 4 and were similar to those observed in the crystallographic structure (PDB ID: 6CJ9; Leal et al., 2018). For the N-glycans, DlyL interacted mainly via a terminal mannosyl residue with secondary interactions involving other regions of the ligand. The DlyL-MAN3 complex (Figure 1B) demonstrated interactions involving the Tyr12, Asn14, Leu99, Tyr100, Asp208 and Arg228 residues and the O3, O4, O5 and O6 atoms of a terminal mannosyl residue. In addition, hydrogen interactions also occur between Tyr12 and Tyr100 with the O6 atom of the first GlcNAc residue from glycan chain. Thr15, Asp16, Gly99, Ala207 and Gly227 amino acid residues are involved in non-polar interactions (Supplementary Figure 5). For DlyL-MAN5 complex (Figure 1C), most of the hydrogen interactions occurred in the terminal mannosyl residue of the smallest branch. The Tyr12, Asn14, Leu99, Tyr100, Asp208 and Arg228 amino acid residues formed hydrogen bonds with the O2, O3, O4, O5 and O6 atoms of the terminal mannosyl residue of the smallest branch. Unlike complexes with Xman or MAN3, in this case, all available oxygen atoms participated of the interactions. In addition, Tyr12 and Tyr100 formed hydrogen interactions with the O4 and O6 atoms of the second GlcNAc residue of the glycan chain, and Arg228 also formed with the O3 atom of a terminal mannosyl residue of the larger branch. Furthermore, Thr15, Asp16, Gly98, Arg204, Asp205, Ala207 and Gly227 residues are involved in non-polar interactions (Supplementary Figure 6). The hydrogen interactions of the DlyL-MAN9 complex (Figure 1D) were more distributed and occurred mainly in two mannosyl residues present in terminal region of one of the ramifications from larger branch. The Ser168 residue forms interactions with O3 and O4 atoms of the terminal mannosyl residue, while Asn14, Leu99 and Asp208 form interactions with O4 and O5 atoms of the second mannosyl residue. In addition, Tyr100 forms interaction with O4 atom of a mannosyl residue from the other ramifications of the larger branch. Tyr12, Asp16, Ala207, Gly224, Gly226 and Arg228 participate in non-polar interactions (Supplementary Figure 7). It is worth mentioning that this was the only case where Arg228 did not form hydrogen bonds and that Ser168 participated in the interactions with ligands.

Molecular docking data provided information on the CRD residues that form interactions with ligands, but in order to evaluate these complexes in more detail as well as to rule out possible random interactions that may take place in the surface of the protein, the coordinates generated from the molecular docking were submitted to molecular dynamics simulations.

### 3.1.2. Molecular dynamics

Simulations of bound and unbound forms of DlyL were prepared in CHARMM-GUI and performed in the AMBER20 suite. For minimization, all systems were subjected to 7500 steps of steepest descent method and 3,400, 4,000, 2,700 and 2,000 steps of conjugate gradient method for DlyL, DlyL-XMN, DlyL-MAN3, DlyL-MAN5 and DlyL-MAN9, respectively. The DlyL and DlyL-XMN systems reached an energy value of $-3.02e+05$ kcal/mol, DlyL-MAN3, DlyL-MAN5 and DlyL-MAN9 systems reached the values of $-3.25e+05$, $-3.57e+05$ and $-3.33e+05$ kcal/mol, respectively.

All simulations were set for a time of 100 ns, with about 20,000 frames, at pressure of 1 bar and temperature of 300K. Furthermore, pre-analysis of the trajectories demonstrated that they were correctly and completely processed, and in the case of the bound form of DlyL, the ligands Xman, MAN3, MAN5 and MAN9 continued to interact with the CRD cavity throughout the trajectories. Then, analyses were performed to understand the structural stability of the protein, such as RMSD, RMSF, RoG and SASA, as well as to evaluate the interactions of DlyL with the ligands through contact frequency of the residues (interactions above 4 Å) and the hydrogen bonds (HB) number and profile along the

| Ligand | Score | H-bonds contribution | Non-Polar contribution | Buried contribution | Ligand penalty |
|-------|-------|----------------------|------------------------|-------------------|----------------|
| XMN  | -46.03 | -8.63                | -34.31                 | 3.42              | 0.33          |
| MAN3 | -44.57 | -10.68               | -30.06                 | 4.69              | 4.80          |
| MAN5 | -49.73 | -11.08               | -33.14                 | -11.11            | 5.60          |
| MAN9 | -46.35 | -12.60               | -39.34                 | -3.31             | 8.90          |

### Table 1. Summary of docking results of DlyL interacting with carbohydrates (in arbitrary units).
trajectory. From this series of assessments, it was possible to observe the stability of the complexes, the molecular behavior of the lectin as it interacted with different ligands, allowing to make a determination of key CRD residues that interacted with simpler carbohydrates, such as Xman, as well as N-glycans, such as MAN3, MAN5 and MAN9.

