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VINÍCIUS JOSÉ DA SILVA OSTERNE

CONSTRUCTION AND APPLICATION OF A CONTROLLED/SLOW RELEASE SYSTEM BASED ON BIOCONJUGATES OF CALCIUM CARBONATE AND LECTIN FROM *Dioclea violacea*: EFFECT ON CERVICAL CANCER CELLS

FORTALEZA

2020

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Thesis presented to the Graduation Program in Natural Resources Biotechnology and Graduation Program and Applied Biological Sciences: Cell And Gene Biotechnology as partial requirement for obtaining the following titles of Doctor in Natural Resources Biotechnology by UFC and Doctor in Applied Biological Sciences: Cell and Gene Biotechnology by UGent.

Supervisors: Prof. Dr. Benildo Sousa Cavada (UFC)
Prof. Dr. Els Van Damme (UGent)

Co-supervisor: Prof. Dr. Kyria Santiago do Nascimento (UFC)

FORTALEZA
2020

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca Universitária
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

O94c Osterne, Vinicius José da Silva.
Construction and application of a controlled/slow release system based on bioconjugates of calcium carbonate and lectin from Dioclea violacea : effect on cervical cancer cells / Vinicius José da Silva Osterne. – 2020.
116 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Centro de Ciências Agrárias, Programa de Pós-Graduação em Biotecnologia de Recursos Naturais, Fortaleza, 2020.
Orientação: Prof. Dr. Benildo Sousa Cavada.
Coorientação: Prof. Dr. Kyria Santiago do Nascimento.

1. Lectin. 2. Dioclea violacea. 3. Anticancer. 4. Particles. 5. Controlled release. I. Título.
CDD 660.6

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To everyone that helped me in my journey!!

AGRADECIMENTOS

First of all, I would like to thank my family, in special my grandmother Lucia, my mother Alzenir, my brother Victor, my father Wladimir, my second mother Neide, my beloved little brother Enzo, my cousins Biel and Digo, my aunt Herbene among several others for the deep support that are given to me. I am extremely grateful to you all.

My deepest thanks to Prof and Profa (they know the code) for offering to me the opportunity for working on their lab, the orientation for several years and huge support. What was said for my family can be also said here because I consider you two as such.

My thanks to Profa Els Van Damme for accepting me in your group. Those moments have been amazing. Like I said when we met, you are my idol! Meeting you was one of the highlights of my career that I have no qualms bragging about :)

My thanks to Prof. Andre Skirtach and Prof. Guy Smagghe for giving me the opportunity to work on their labs. A second thanks to Prof Andre for accepting my invitation to the Jury.

To Profs Wandemberg, Rodrigo and Pierre for accepting my invitation to the Board.

My dear friends, Vanir, Mayara, Adolph and JV, you better believe you people are super damn amazing. Love y'all.

Thanks to my amazing second family of BioMol. Our group is superb, each one of you are special to me. Even Claudio (shockingly). Unfortunately, there are people that still do not drink coffee, but there is time to change this!!!

My deepest thanks to the Glyco group and the other groups of UGent. Gernot, Gosia, Mariya, Simin, Andrea, Sinem, Jeroen, Jeroen Lambin, Pieter, Isabel, Koen, Chen, Kristof, Bogdan, Johannes (this name is super cool you know?!) as well as everyone else that I had the pleasure to meet.

To FUNCAP, CAPES, and CNPq for the financial resources required for a successful research.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

“What we anticipate seldom occurs: but what we least expect generally happens.”

Benjamin Disraeli

RESUMO

As lectinas são proteínas que se ligam especificamente à carboidratos e são capazes de formar complexos com moléculas contendo sacarídeos sem alterar sua estrutura. Devido às muitas atividades biológicas exibidas por essas proteínas, elas possuem grande aplicabilidade na biotecnologia. A tecnologia de liberação controlada/lenta de medicamentos é uma das aplicações mais promissoras da nanotecnologia no tratamento e diagnóstico de doenças, visto que supera diversos problemas associados ao uso de macromoléculas, como proteínas e medicamentos. Nesse contexto, o presente trabalho teve como objetivo desenvolver um sistema para a liberação controlada da lectina de *Dioclea violacea* (DVL), uma lectina de leguminosa específica para manose/glicose com propriedades anticâncer, através da aplicação de dois tamanhos diferentes de partículas de $CaCO_3$, um composto de baixo custo, amplamente disponível e biocompatível. Os resultados mostraram que os sistemas são viáveis e apresentam propriedades desejáveis de adsorção e liberação. A aplicação dos bioconjugados nas células HeLa mostrou que o sistema proposto é eficaz como agente citotóxico contra esse tipo de célula, mais potente inclusive que lectina livre. Os dados obtidos demonstram que o sistema tem uma aplicação potencial no tratamento do câncer e a metodologia permite o uso de outras lectinas/proteínas anticâncer.

Palavras-chave: Lectina. *Dioclea violacea*. Anticâncer. Partículas. Liberação controlada.

ABSTRACT

Lectins are proteins that bind specifically to carbohydrates and are able to form complexes with molecules containing saccharides without changing their structure. Because of the many biological activities displayed by these proteins, they have great applicability in biotechnology. Controlled/slow drug release technology is one of the most promising applications of nanotechnology for disease treatment and diagnosis because it overcomes several problems associated with the use of macromolecules such as proteins as drugs. In this context, the present investigation aimed to develop a system for the controlled release of *Dioclea violacea* lectin (DVL), a mannose/glucose-specific legume lectin with anticancer properties, by applying two different sizes of $CaCO_3$ particles, a low-cost, broadly available and biocompatible compound. Results show that the systems are feasible and present desirable properties of adsorption and release. The application of the bioconjugates on HeLa cells indicates that the proposed system is effective as a cytotoxic agent against this cell type, even more than the free lectin. The present data demonstrate that the system has a potential application in cancer treatment and the methodology enables the use of other anticancer lectins/proteins.

Keywords: Lectin. *Dioclea violacea*. Anticancer. Particles. Controlled release.

LIST OF FIGURES

LIST OF TABLES

.