The RMSD graph (Figure 2A) indicates that all trajectories reached equilibrium close to 12.5 ns, with values varying between 1.5 and 2.5 Å. This demonstrates that the molecule has good stability when transitioning from its minimized state to a solvated molecule. This behavior was maintained when the protein was in complex with ligands. The average
RMSD values for DlyL, DlyL-XMN, DlyL-MAN3, DlyL-MAN5 and DlyL-MAN9 are 1.80 Å, 1.93 Å, 1.98 Å, 2.02 Å and 1.91 Å, respectively. These results demonstrate small non-significant deviations; therefore, it can be concluded that DlyL in complex with ligands has an RMSD profile similar to that of unbound DlyL. This profile was also seen in simulations of lectins from *Dioclea reflexa* (DrlL), *Dioclea sclerocarpa* (DSL), *Canavalia bonariensis* (CaBo), *Canavalia brasiliensis* (ConBr) and ConA (Cavada et al., 2019, 2020a; Pinto-Junior et al., 2017b). RMSF, using theoretical B-factors from systems, is a structural parameter to assess the flexible and nonflexible regions of the protein in its unbound form and in complex with ligands. In the RMSF plot (Figure 2B), region 112-125, which does not contain amino acids involved with the CRD, we observed some differences in fluctuations also noticed in simulations of the same Diocleinae lectins mentioned above, but also with other legume lectins, such as *Pisum arvense* (PAL) and *Centrolobium microchaete* (CML) (Neco et al. 2018; Pinto-Junior et al., 2017c). In general, when the protein is complexed with Xman, several regions have less flexibility. When in complex with N-glycans, however, we see a very slight increase in flexibility, but no major differences. This slight increase probably results from the greater number of interactions between proteins and N-glycans, which could affect the molecule in different regions.

To evaluate the folding or compacting of the lectin in complex with carbohydrates, the radius of gyration (RoG) of the protein was evaluated during the trajectories. In this study, as shown by RoG plot (Figure 3A), the interaction with carbohydrates does not generate significant changes in the compaction of the molecule. The average RoG values for DlyL, DlyL-XMN, DlyL-MAN3, DlyL-MAN5 and DlyL-MAN9 are 17.32 Å, 17.35 Å, 17.38 Å, 17.40 Å and 17.36 Å, respectively. When evaluating the graph with RoG vs. total energy of the complexes (Figure 3B), we see no changes in RoG values when interacting with Xman. However, DlyL adopts a different energetic state compared to unbound DlyL, or in complex with N-glycans (−4400 to −3700 kcal/mol), reaching values of energy between −3500 and −2700 kcal/mol. The average total energy values in kcal/mol for DlyL, DlyL-XMN, DlyL-MAN3, DlyL-MAN5 and DlyL-MAN9 are −4040.59, −3086.69, −3878.27, −4041.05 and −4128.26, respectively. The DlyL-MAN3 complex at times during the trajectory also shifts to a state similar to that of the Xman complex, most likely by its smaller size compared to MAN5 and MAN9. The behavior and interactions between DlyL and MAN3 should be similar when in complex with Xman at some points.

SASA analysis of the systems was performed to understand the behavior of the protein solvent and evaluate the impact of interaction with carbohydrates on this behavior (Figure 4A). The solvent can interact with the protein by polar and nonpolar interactions, but the interaction with carbohydrates can change the conformation of the protein in a way that affects solvent accessibility. As expected, DlyL undergoes a small reduction in SASA after interacting with ligands since carbohydrates interact with the surface of the molecule in CRD cavity. However, no significant variations were noted when comparing the SASA values for simpler molecules, such as Xman, or larger molecules, such as MAN9, most likely because they affected accessibility of the same residues in the same region of the protein. The average SASA values for DlyL, DlyL-XMN, DlyL-MAN3, DlyL-MAN5 and DlyL-MAN9 are 10199.62 Å, 9898.48 Å, 9874.55 Å, 9904.90 Å and 9762.55 Å, respectively. By delimiting the analysis to the DlyL CRD region, we can better observe the impact of the interaction with the ligands (Figure 4B). The average SASA-CRD values for DlyL, DlyL-XMN, DlyL-MAN3, DlyL-MAN5 and DlyL-MAN9 are 904.31 Å, 711.26 Å, 583.19 Å, 613.78 Å and 526.86 Å, respectively. With the exception of MAN5, it is possible to observe that the larger the ligand, the lower the SASA values. For a better understanding of the behavior of MAN5 and other ligands, it is necessary to observe the interactions and contacts between DlyL and carbohydrates.

All data from the structural stability analysis show that DlyL has a very stable structure without significant changes in its folding properties. Some change in the CRD region was observed, depending on the interacting ligand, but nothing that would affect the folding of the protein. However, subtle changes in the interaction with Xman were enough to change its energy state. This stability has been reported in
other studies involving Diocleinae lectins, such as DrfL, DSL, ConA and ConBr (Cavada et al., 2019; Pinto-Junior et al., 2017b). These lectins are very similar structurally, but many studies show that they present differences in intensity of effects in several biological assays, e.g. inflammatory response, vasorelaxation or cytotoxicity against tumor cells (Cavada et al., 2018, 2019, 2020a; Leal et al. 2018; Pinto-Junior et al., 2017b). It was possible to observe that the DlyL fold is very stable and does not present significant changes when interacting with carbohydrates, a behavior profile similar to other Diocleinae lectins. Thus, the pH-dependent oligomerization and the behavior of CRD when interacting with glycosylated receptors are the most likely factors to explain these differences in intensity and the resultant effects on biological activities (Leal et al. 2018; Nagano et al., 2008; Zamora-Caballero et al., 2015).

### 3.1.3. Binding analysis

The interactions of hydrogen and other contacts of DlyL with Xman, MAN3, MAN5 and MAN9 showed interesting changes, depending on the structural complexity of the ligand, revealing residues not reported in previous works involving Diocleinae lectins. The average number of hydrogen bonds for DlyL, DlyL-XMN, DlyL-MAN3, DlyL-MAN5 and DlyL-MAN9 is 3.2, 3.6, 4.3 and 6, respectively (Figure 5). With the increase of ligand size, the number of hydrogen interactions increases, but the interaction with Xman shows a more stable behavior during the trajectory, while the N-glycans show sudden variations in the number of interactions along the dynamics. Although, at some points, some ligands reach 0 interactions, at no point in all trajectories the carbohydrates have stopped interacting with the CRD. The list of hydrogen pairs and the frequency with which interactions occur are found in Supplementary Table 1.

Regardless of the ligand, we see the high frequency of hydrogen interactions for Asp208 atoms, as demonstrated in several studies involving Diocleinae lectins. This residue is part of a cis-peptide stabilized by the calcium ion, and it is located at the base of the CRD. Asp208 is essential for stabilizing the ligands within the site, once again demonstrating its importance with a frequency of interactions above 90% in all carbohydrates tested in this work (Cavada et al. 2018). For Xman, the most
frequent residues, in addition to Asp208, were Asn14, Leu99, Arg228, Tyr100 and Gly98, all residues, the participation of which is well documented in the literature, even statistical analysis from crystallography data (Cavada et al. 2018; Leal et al. 2018; Pinto-Junior et al., 2017b). These residues also participated in the interaction with N-glycans, albeit with differences in frequency, depending on the simulations in which they occur. Particular cases can also be reported. For example, with MAN3, it was possible to detect new residues interacting with this glycan, mainly Asp16 with a frequency above 50%, but it also had Thr15 and Tyr12 with frequencies above 5%. In the complex with MAN5, a high frequency of hydrogen interactions involving Tyr12 (25%), but low frequency for Asp16 (11%), was observed. In addition, Pro13 and Thr15 showed a frequency in the range of 11% and 10%, respectively. In the case of MAN9, Asp16 presented a frequency similar to that observed with MAN3 (58%), but Thr15 had a high frequency, around 47%, quite different from that observed for MAN3 and MAN5. Pro13 and Tyr12 also showed an increase in frequency compared to previous glycans, with values of 33% and 23%, respectively. In the DylL-MAN9 complex, a new interaction involving Ser168 was observed. It proved to be relevant presenting a frequency of 42%. Although the tables show all hydrogen interactions during trajectories, interactions with frequency below 5% were not considered. These data demonstrate that the DylL CRD goes farther than residues defined in the work of Leal et al. (Leal et al. 2018). When interacting with N-glycans, the site is expanded, with interactions that proved to be non-random. In addition, the interactions of the CRD with the tested N-glycans do not necessarily follow the same interaction frequency profile, despite being in the same class of N-glycans and despite having the same residues as participants in the interactions.

Frequency of contact for interactions above 4 Å (van der Waals and hydrophobic) also showed a significant participation of loops 12-16, 97-100, and 223-228, as well as residues 97-100, 168 and 204-208 (Figure 6). Again, residues Pro13, Thr15 and Asp16 were not relevant to the interaction with Xman; instead, they had more relevance to contact frequency with N-glycans. In addition, Ser21, Arg204 and Asp205 had a frequency above 20%, but only with MAN3, while Ser168 (85% of frequency), Ser223, Gly224 and Gly226 interacted with MAN9. MAN5 did not present any exclusive contact. The remaining residues show similar frequencies with few variations.

From these data, we can define the DylL CRD as formed by residues Tyr12, Pro13, Asn14, Thr15, Asp16, Ser21 (only MAN3), Thr97, Gly98, Leu99, Tyr100, Arg204 (only MAN9), Asp205 (only MAN9), Ala207, Asp208, Ser168 (only MAN9), Ser223 (only MAN9), Gly224 (only MAN9), Gly226, Gly227 and Arg228. All can interact with carbohydrates via polar and nonpolar interactions, indicating a domain more expansive and dynamic than that previously reported in structural studies of Diocleinae lectins (Cavada et al. 2018).

### 3.1.4. MM/Gbsa analysis

The binding free energy calculated by MMGBSA for the interactions of DylL with the carbohydrates tested in the simulations were $-30.12 \pm 3.39$, $-30.48 \pm 5.74$, $-25.84 \pm 5.58$ and $-38.90 \pm 12.35$ kcal/mol for Xman, MAN3, MAN5 and MAN9, respectively, and the detailed values can be found in Table 2. Although the free energy values of Xman, MAN3 and MAN5 are technically equal, it is possible to observe a profile different from that obtained in the molecular docking, where MAN5 had a higher docking score, but a lower free energy value, in MM/GBSA analysis. This could be explained by the docking used in the GOLD software which is semi-flexible in selected residues, whereas molecular dynamics allows for much higher flexibility based on the restriction of the connections and of the force field used. The DylL-MAN9 complex, on the other hand, obtained a high value of free energy, demonstrating greater stability compared to the other complexes.