LISTA DE ABREVIATURAS E SIGLAS

Asn	Asparagine
CaBo	*Canavalia bonariensis* lectin
ConA	*Canavalia ensiformis* lectin
CRD	Carbohydrate-recognition domain
DLL	*Dioclea lasocarpa* lectin
DMEM	Dulbecco's Modified Eagle Medium
DVL	*Dioclea violacea* lectin
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FTIR	Fourier-transform infrared spectroscopy
GalNAc	*N*-acetyl-D-galactosamine
GlcNAc	*N*-acetyl-D-glucosamine
LCA	*Lens culinaris* agglutinin
LLs	Legume lectins
MPs	Bigger $CaCO_3$ particles
NPs	Smaller $CaCO_3$ particles
RIPs	Ribosome-inactivating protein
Ser	Serine
Thr	Threonine
WGA	Wheat germ agglutinin

TABLE OF CONTENTS

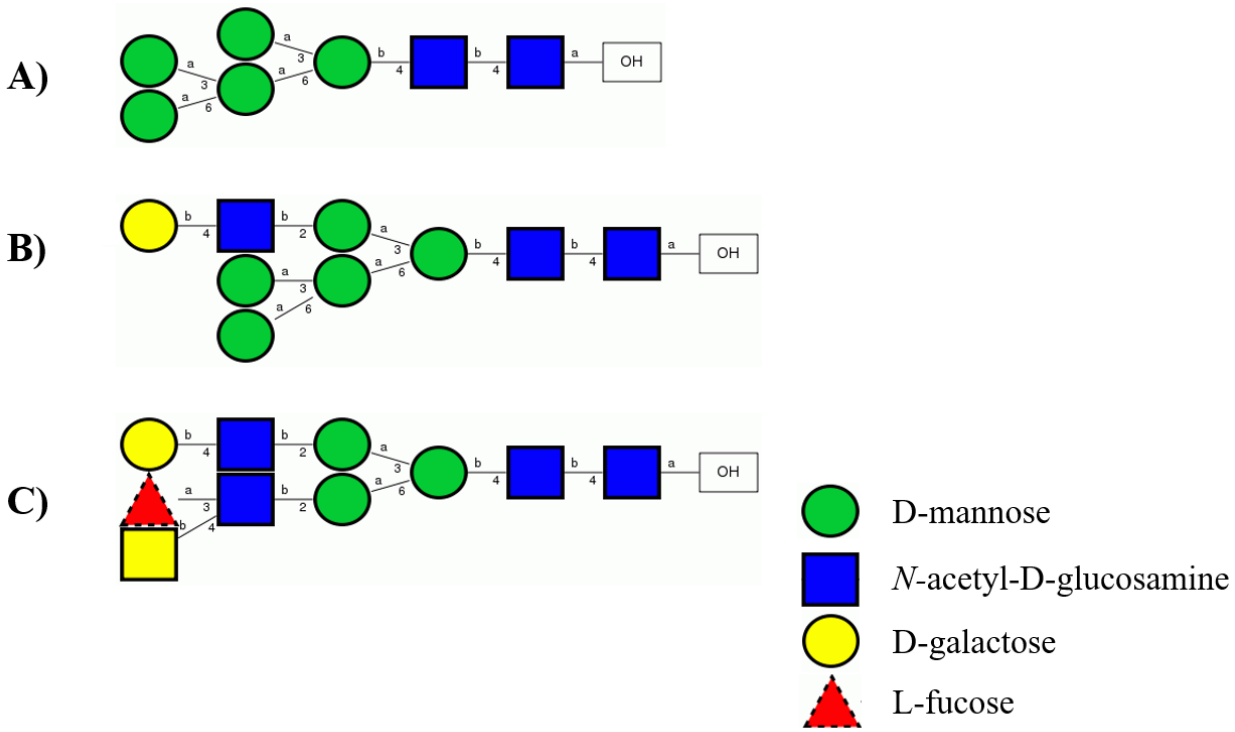
1 INTRODUCTION

1.1 Carbohydrates and glycans

The functions of the carbohydrate portion of glycoconjugates are as numerous as their structural diversity. Two important functions can be highlighted: the structural function and the role in molecular recognition. These physical properties of carbohydrates such as high electrostatic charge and high solubility, give them a role in structural stabilization, solubilization and protection against proteolysis in glycoproteins (JAYAPRAKASH; SUROLIA, 2017). Protein glycosylation is one of the most common post-translational modifications, strongly influencing many other functional aspects, including cell localization, degradation and quality control of the protein (SOLÁ; GRIEBENOW, 2009; VARKI et al., 2017). Glycosylation has a variety of important roles in many cellular events ranging from a structural role, to signaling and recognition. Key to understanding these functions is knowledge of the primary structure of glycoconjugates. There are two main types of glycosylation: *N*-linked and *O*-linked glycosylation. In *N*-glycosylation, an *N*-acetylglucosamine residue (GlcNAc) is linked by an amide bond to an asparagine residue belonging to an Asn-X-Ser/Thr consensus sequence, where X can be any amino acid except proline. The presence of the consensus sequence is required for *N*-glycosylation, however, the occupation of the potential site is not mandatory. Therefore, a glycoprotein may contain a number of potential *N*-glycosylation sites, each of which may or may not be glycosylated (STANLEY; TANIGUCHI; AEBI, 2017). *N*-linked glycans are synthesized in the ER. When the nascent protein enters the endoplasmic reticulum (ER) a block of sugars (Glc3Man9GlcNAc2) is transferred to the amino group on the asparagine side chain and the glycan is further processed and modified in the endoplasmic reticulum and Golgi complex, generating the three *N*-glycan types found in mature proteins: high-mannose, complex and hybrid type (STANLEY; TANIGUCHI; AEBI, 2017) (Figure 1). Usually, *N*-glycan chains found in glycoproteins have a common core Manα1–6 (Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn-X-Ser/Thr . High-mannose glycans are those in which mainly mannose residues are attached to the core; complex *N*-glycans are those in which branches initiated with a *N*-acetyl-glucosamine (GlcNAc) are attached to the core, normally with subsequent addition of residues in β1–4 bond with GlcNAc, forming a *N*-acetyllactosamine (LacNAc). Poly-LacNAc chains are found in several cell types and may serve as a scaffold for the insertion of other glycosyl moieties. It is not uncommon also the formation of GalNAcβ1–

4GlcNAc branches. Hybrid *N*-glycans have characteristics of the two previous types with some branches presenting unsubstituted mannose residues and others with GlcNAc linkage (MARQUES et al., 2017; PINTO-JUNIOR et al., 2017; STANLEY; TANIGUCHI; AEBI, 2017).

Figure 1 – Examples of the structure of the three major *N*-glycan types. A) High-mannose type, B) Hybrid type and C) Complex type



Source: Elaborated by the author.

The glycoforms of a protein, or the structural and complex variation associated with the glycoprotein, ultimately define its function and activity. Additionally, cell communication, such as cell-cell, cell-matrix, protein-protein, and sugar-sugar interactions, are controlled through specific interactions between glycans and their targets (VARKI, 2017; VARKI et al., 2017). Modulation of protein glycosylation by variations in occupancy of sites in the polypeptide chain or variation of oligosaccharide structures occupying a particular protein glycosylation site modulates the biological activity of glycoproteins, and abnormal glycosylation of glycoconjugates have been identified in many mammalian diseases, including mammalian hereditary disorders, immunodeficiencies, cardiovascular diseases and cancer (TONG et al., 2003). Studies have already shown that the identification of abnormal patterns of glycosylation in cells can be used to diagnose some diseases, such as cancer

(PERACAULA et al., 2008; STOWELL; JU; CUMMINGS, 2015). The process of transforming normal cells into neoplastic cells is related to changes in the composition of glycans present in the cell membrane, which determine characteristics related to differentiation and maturation phenomena. Changes are detected more frequently in metastasis processes, since in the primary tumor, these are minimal, suggesting that these alterations may provide advantages to tumor cells throughout their tumor progression process (STOWELL; JU; CUMMINGS, 2015). Changes in glycoconjugates on the cell surface can cause ramifications in complex carbohydrate structures and generate structurally unusual glycans. Thus, lectins have been used as simple tools to recognize these changes, identify and sort malignant and benign tumors, in addition to assessing the degree of glycosylation associated with metastasis (PERACAULA et al., 2008; TAYLOR-PAPADIMITRIOU; EPENETOS, 1994). Also, some lectins present antitumor activity in certain types of neoplasms, inducing cell death (DE MEJÍA; PRISECARU, 2005). One of the most frequent types of changes on the surface of neoplastic cells is the increase in the presence of epitopes containing residues of *N*-acetylgalactosamine, resulting from changes in the glycosylation pathways. Among these epitopes, the Tn antigen (GalNAc-α-O-Ser / Thr) is one of the most specific structures associated with cancer in humans and is often used as a biomarker. This epitope is present in *O*-glycosylated mucins on epithelial cells and is spatially accessible on the cell surface in most carcinomas, unlike normal cells and benign tumors, in which it is inaccessible (FU et al., 2016), The addition of carbohydrate residues to these disaccharides gives rise to the structures normally found in healthy cells and makes the Tn antigen inaccessible, functioning as a kind of molecular blocker. The Tn antigen is considered a biological marker of great potential for cancer diagnosis and therapy, given its wide distribution and an early appearance in different types of cancer in humans and animals, as well as its ability to induce responses in T cells (JU et al., 2014). These were a few examples of changes in the glycosylation pattern that takes place in malignant cells.

1.2 Overview of lectins

There is a huge variety of glycans expressed by organisms. Only taking into account *N*-glycosylation, it is estimated that approximately three-quarters of the proteins deposited in the SWISS-PROT database are modified in such a way (APWEILER, 1999). Taking advantage of this variety, organisms make use of glycans in a series of physiological processes, many of them with high specificity. The high structural diversity of glycans

generates a specific code, the so-called glycocode, involved in several biological processes (AMBROSI; CAMERON; DAVIS, 2005). To decipher this code, organisms have an arsenal of proteins capable of binding carbohydrates.

Carbohydrate-binding proteins are defined as those that recognize and bind to them without exhibiting enzymatic activity at the same binding site. With the exception of antibodies generated with specificity to certain glycans, most proteins with this capacity are called lectins (PEUMANS; VAN DAMME, 1995; VAN DAMME et al., 1998), which can be grouped into a series of phylogenetically and structurally distinct families (VAN DAMME et al., 1998). Lectins bind to mono- or oligosaccharides, usually with high specificity, but low affinity, an affinity that is normally enhanced by applying multivalence (LAGARDA-DIAZ; GUZMAN-PARTIDA; VAZQUEZ-MORENO, 2017; ROY; MURPHY; GABIUS, 2016).

First discovered in plants over a hundred years ago, guided by their ability to agglutinate erythrocytes, lectins are found in all taxonomic groups and are also present in viruses (GILBOA-GARBER; AVICHEZER; GARBER, 1996; WEIS, 1997). In fact, in viruses, lectins or hemagglutinins are essential for cell-virus fusion and, therefore, are important for the medical field, which led to the fact that one of the first 3D structures of a lectin determined experimentally was that of the influenza virus hemagglutinin (WILSON; SKEHEL; WILEY, 1981).

Historically, the first report of a binding activity with the involvement of lectins was made in 1860 by the psychiatrist and neurologist Silas Weir Mitchel, who found that a drop of the *Crotalus durissus* venom was able to quickly agglutinate a drop of blood from a pigeon (VAN DAMME et al., 1998). In 1902, the agglutinating activity was confirmed by Simon Flexner and H. Noguchy, when they reported in more detail the hemagglutination process (KILPATRICK, 2002). Regarding lectins from plants, the first report dates from 1888 by Peter Hermann Stillmark who, while studying the toxic effects of castor bean (*Ricinus communis*), verified that when mixing erythrocytes with the extract of this seed hemagglutination occurred. The toxic agglutinin was isolated and called Ricin. Later, in 1891, H. Hellin showed that the *Abrus precatorius* seed extract demonstrated the same activities as ricin, and, based on this observation, the toxic hemagglutinin was isolated and called Abrin (SHARON; LIS, 2004). In 1891, Paul Ehrlich made use of the two toxic hemagglutinins Ricin and Abrin and noted that mice became immune to a lethal dose of ricin or abrin through repetitive application of small sublethal doses, he also found out that the anti-ricin antibody did not protect the animals against the toxic effect of abrin and neither the anti-abrin protected them against ricin. These results were clear evidence of the immune response specificity.

These antibodies were used for several experiments and, with that, it was possible to establish several of the fundamental principles of immunology such as: the specificity of the antibody response, the phenomenon of immunological memory and the transfer of the mother's humoral immunity to her progeny (SHARON; LIS, 2004).

In the year 1907, in a work carried out by Landsteiner and Raubischek, it was found that extracts of *Phaseolus vulgaris, Pisum sativum, Lens culinaris* and *Vicia sativa* had distinct hemagglutinating activity against the blood of various animals indicating that lectins had differences in their ability to agglutinate, in addition these extracts were not toxic which demonstrated that toxicity is not an intrinsic property of lectins (SHARON; LIS, 2004).

In 1919, there was the first report of the purification of a plant hemagglutinin, when James B. Summer purified the lectin called Concanavalin A or ConA from the seeds of pork beans (*Canavalia ensiformis*). Two decades later, in the year 1936, Summer and Howell reported that *Canavalia ensiformis* lectin (ConA) had the ability to agglutinate cells such as erythrocytes and yeasts and was also capable of precipitating glycogen in solution. These researchers also demonstrated the inhibition of ConA hemagglutinating activity with the addition of sucrose, this being the first report that lectins are capable of binding to carbohydrates. From this data it was suggested that lectin was able to interact with the carbohydrates found on the cell surface (DAN; LIU; NG, 2016). Following, Boyd and Shapleigh (1954) found that protein extracts from several plants had different hemagglutination profiles when tested with different human blood groups. Plant lectins were previously designated as agglutinins or phytoagglutinins but after the observation that these proteins had a certain specificity by blood type, the name of lectins, from the Latin ‘legere’, which means to select was proposed (LAM; NG, 2011). The number of purified lectins has grown significantly since the 1970s with the development of affinity chromatography applied in the purification of these proteins (AGRAWAL; GOLDSTEIN, 1965). There was an increase in interest in lectins when these proteins were shown to have several biotechnological applications such as: detection and characterization of glycoconjugates (WU et al., 2009); analysis of changes that occur on the cell surface during physiological and pathological processes (AUB; SANFORD; COTE, 1965) and mitogenicity for lymphocytes (ASHRAF; KHAN, 2003). Due to the diverse biotechnological applications of lectins, today we already have a large number of lectins from plants, microorganisms, algae and animals, all purified and characterized.