The analysis of energy decomposition to assess the contribution of each protein residue to free binding energy (Figure 7) again showed the importance of Tyr12, Pro13, Asn14, Thr15, Asn16, Thr97, Gly98, Leu99, Tyr100, Ala207, Asp208, Gly227 and Arg228 residues for their interaction with carbohydrates, all with a favorable contribution to a negative free energy. However, differences were noted in the energy profile, depending on the ligand. For instance, loop 12-16 contributes less to the interaction with Xman and more to interaction with N-glycans. The energy contribution of residue Asn14, which is considered one of the main residues of the CRD from Diocleinae lectins, could be observed for Xman, but it decreases considerably during interaction with N-glycans, despite having a high frequency of contact by polar and nonpolar interactions (Supplementary Table 1; Figure 6). With N-glycans, residues Tyr12, Pro13, Thr15 and Asp16 gain more prominence, with higher values of energy contribution. These residues have a high frequency of contact in interactions above 4 Å, with a majority above 50% (Figure 6), and in the case of hydrogen interactions, they show high frequency and/or a greater number of hydrogen pairs formed during the trajectory (Supplementary Tables 2-4). The 97-100 loop exerted considerable energy contribution to all binders. Residue Thr97 was subtler as its contribution was less than the other residues in this region and occurred only in the trajectories of Xman and MAN3 (Figure 7A and B). Residue Gly98 contributes in all cases, but its contribution decreases with increase in the complexity of the ligand. Residues Leu99 and Tyr100 contributed most, with Leu99 having the greatest contribution in the trajectories of Xman and MAN9 (Figure 7A and D) and Tyr100 having the greatest contribution in MAN3 and MAN5 (Figure 7B and C). The contact frequency of Thr97 was much lower than the others, which explains the low contribution. Gly98, Leu99 and Tyr100 had very similar contact frequencies, and in the case of hydrogen interactions, Leu99 had a higher frequency of contact with Xman and MAN3. However, this last Tyr100 obtained a greater number of hydrogen pairs formed with high frequency, justifying the greater energy contribution (Supplementary Tables 1 and 2). In the trajectories of MAN5 and MAN9, Tyr100 obtained hydrogen interaction with a higher frequency of contact, which could be an outlier because its energy contribution was less than that of Leu99, while the amount of hydrogen pairs formed was equivalent.
(Supplementary Tables 3 and 4). Because MAN9 is a much larger molecule than the others, it is likely that the interactions involving Leu99 were crucial for the stabilization of the complex. The cis-peptide Ala207-Asp208 participated in all ligands, again demonstrating its preeminence as the key residue for carbohydrate stabilization in CRD (Cavada et al., 2018). In all cases, they had a high frequency of contact, and the hydrogen interactions involving Asp208 were the most frequent in all trajectories. This has already been demonstrated for Diocleinae lectins and several other legume lectins, but in the present study, we provide more detail about the performance of this residue (Cagliari et al., 2018; Cavada et al., 2020a; Nascimento et al., 2020). Residues Gly227 and Arg228 also contribute to interaction, but their contribution decreases with increasing complexity of the ligand. The Ser168 residue contributed only to MAN9, as previously seen; this residue only forms interactions with this ligand, but does not appear to be random.

These energy data, together with the contact frequency data, reinforce the idea that DlyL is capable of interacting with these N-glycans, but in a different way from its interaction with simpler ligands such as Xman, the carbohydrate in which DlyL was cocry stallized with. In addition, interactions show no significant impact on folding and structural stability of the entire protein; the influence of the interaction only affects the CRD. It was only possible to demonstrate this through molecular dynamics simulations that are superior to static analyses derived only from crystallographic data or molecular docking. By detailing which residues are important for the interaction of each type of glycan, it is possible
to understand how lectins interact with glycosylated receptors, as well as generate useful information for engineering lectins and their applications, such as biosensors (Belicky et al., 2016; Hirabayashi & Arai, 2019).

### 3.2. Vasorelaxant properties

Phenylephrine induced stable tonic contractions of 0.36 ± 0.13 g in aortic ring preparations possessing an intact endothelium (n = 8) and of 0.68 ± 0.11 g in those in which endothelium had been mechanically removed (n = 6). In preparations with preserved endothelium, DlyL (IC50 = 9.8 ± 0.9 µg/ml) elicited relaxation at 10, 30 and 100 µg/ml in 42% ± 5.3, 89.7% ± 5.5, and 126.1% ± 11.9, respectively. In contrast, in preparations of denuded endothelium, cumulative addition of DlyL was not able to induce relaxant effect (Figure 8 and 9A). In addition, L-NAME and ODQ completely blocked the lectin effect, while the association of DlyL (30 µg/ml) with glucose reduced lectin relaxation to 40.9%. However, the muscarinic receptor antagonist did not change the relaxing effect of DlyL (30 µg/ml) (Figure 9B).