1.3 Classification of Lectins

Plant lectins are ubiquitous and very diverse in nature, making it very difficult to classify them in different groups. Attempts in the past have subdivided plant lectins into groups based on various criteria. In an initial effort Peumans and Van Damme (1998) proposed a lectin classification based on their sequence and evolutionary relationships. According to this division, lectins are separated in the seven families: legume lectins, chitin-binding lectins composed of hevein domains, the type 2 ribosome-inactivating proteins, monocot mannose-binding lectins, jacalin-related proteins, *Amaranthaceae* lectins and *Cucurbitaceae* phloem lectins.

a) Legume lectins: is the most well-studied lectin family, proteins from this group share high similarities both in sequence and three-dimensional structure. Inside this group one can find the lectins from *Canavalia* (CAVADA, BENILDO SOUSA; OSTERNE; PINTO-JUNIOR; et al., 2019), *Dioclea* (PINTO-JUNIOR et al., 2017), *Vatairea* (SOUSA et al., 2016), among several others. The lectin from *Canavalia virosa* is shown in Figure 2A.

b) Monocot mannose-binding lectins: a group of very similar lectins that were found in several families of monocots: *Amaryllidaceae, Alliaceae, Araceae, Orchidaceae, Liliaceae* and *Bromeliaceae*. When comparing the sequence of this group of lectins, it appears that these lectins belong to a single superfamily of evolutionarily related proteins. Structurally, these lectins are formed by 1 to 4 subunits of approximately 12 kDa and are mannose-specific (PEUMANS; VAN DAMME, 1995; VAN DAMME et al., 1998). Figure 2B shows the structure of the mannose-specific lectin from *Gastrodia elata*.

c) Chitin-binding lectins composed of hevein domains: a group of lectins that have specificity for GlcNAc and their main characteristic is the presence of hevein domains. Hevein is a chitin-binding merolectin with 43 amino acid residues extracted from the rubber tree latex (*Hevea brasiliensis*) (ITAKURA et al., 2017). This group of lectins has as main representative the wheat germ lectin (WGA), which is composed of two subunits of 18 kDa and consists of four domains structurally similar to Hevein (PORTILLO-TÉLLEZ et al., 2011). The structure of the WGA lectin is shown in figure 2C.

d) Type 2 ribosome-inactivating proteins: Type 2 RIPs are formed by two polypeptide chains (chains A and B). Chain A is formed by a polypeptide consisting of an adenosine glycosidase domain and chain B has lectin properties that allow the binding of these proteins to cell surface carbohydrates. The A and B chains are initially linked by disulfide bonds, but when the protein enters the cells, the bond is broken and the A chain acquires a strong N-

glycosidase activity and, with this, the protein is able to inactivate prokaryotic and eukaryotic ribosomes (SCHROT; WENG; MELZIG, 2015). The inactivation activity of ribosomes by these proteins occurs due to their ability to remove a specific adenine residue from a loop in the larger subunit of the 28 S ribosomal RNA (ENDO, 1988). Classical examples include Ricin and Abrin extracted from *Ricinus communis* and *Abrus precatorius*, respectively. The structure of Abrin is shown in figure 2D.

e) Jacalin-related lectins: jacalin is one of the two lectins present in jackfruit seeds (*Artocarpus integrifolia*). The term "jacalin-related lectins" is used for all lectins that are structurally and evolutionarily related to jacalin. Based on specificity for carbohydrates, these lectins are classified into two groups: the galactose and the mannose binding lectins. Lectins belonging to this group are found in species of Moraceae and Convolvulaceae (VAN DAMME et al., 1998). The structure of the jacalin is shown in figure 2E.

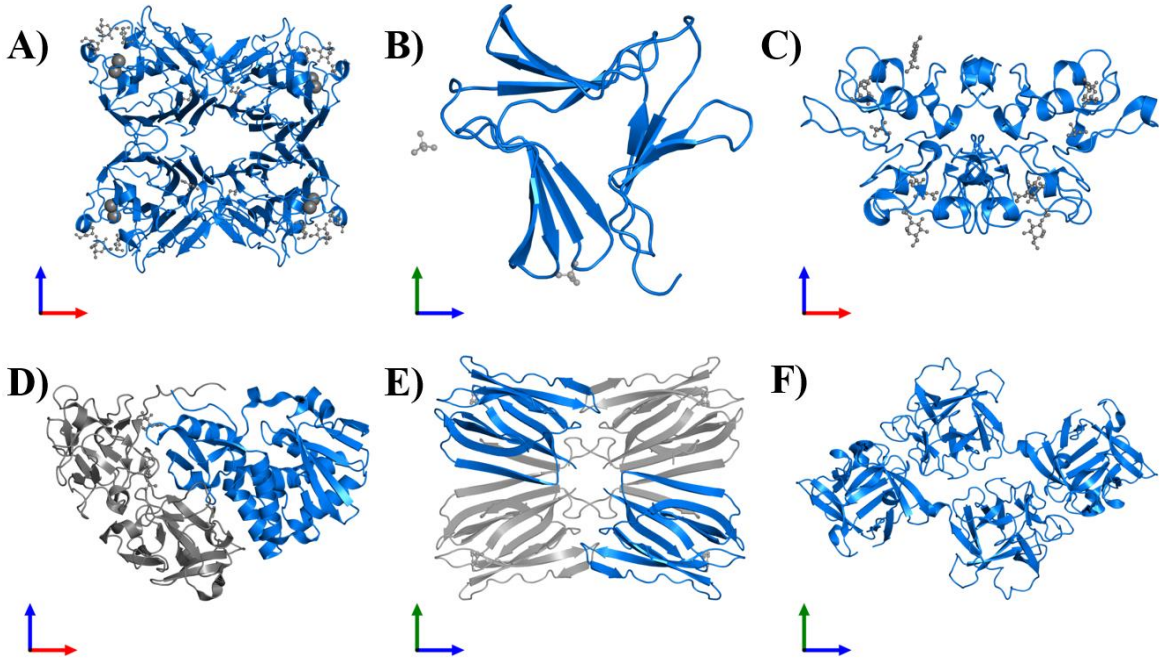
f) *Amaranthaceae* lectins: this group has as its main representative Amarantin, a lectin extracted from *Amaranthus caudatus*, which does not resemble any other plant lectin both in the sequence or three-dimensional structure. Structurally, they are homodimeric proteins composed of two subunits of approximately 30 kDa. This group contains several other lectins extracted from plants of the genus *Amaranthus*, all of them being specific to GalNAc (KAUR et al., 2006; VAN DAMME et al., 1998). The structure of Amarantin is shown in figure 2F.

g) *Cucurbitaceae* phloem lectins: a small family of chitin-binding lectins that occur in the phloem of some plants in the *Cucurbitaceae* family. Structurally, this group contains dimeric lectins composed of two 24 kDa subunits. These lectins have great sequential similarity to each other but are very different from other plant lectins (LANNOO; VAN DAMME, 2014; VAN DAMME et al., 1998). So far, there are no resolved structures of *Cucurbitaceae* phloem lectins.

Following, attempts were made to classify lectins into four groups based on their structure (VAN DAMME et al., 1998). In this system, merolectins are lectins with only one carbohydrate-recognition domain (CRD) in their structure and, therefore, monovalent with respect to carbohydrate binding thus not possessing the capacity for agglutination. Hololectins have at least two CRDs being bi or multivalent and therefore capable of agglutinating cells, as well as precipitating glycoconjugates. Most of the plant lectins belong to the hololectin group. Chimerolectins combine one or more carbohydrate-recognition domains with unrelated domains. These domains work independently and can have different biological functions. Superlectins are lectins that have two or more CRDs that bind structurally unrelated carbohydrates. Figure 3 shows a scheme as well as examples of lectins that can be classified

in each group.

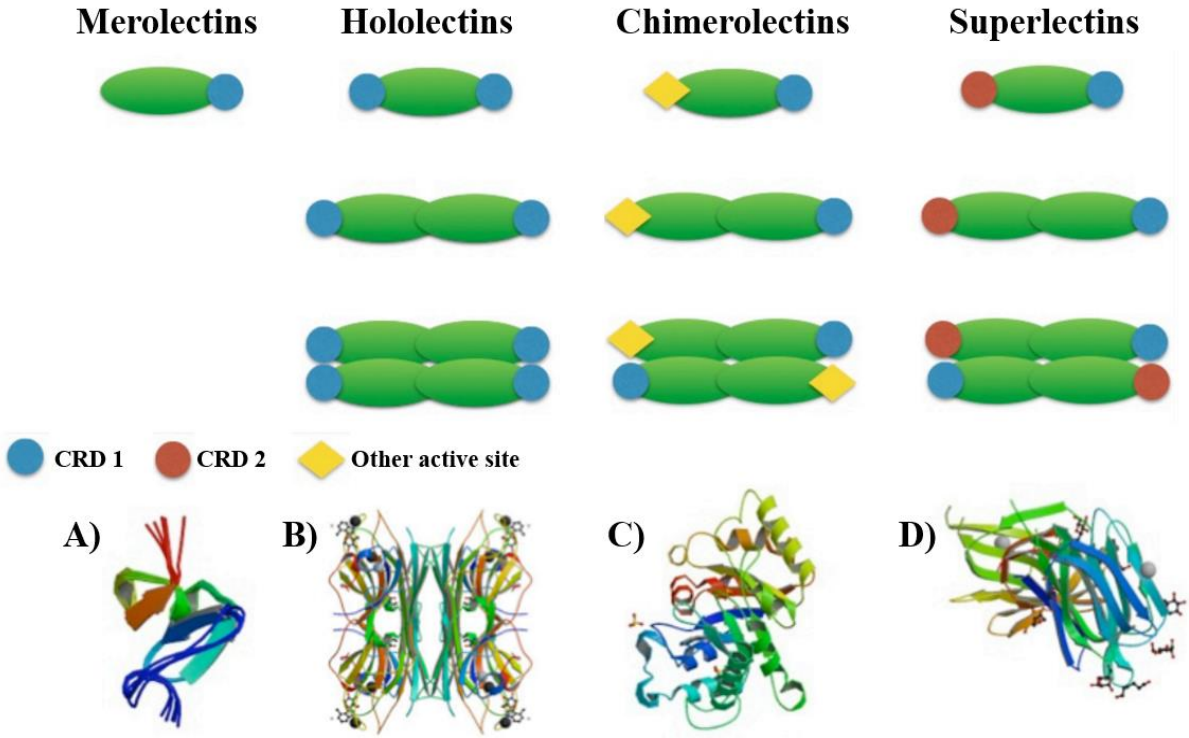
Figure 2 – Three-dimensional structure of a lectin of each evolutionary-related family. A) Legume lectin - ConV (PDB id: 5F5Q), B) Monocot mannose-binding lectins - *Gastrodia elata* lectin (PDB id: 1XDG), C) Chitin-binding lectins composed of hevein domains - Wheat germ agglutinin (PDB id:2UVO), D) Type 2 ribosome-inactivating protein - Abrin (PDB id: 2ZR1), E) Jacalin-related lectins- Jacalin (PDB id: 1UGW), F) *Amaranthaceae* lectins - Amarantin (PDB id: 1JLY)



Source: Elaborated by the author.

Another method for the classification of lectins relates to their carbohydrate specificity such as: mannose, mannose/glucose, GlcNAc, mannose/maltose, galactose/GalNAc, and fucose-specific lectins (VAN DAMME et al., 1998). This classification system is useful for the selection of lectins for specific applications, but it is artificial and does not provide relevant information about their similarity or evolutionary history. More recently, plant lectins have been grouped according to their evolutionary relationship and sequence similarities and considering the conservation of the CRD, taking advantage of the rapid progress in structural analysis and DNA sequencing (JIANG; MA; RAMACHANDRAN, 2010). Comparison of sequences and phylogenetic analysis of available plant lectins distinguished 12 families, as shown in Table 1 (VAN DAMME et al., 2008; DE SCHUTTER; VAN DAMME, 2015)

Figure 3 – Structural classification of plant lectins in merolectins, hololectins, chimerolectins and superlectins. A) Hevein (PDB id: 1HEV), B) *Dioclea wilsonii* lectin (PDB id: 3SH3), C) *Parkia platycephalla* lectin 2 (PDB id: 2GSJ) and D) *Musa acuminata* lectin (PDB id: 3MIT)



Source: Elaborated by the author.

The ubiquitous occurrence of lectins and carbohydrate structures in nature suggests that lectins probably have several functions. In fact, in many microorganisms such as viruses, bacteria, and fungi play roles important host infections and contribute to pathogenicity (SHARON; LIS, 2007). Plant lectins contribute to growth, development, defense and symbiosis of plants with nitrogen-fixing bacteria. Animal lectins are involved in the control of glycoprotein biosynthesis, immunity, regulation of cell growth and apoptosis, cell cycle regulation, hormone clearance sulfated glycoproteins, targeting lysosomal enzymes, homing of leukocytes, cell-cell interactions in the immune and neural system and interaction sperm-egg (SHARON; LIS, 2004).

Table 1 – Plant lectin families classified according to structural and evolutionary relationships and CRD conservation.