The present study demonstrated the importance of the endothelium in the relaxation induced by DlyL in rat aorta, as well as the role of the lectin domain and nitric oxide in this effect. In all the lectins studied so far, the mechanism of relaxation is endothelium-dependent and reversible by addition of the respective binding sugar (Assreuy et al., 2009; Bezerra et al. 2013; Gadelha et al., 2005). NO is the principal mediator of endothelium-dependent relaxation by acetylcholine in vascular smooth muscles (Furchgott & Vanhoutte, 1989) and it seems to be that the endothelium-derived relaxing factor (EDRF) nitric...
oxide responsible for the lectin effect. In fact, our results showed that L-NAME and ODQ blocked the vasorelaxant effects of DlyL. After its synthesis by NOS, NO diffuses to adjacent smooth muscle cells and acts through activation of soluble guanylate cyclase, thereby increasing the intracellular GMPc concentration and promoting relaxation (Bauer & Sotnikova, 2010). Thus, the role of nitric oxide can be determined if relaxant responses are reduced in the presence of an inhibitor of nitric oxide synthase (Hogan et al., 2005) or by inhibition of NOS by several substituted L-arginine analogues, for example, by L-NAME (Epstein et al., 1993). In order to verify the participation of the enzyme and the NO/GMPc pathway, we incubated the preparations with L-NAME, a non-selective inhibitor of NOS, and ODQ, an inhibitor of the NO soluble guanylate cyclase (Garthwaite et al., 1995). Both inhibitors blocked the DlyL relaxant effect, confirming the participation of NO.

The aorta was incubated with atropine, a muscarinic receptor antagonist; however, modification of the relaxing effect of lectin was not observed, indicating that it must use other means to activate NOS. One possibility might be phosphorylation of NOS whereby, for example, protein kinase A (PKA) and protein kinase B (Akt) activate eNOS by phosphorylating Ser1177 independent of the calcium concentration (Zhao et al., 2015).

The literature has shown that other lectins of the Diocleinae subtribe present relaxant effects in rat aorta precontracted with phenylephrine via NO and lectin domain,
both in *Dioclea* (Bezerra et al. 2013; da Nóbrega et al., 2012; do Nascimento et al., 2012) and *Canavalia* (Assreuy et al., 2009; Gadelha et al. 2005; Kleha et al., 1991). In our study the relaxation induced by DlyL occurs via the lectin domain since the association with glucose was capable of reversing this effect.

High mannose N-glycans are found in the surface of endothelial cells and participate in some molecular processes of cell interaction and recognition (Gu et al., 2012; Schwarz & Aebi, 2011). MAN3, MAN5 and MAN9 glycans, for example, are anchored to glycoproteins, most of which are involved in cell recognition processes, immune system regulation and other metabolic processes (Parker et al., 2013; Rudd, 2001; Sparrow et al., 2008). Some biological effects, such as inflammation (Pinto-Junior et al., 2017a) and vasorelaxation, may be related to interactions of the lectin with glycosylated receptors in endothelial cells. All molecular dynamics simulations demonstrate that DlyL can recognize and interact with high mannose N-glycans with different interaction profiles, commonly found in endothelial cells.

### 4. Conclusion

In this work, the behavior of DlyL in its unbound form and its interaction with different carbohydrates were determined in detail. The results showed that DlyL is a very stable protein, like other Diocleinae lectins, and that its overall structure is not affected by interaction with the tested ligands. The impacts were seen at CRD level, which was a reflection of the different interaction profiles with different ligands. Despite these differences, DlyL was able to interact stably with simple carbohydrates and with high-mannose N-glycans. Furthermore, DlyL relaxes endothelialized rat aorta with mechanism involving NO and the carbohydrate-binding properties. The interaction of lectin with glycosylated receptors present on the surface of endothelial cells is the most accepted hypothesis to explain the vasorelaxant effect of DlyL and that of other Diocleinae lectins, however, further investigations are needed to fully understand the mechanism.

### Disclosure statement

No potential conflict of interest was reported by the authors.

### Funding

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). Cavada BS, Ferreira WP, Sousa JS, Assreuy AMS and Nascimento KS are senior investigators of CNPq/Brazil. David Martin helped with the English editing of the manuscript.

### References

- Assreuy, A. M. S., Fontenele, S. R., Pires, A. D. F., Fernandes, D. C., Rodrigues, N. V. F. C., Bezerra, E. H. S., Moura, T. R., do Nascimento, K. S., & Cavada, B. S. (2009). Vasodilator effects of Diocleinae lectins from the Canavalia genus. *Naunyn-Schmiedeberg’s Archives of Pharmacology*, 380(6), 509–521. https://doi.org/10.1002/j.0021-0009-0465-1
- Bauer, V., & Sotnikova, R. (2010). Nitric oxide – the endothelium-derived relaxing factor and its role in endothelial functions. *General Physiology and Biophysics*, 29(4), 319–340. https://doi.org/10.4149/gpb_2010_04_319
- Belčík, Š., Katriš, J., & Tkáč, J. (2016). Glycan and lectin biosensors. *Essays in Biochemistry*, 60(1), 37–47. https://doi.org/10.1042/EB20150005
- Berendsen, H. J. C., Postma, J. P. M., van Gunsteren, W. F., DiNola, A., & Haak, J. R. (1984). Molecular dynamics with coupling to an external bath. *The Journal of Chemical Physics*, 81(8), 3684–3690. https://doi.org/10.1063/1.448118
- Bezerra, M. J. B., Rodrigues, N. V. F. C., Pires, A. D. F., Bezerra, G. A., Nobre, C. B., Alencar, K. L. D. L., Soares, P. M. G., Nascimento, K. S. D., Nagano, C. S., Martins, J. L., Gruber, K., Sampaio, A. H., Delatorre, P., Rocha, B. A. M., Assreuy, A. M. S., & Cavada, B. S. (2013). Crystal structure of Dioclea violacea lectin and a comparative study of vasorelaxant properties with Dioclea rostrata lectin. *The International Journal of Biochemistry & Cell Biology*, 45(4), 807–815. https://doi.org/10.1016/j.ijbiocel.2013.01.012
- Cagliari, R., Kremer, F. S., & Pinto, L. D. S. (2018). Bauhinia lectins: Biochemical properties and biotechnological applications. *International Journal of Biological Macromolecules*, 119, 811–820. https://doi.org/10.1016/j.ijbiomac.2018.07.156
- Case, D. A., Belfon, K., Ben-Shalom, I., Brozell, S. R., Cerutti, D., Cheatham, T., Cruzeiro, V. W. D., Darden, T., Duke, R. E., Giambasu, G., & Gilson, M. (2020). *AMBER* 20. University of California.
- Case, D. A., Cheatham, T. E., Darden, T., Gohlke, H., Luo, R., Merz, K. M., Onufriev, A., Simmerling, C., Wang, B., & Woods, R. J. (2005). The Amber biomolecular simulation programs. *Journal of Computational Chemistry*, 26(16), 1668–1688. https://doi.org/10.1002/jcc.20290
- Cavada, B. S., Barbosa, T., Arruda, S., Grangeiro, T. B., & Barral-Netto, M. (2001). 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 & Peptide Science*, 2(2), 123–135. https://doi.org/10.2174/1389203013381152
- Cavada, B. S., Osterne, V. J. S., Lossio, C. F., Pinto-Junior, V. R., Oliveira, M. V., Silva, M. T. L., Leal, R. B., & Nascimento, K. S. (2019). One century of ConA and 40 years of ConBr research: A structural review. *International Journal of Biological Macromolecules*, 134, 901–911. https://doi.org/10.1016/j.ijbiomac.2019.05.100
- Cavada, B. S., Pinto-Junior, V. R., Osterne, V. J. S., Oliveira, M. V., Lossio, C. F., Silva, M. T. L., Bari, A. U., Lima, L. D., Souza-Filho, C. H. D., & Nascimento, K. S. (2020). Comprehensive review on Caelsalpinioideae lectins: From purification to biological activities. *International Journal of Biological Macromolecules*, 162, 333–348. https://doi.org/10.1016/j.ijbiomac.2020.06.161
- Cavada, B. S., Silva, M. T. L., Osterne, V. J. S., Pinto-Junior, V. R., Nascimento, A. P. M., Wolin, I. A. V., Heinrich, I. A., Nobre, C. A. S., Moreira, C. G., Lossio, C. F., Rocha, C. R. C., Martins, J. L., Nascimento, K. S., & Leal, R. B. (2018). Canavalia bonariensis lectin: Molecular bases of glycoconjugates interaction and antiglioma potential. *International Journal of Biological Macromolecules*, 106, 369–378. https://doi.org/10.1016/j.ijbiomac.2017.08.023
- Cavada, B. S., Silva, M. T., Osterne, V. J. S., Pinto-Junior, V. R., Lossio, C. F., Madeira, J. C., Pereira, M. G., Leal, R. B., Ferreira, W. P., Nascimento, K. S., & Assreuy, A. M. (2020a). Exploring the carbohydrate-binding ability of Canavalia bonariensis lectin in inflammation models. *Journal of Molecular Recognition*, 33(11), e2870. https://doi.org/10.1002/jmr.2870
- Cavada, B., Pinto-Junior, V., Osterne, V., & Nascimento, K. (2018). ConA-like lectins: High similarity proteins as models to study structure/biological activities relationships. *International Journal of Molecular Sciences*, 20(1), 30. https://doi.org/10.3390/ijms20010010
- da Nóbrega, R. B., Rocha, B. A., Gadelha, C. A. A., Santi-Gadelha, T., Pires, A. F., Assreuy, A. M. S., Nascimento, K. S., Nagano, C. S., Sampaio, A. H., Cavada, B. S., & Delatorre, P. (2012). Structure of Dioclea virgata...
Humphrey, W., Dalke, A., & Schulten, K. (1996). VMD: Visual molecular dynamics. Journal of Molecular Graphics, 14(1), 33–38, 27–8. https://doi.org/10.1016/0267-8558(96)00018-5