Lectins families	**Specificity**
Agaricus bisporus lectin family	GlcNAc/GalNAc, Galactose
Chitinase related agglutinin family	High mannose *N*-glycans
Amaranthin family	GalNAc
Cyanovirin family	Mannose
Euonymus europaeus lectin family	Galactosides, high-mannose *N*-glycans
Galanthus nivalis lectin family	Mannose
Hevein family	Chitin
Jacalin family	Mannose- and galactose-specific subgroup
Legume lectin family	Mannose
LysM family	Chitin, peptidoglycan
Nicotiana tabacum lectin family	(GlcNAc)n, high-mannose and complex *N*-glycans
Ricin-B family	Gal/GalNAc, Sialylated Gal/GalNAc

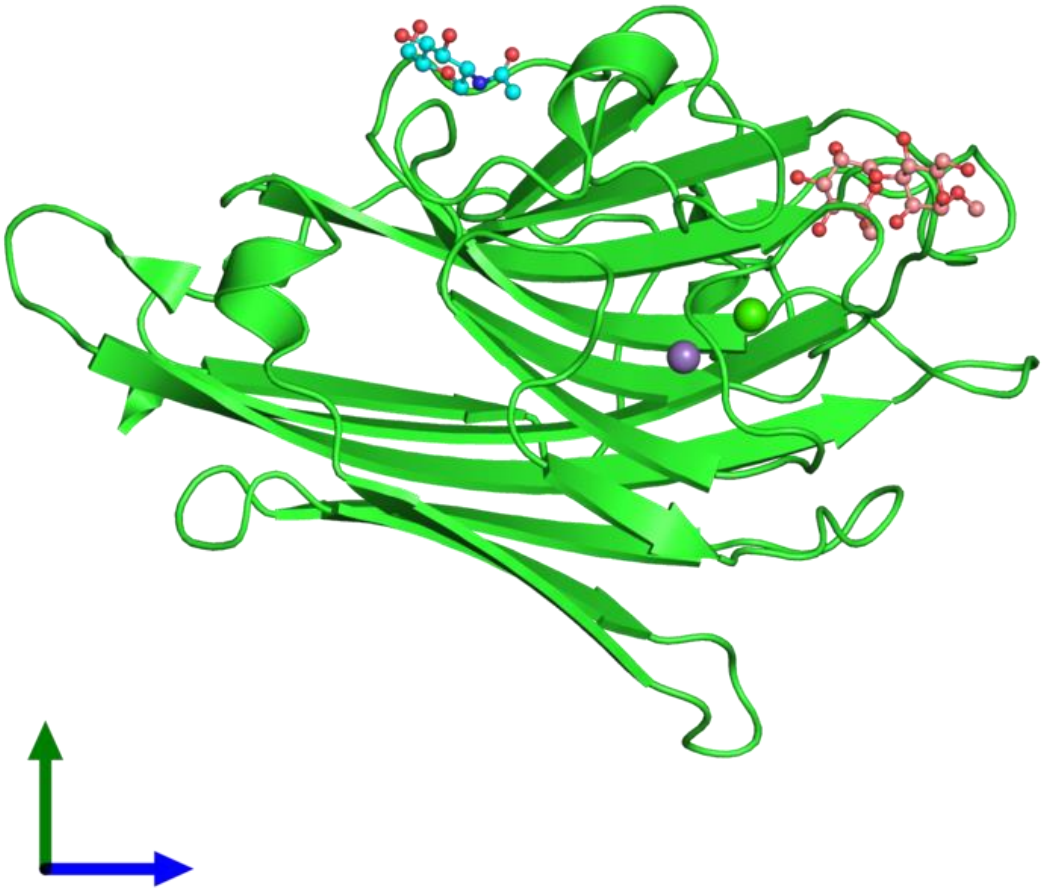
Source: Adapted from DE SCHUTTER; VAN DAMME, 2015.

1.4 Legume lectins

The most well-studied by far are those lectins extracted from plants of the family Leguminosae (Legume lectins - LL). Among all lectins, LLs form a large group of related lectins that, despite their differences in specificity for carbohydrates, share a high degree of sequence identity and conserved regions (GAUTAM et al., 2018; LAGARDA-DIAZ; GUZMAN-PARTIDA; VAZQUEZ-MORENO, 2017). In addition, LLs are usually abundant in seeds, and significantly less abundant in other tissues. LLs usually bind to simple sugars, such as glucose/mannose, *N*-acetyl-galactosamine/galactose, fucose and *N*-acetyl-glucosamine as well as more complex sugars like glycans of varying sizes (FERNANDES et al., 2011; GAUTAM et al., 2018). Many LLs are hololectins that, have the potential to agglutinate cells and precipitate glycoconjugates. The carbohydrate recognition domains in LLs are highly conserved and composed of 4 different loops in the protein structure (SHARMA, V.; SUROLIA, 1997). Characteristically also, carbohydrate-binding activity does not occur without the concomitant presence of calcium and another transition ion in the vicinity of the CRD (LORIS et al., 1998). In addition to the ability to bind carbohydrates, these lectins are also capable of interacting with other ligands such as hydrophobic molecules in a region distinct from the CRD (DELATORRE et al., 2013; ROBERTS; GOLDSTEIN, 1983). Many, but not all, LLs are glycosylated and have at least a high-mannose or complex glycosylation. The glycosylation in these lectins normally result in the formation of quaternary LL complexes stabilizing or destabilizing the non-covalent association of the subunits to assist in the formation of dimers or prevent formation of tetramers (PEUMANS et al., 2001). Until now, there is no evidence that glycans play a structural role in ligand recognition.

Legume lectins are well characterized in terms of their tertiary structures and the interaction with carbohydrates being very conserved in terms of topology structural similar to a jelly roll or β-sandwich architecture. Each structural unit contains approximately 250 amino acids and is constructed with 13 β-strands, seven on the frontal face and six on the rear face interconnected by loops and β-turns. α-helix are not found and approximately half of the residues in the domain consist of loops and turns. A figure of the jellyroll is presented in figure 4. The antiparallel arrangement of β-strands on the frontal and rear faces has a consequence, a very strong connectivity within the monomer due to the various hydrogen bonds between them (BRINDA et al., 2004; HAMELRYCK, THOMAS W. et al., 1998; LORIS et al., 1998; OSTERNE et al., 2017).

Figure 4 – Scheme of a jellyroll from the structure of *Centrolobium microchaete* lectin (PDB id: 5EYY)



Source: Elaborated by the author.