Jo, S., Kim, T., Iyer, V. G., & Im, W. (2008). CHARMm-GUI: A web-based graphical user interface for CHARMM. Journal of Computational Chemistry, 29(11), 1859–1865. https://doi.org/10.1002/jcc.20945

Kirschner, K. N., Yongye, A. B., Tschampel, S. M., Gonzalez-Outleteiro, J., Daniels, C. R., Foley, B. L., & Woods, R. J. (2008). GLYCAM06: A generalizable biomolecular force field. Carbohydrates Journal of Computational Chemistry, 29(4), 622–655. https://doi.org/10.1002/jcc.20820

Kleha, J. F., Devesly, P., & Johns, A. (1991). The effects of lectins on the release of EDRF from rabbit aorta. British Journal of Pharmacology, 104(2), 287–288. https://doi.org/10.1111/j.1365-2468.1991.tb12421.x

Korb, O., Stutzle, T., & Exner, T. E. (2009). Empirical scoring functions for advanced protein – ligand docking with PLANTS. Journal of chemical information and modeling, 49(1), 84–96. https://pubs.acs.org/doi/abs/10.1021/ci800298z

Kumar, K., Reddy, G., Reddy, B. V. R., Shekar, P., Sumanthi, J., & Chandra, K. P. (2012). Biological role of lectins: A review. Journal of Orofacial Sciences, 4(1), 20. https://doi.org/10.1016/j.joros.2009.09.003

Lee, J., Hitzenberger, M., Rieger, M., Kern, N. R., Zacharias, M., & Im, W. (2018). CHARMm-GUI supports the Amber force fields. The Journal of Chemical Physics, 153(3), 035103. https://doi.org/10.1063/1.5002280

Miller, B. R., McGee, T. D., Swails, J. M., Homeyer, N., Gohlke, H., & Roitberg, A. E. 3rd. (2012). MMPBSA.py: An efficient program for end-state free energy calculations. Journal of Chemical Theory and Computation, 8(9), 3314–3321. https://doi.org/10.1021/ct300418h

Mishra, A., Behura, A., Mawatwal, S., Kumar, A., Naik, L., Mohanty, S. S., Mann, D., Dokania, P., Mishra, A., Patra, S. K., & Dhiman, R. (2019). Structure-function and application of plant lectins in disease biology and immunity. Food and Chemical Toxicology, 134, 110827. https://doi.org/10.1016/j.fct.2019.110827

Nagano, C. S., Calvette, J. J., Barettoni, D., Perez, A., Cavada, B. S., & Sanz, L. (2008). Insights into the structural basis of the pH-dependent dimer-tetramer equilibrium through crystallographic analysis of recombinant Diociaceae lectins. The Biochemical Journal, 409(2), 417–428. https://doi.org/10.1042/BJ20070942

Nascimento, K. S., Silva, M. T. L., Oliveira, M. V., Lossio, C. F., Pinto-Junior, V. R., Osterne, V. J. S., & Cavada, B. S. (2020). Dalbergiaceae lectins: A review of lectins from species of a primitive Papilionoideae (leguminous) tribe. International Journal of Biological Macromolecules, 144, 509–526. https://doi.org/10.1016/j.ijbiomac.2019.12.117

Neco, A. H. B., Pinto-Junior, V. R., Araipe, D. A., Santiago, M. Q., Osterne, V. J. S., Lossio, C. F., Nobre, C. A. S., Oliveira, M. V., Silva, M. T. L., Martins, M. G. Q., Cajaíbes, J. B., Marques, G. F. O., Costa, D. R., Nascimento, K. S., Assreuy, A. M. S., & Cavada, B. S. (2018). Structural analysis, molecular docking and molecular dynamics of an edematogenic lectin from Centrolobium microchaete seeds. International Journal of Biological Macromolecules, 117, 124–133. https://doi.org/10.1016/j.ijbiomac.2018.05.166

Parker, B. L., Thaysen-Andersen, M., Solis, N., Scott, N. E., Larsen, M. R., Graham, M. E., Packer, N. H., & Cordwell, S. J. (2013). Site-specific glycan-peptide analysis for determination of N-glycoproteome heterogeneity. Journal of Proteome Research, 12(12), 5791–5800. https://doi.org/10.1021/pr400783j

Peumans, W. J., & Van Damme, E. J. M. (1995). Lectins as plant defense proteins. Plant Physiology, 109(2), 347–352. https://doi.org/10.1104/pp.109.2.347

Pinto-Júnior, V. R., de Santiago, M. Q., Osterne, V. J. D. S., Correia, L. J. A., Pereira-Júnior, F. N., Cajaíbes, J. B., de Vasconcelos, M. A., Teixeira, E. H., do Nascimento, A. S. F., Miguel, T. B. A. R., Miguel, E. D. C., Sampaio, A. H., do Nascimento, K. S., Nagano, C. S., & Cavada, B. S. (2013). Purification, partial characterization and immobilization of a mannose-specific lectin from seeds of Dioclea laisiphylla mart. Molecules (Basel, Switzerland), 18(9), 10857–10869. https://doi.org/10.3390/molecules180910857