At quaternary structure level, LLs are mostly found in the forms of dimer or tetramer. Some of these folds also have a number of binding pockets that can accommodate hydrophobic ligands like adenine and other hormones. The variation in the association of subunits also results in a higher level of binding specificity and affinity (HAMELRYCK, T. W. et al., 1999; MANOJ; SUGUNA, 2001; SHETTY et al., 2013). The carbohydrate-recognition domain (CRD) is normally a shallow cavity in the lectin surface with sequences containing some invariant amino acids that are common to all legume lectins such as an aspartic acid, an asparagine, a glycine and an aromatic residue or a leucine (SHARON; LIS, 2001). Despite the presence of key residues, there is variability in specificity inside this group with lectins such as ConA binding mannose and derivatives while other lectins such as peanut agglutinin and soybean agglutinin bind galactose and derivatives. This clearly shows that the invariant amino acids are very important for the binding, but they are not responsible for the specificity that arises from other regions of the binding domain. The CRD structure is formed by four loops

usually designated as A, B, C and D with the latter one being quite variable in length, sequence and conformation and is supposed that it is an important factor in the specificity determination of the lectin (AMBROSI; CAMERON; DAVIS, 2005; SHARMA, V.; SUROLIA, 1997; SHARON; LIS, 2001). In the vicinity of the CRD, LLs present a metal-binding site containing one Ca^{2+} and one Mn^{2+} per monomer. These cations are not directly involved with the carbohydrate-binding but are essential for the positioning of residues for interaction with the carbohydrates (LORIS et al., 1998). The interaction with carbohydrates takes place through an intricate network of hydrogen bonds and hydrophobic interactions between the protein and the ligand (AMBROSI; CAMERON; DAVIS, 2005; LORIS et al., 1998).

Plant lectins, especially LLs, have attracted interest from the scientific community given that they are very useful tools in biotechnology, the chemical industry and medical sciences. This led to a situation where a lot is known about these proteins, their specificities/affinities, structure, etc., but virtually nothing is known with respect to their biological functions in the organism from which they are extracted. Therefore, while the exact functions of these proteins in plants remain unclear, literature data suggest a variety of roles such as mediating the binding of bacteria in the roots (BREWIN; KARDAILSKY, 1997; HIRSCH, 1999, RAMOS et al., 2000), storage proteins (GAUTAM et al., 2018), defense against pathogens (LANNOO; VAN DAMME, 2014; PEUMANS; VAN DAMME, 1995), among others.

Regarding their applicability, lectins can be applied in several biological activities and/or biotechnological tools. Not surprisingly, the vast majority of applications are dependent on the interaction lectin-carbohydrate. Some of their applications are: immunotoxins (FRANZ; PFÜLLER, 1983), tools for the investigation of glycosylated receptors (BELICKÝ; KATRLÍK; TKÁČ, 2016), microorganism and blood typing (HART, 1980; KHAN, FAUZIA et al., 2002), insecticidal activity (MACEDO; OLIVEIRA; OLIVEIRA, 2015; REYES-MONTAÑO; VEGA-CASTRO, 2018), antifungal (GOMES et al., 2012), bactericidal and antibiofilm (ALYOUSEF; ALQASIM; ALOAHD, 2018; VASCONCELOS et al., 2014), antiviral (MAZALOVSKA; KOUOKAM, 2018), inflammatory effect (ALMEIDA et al., 2016; ASSREUY et al., 1997), vasorelaxant effect (ALVES et al., 2015), cancer mapping and diagnosis (HASHIM; JAYAPALAN; LEE, 2017), immunomodulatory lectins (SOUZA et al., 2013), capture of glycoconjugates for glycoproteomics studies (ALVES et al., 2015) as well as their applications in nanobiotechnology aiming the use in therapy and research (SHARMA, ANJALI; SHARMA;

KHULLER, 2004; VENUGOPAL et al., 2016).

1.5 Diocleinae lectins

Legume lectins extracted from plants of the Diocleinae subtribe normally have specificity for mannose/glucose and related carbohydrates. This group of lectins, also called ConA-like lectins, designated because of their similarity with the lectin from *Canavalia ensiformis* (ConA), is quite well-studied and several works reported on their purification, physicochemical and structural characterizations in addition to their applications and several biological activities (CAVADA et al., 2018; CAVADA et al., 2019; OSTERNE et al., 2017). An interesting feature about this group of proteins is their high degree of similarity at primary and tertiary structure level, which does not prevent them from showing very different results when tested for biological activities. The explanation for this is the fact that the replacement of some amino acids alters the position of key residues, which results in changes in the tertiary and quaternary structure, slightly altering the interaction with carbohydrates and, consequently, the biological activities dependent on this interaction (CAVADA et al., 2019; CAVADA et al., 2001).

ConA-like lectins have the interesting property of having a set of very characteristic subunits, resulting from a post-translational processing called circular permutation (GOODSELL, 2010; HEMPERLY; CUNNINGHAM, 1983). In this processing, the lectin gene is translated into a glycosylated preprotein. In the endoplasmic reticulum, the lectin precursor loses its signal peptide and is now called pro-lectin. In the Golgi complex, the pro-lectin undergoes proteolytic cleavage and loses a glycosylated segment of fifteen amino acids in the center of its structure and a peptide in its C-terminal region, this process releases a sufficient amount of energy for later rewiring. With this loss in the initial chain, the polypeptide is now cleaved into two, β and γ, peptides which are now linked together, but in different order. The subunit that was previously in the N-terminal region is linked to the β subunit having an inversion between the N and C-terminal regions forming the α subunit also called mature lectin. This process is catalyzed by an asparaginyl endopeptidase enzyme that does not have 100% yield, since some unbound β and γ chains are always present in the preparation of these lectins. Therefore, in an SDS-PAGE gel, three bands are observed. It is believed that most ConA-like lectins undergo the same processing (CARRINGTON; AUFFRET; HANKE, 1985; OSTERNE et al., 2014; SHARON; LIS, 2007). The determination of the primary and tertiary structures was used as a way to understand this

difference in specifying purified proteins from closely related phylogenetically specimens and to obtain information about their evolutionary origin.

Another property of some ConA-like lectins is the pH-dependent oligomerization, in which the proportion of proteins in dimeric and tetrameric oligomerization can be changed according to the pH of the medium (NAGANO et al., 2008). The changes in this dimer/tetramer balance can directly influence the biological activities, because these lectins are able to interact with glycans of glycoconjugates with greater affinity when in the tetrameric form (OLIVEIRA et al., 2008; SOL et al., 2007; WAH et al., 2001).

Several lectins from the Diocleinae subtribe can elicit biological activities such as inflammatory, vasoactive, antiproliferative, immunomodulatory, among others. These activities are directly related to their capacity to interact with glycan ligands on the cell surface and because of this, the effect is closely affected by the affinity and the mode of binding between carbohydrate and lectin generating variability in effect (CAVADA et al., 2019a, 2019b).

1.6 *Dioclea violacea lectin*

Dioclea violacea is a legume native to Brazil popularly known as bull's-eye. The plant is a woody vine whose leaves are composed of three oval elliptical leaflets, it has an inflorescence in the shape of an ear, long with purple flowers and yellowish-white petals at the base. The fruits are pods containing large, hard and brown colored flat globose seeds (QUEIROZ, 2015; ZAPPI et al., 2015). This plant is used in folk medicine as a tranquilizer, tonics, parasiticide and ant killer. It is also verified its application in the prevention of strokes and removal of sequelae resulting from this problem, besides being applied in cases of epilepsy (FERN, 2020). Images of the seed and plant as well as the taxonomy of *Dioclea violacea* are shown in figure 5.