Pinto-Junior, V. R., Osterne, V. J. S., Santiago, M. Q., Correia, L. J. A., Pereira-Júnior, F. N., Leal, R. B., Pereira, M. G., Chicas, L. S., Nagano, C. S., Rocha, B. A. M., Silva-Filho, J. C., Ferreira, W. P., Rocha, C. R. C., Nascimento, K. S., Assreuy, A. M. S., & Cavada, B. S. (2017b). Structural...
studies of a vasorelaxant lectin from Dioclea reflexa Hook seeds: Crystal structure, molecular docking and dynamics. International Journal of Biological Macromolecules, 98, 12–23. https://doi.org/10.1016/j.ijbiomac.2017.01.092

Pinto-Junior, V. R., Osterne, V. J. S., Santiago, M. Q., Lossio, C. F., Nagano, C. S., Rocha, C. R. C., Nascimento, J. C. F., Nascimento, F. L. F., Silva, I. B., Oliveira, A. S., Correia, J. L. A., Leal, R. B., Assreuy, A. M. S., Cavada, B. S., & Nascimento, K. S. (2017a). Molecular modeling, docking and dynamics simulations of the Dioclea lasiophylla Mart. Ex Benth seed lectin: An edematogenic and hypernociceptive protein. Biochimie, 135, 126–136. https://doi.org/10.1016/j.biochi.2017.02.002

Pinto-Junior, V. R., Santiago, M. Q., Nobre, C. B., Osterne, V. J. S., Leal, R. B., Cajazeiras, J. B., Lossio, C. F., Rocha, B. A. M., Martins, M. G. Q., Nobre, C. A. S., Silva, M. T. L., Nascimento, K. S., & Cavada, B. S. (2017c). Crystal structure of Pism arvense seed lectin (PAL) and characterization of its interaction with carbohydrates by molecular docking and dynamics. Archives of Biochemistry and Biophysics, 630, 27–37. https://doi.org/10.1016/j.abb.2017.07.013

Roe, D. R., & Cheatham III, T. E. (2013). PTRAJ and CPPTRAJ: Software for processing and analysis of molecular dynamics trajectory data. Journal of Chemical Theory and Computation, 9(7), 3084–3095. https://doi.org/10.1021/ct400341p

Rudd, P. M. (2001). Glycosylation and the immune system. Science (New York, N.Y.), 291(5512), 2370–2376. https://doi.org/10.1126/science.291.5512.2370

Ryckaert, J.-P., Ciccotti, G., & Berendsen, H. J. C. (1977). Numerical integration of the cartesian equations of motion of a system with constraints: Molecular dynamics of n-alkanes. Journal of Computational Physics, 23(3), 327–341. https://doi.org/10.1016/0021-9991(77)90098-5

Schwarz, F., & Aebl, M. (2011). Mechanisms and principles of N-linked protein glycosylation. Current Opinion in Structural Biology, 21(5), 576–582. https://doi.org/10.1016/j.sbi.2011.08.005

Sparrow, L. G., Lawrence, M. C., Gorman, J. J., Strike, P. M., Robinson, C. P., McKern, N. M., & Ward, C. W. (2008). N-linked glycans of the human insulin receptor and their distribution over the crystal structure. Proteins: Structure, Function, and Bioinformatics, 71(1), 426–439. https://doi.org/10.1002/prot.21768

Tian, C., Kasavajhala, K., Belfon, K. A. A., Ragouett, L., Huang, H., Migues, A. N., Bickel, J., Wang, Y., Pincay, J., Wu, Q., & Simmerling, C. (2020). ff19SB: Amino-acid-specific protein backbone parameters trained against quantum mechanics energy surfaces in solution. Journal of Chemical Theory and Computation, 16(1), 528–552. https://doi.org/10.1021/acs.jctc.9b00591

Tsaneva, M., & Van Damme, E. J. M. (2020). 130 years of plant lectin research. Glycoconjugate Journal, 37(5), 533–551. https://doi.org/10.1007/s10719-020-09942-y

Van Holle, S., & Van Damme, E. J. M. (2019). Messages from the past: New insights in plant lectin evolution. Frontiers in Plant Science, 10, 36. https://doi.org/10.3389/fpls.2019.00036

Xavier, M. M., Heck, G. S., de Avila, M. B., Levin, N. M. B., Pintro, V. O., Carvalho, N. L., & de Azevedo, W. F. (2016). SAnDReS a computational tool for statistical analysis of docking results and development of scoring functions. Combinatorial Chemistry & High Throughput Screening, 19(10), 801–812. https://doi.org/10.2174/1386207319666160927111347

Zamora-Caballero, S., Pérez, A., Sanz, L., Bravo, J., & Calvete, J. J. (2015). Quaternary structure of Dioclea grandiflora lectin assessed by equilibrium sedimentation and crystallographic analysis of recombinant mutants. FEBS Letters, 589(18), 2290–2296. https://doi.org/10.1016/j.febslet.2015.07.020

Zhao, Y., Vanhoutte, P. M., & Leung, S. W. S. (2015). Vascular nitric oxide: Beyond eNOS. Journal of Pharmacological Sciences, 129(2), 83–94. https://doi.org/10.1016/j.jphs.2015.09.002