Figure 5 – Taxonomy and pictures of *Dioclea violacea* seeds and plants.

Kingdom: *Plantae*

Division: *Magnoliophyta*

Class: *Magnoliopsida*

Order: *Fabales*

Family: *Leguminosae*

Tribe: Phaseolae

Subtribe: Diocleinae

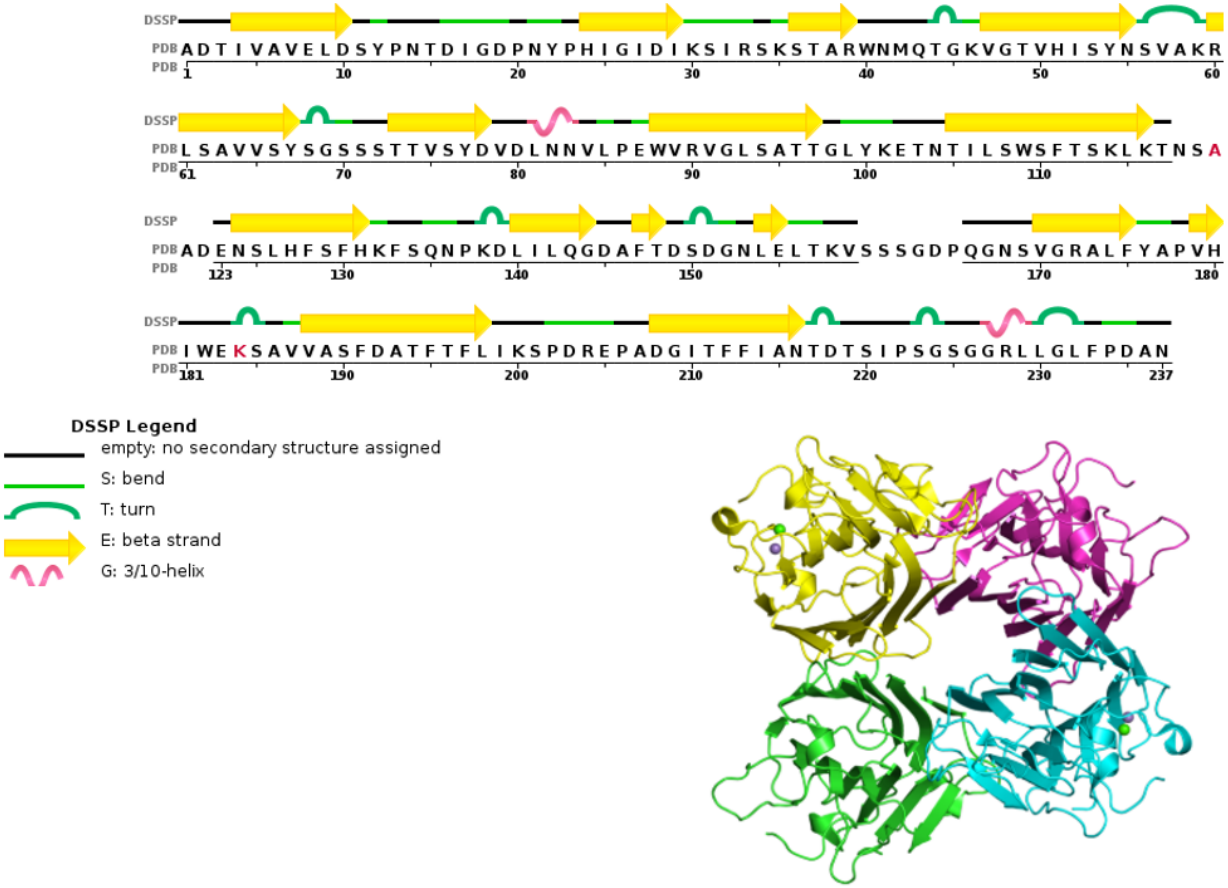
Genus: *Dioclea*

Species: *Dioclea violacea*

Source: Images from: https://faunaefloradorn.blogspot.com/2018/07/olho-de-boi-dioclea-violacea-mart-ex.html

Dioclea violacea lectin (DVL) is a ConA-like lectin with affinity for mannose, glucose and related sugars initially purified by Moreira et al. 1996. ConA and DVL proved to be quite similar, sharing more than 80% similarity at the level of primary structure and also at the level of three-dimensional structure, despite their effects on biological tests being quite different in some works (NASCIMENTO et al., 2018, 2019). Structurally, DVL presents itself in tetrameric oligomerization regardless of the pH of the medium (BEZERRA et al., 2013). In animal models, the administration of DVL was associated with both anti-inflammatory and pro-inflammatory actions (CLEMENTE-NAPIMOGA et al., 2019; DE ALENCAR et al., 2013). In addition, DVL was able to induce relaxation of rat aortas in addition to other biological activities. Regarding the antitumor effect, it has been reported that DVL has an antitumor effect on glioma cells of mice of the C6 line and human glioma of the U87 line, an effect that is involved in the activation of programmed cell death phenomena, basically autophagy and apoptosis (NASCIMENTO et al., 2018, 2019). The sequence and three-dimensional structure are depicted in figure 6.

Figure 6 – Primary, secondary and three-dimensional structures of *Dioclea violacea* lectin (PDB id: 2GDF)



Source: Elaborated by the author.

1.7 Lectin applications in cancer diagnosis and therapy

The term cancer includes different pathologies that have as common characteristics: dysregulation in cell division, loss of the cell differentiation ability, increased cell survival and increased capacity for cell migration (FOUAD; AANEI, 2017). Tumorigenesis in humans is a complex process that reflects genetic changes that drive a progressive transformation of normal cells into highly malignant derivatives (TABASSUM; POLYAK, 2015). The process by which normal cells progressively transform into tumor cells may be the result of endogenous processes such as errors in DNA replication, the intrinsic chemical instability of some DNA bases, the loss of heterozygosity or an attack of free radicals generated during metabolism. DNA damage can also be the result of interactions with exogenous agents such as ionizing radiation, UV radiation and/or chemical carcinogens. The cells have mechanisms to repair such damage, but, for various reasons, errors can occur and,

with this, mutations in the genome of these cells (BERTRAM, 2000).

The transition from normal to tumor cells involves the participation of genes involved in homeostasis mechanisms that control cell proliferation and death. If these changes and mutations induce the activation of genes that stimulate proliferation or protect the cell from death, these genes are referred to as protooncogenes. If mutations inactivate genes that normally inhibit proliferation, these genes are referred to as tumor suppressors (LEE; MULLER, 2010). Cancer marks are six capacities acquired during the development of tumors. They include the maintenance of proliferative signaling, evasion of proliferative suppressors, resistance to cell death, activation of replicative immortality, induction of angiogenesis and capacity for invasion and metastasis (HANAHAN; WEINBERG, 2011).

Current approaches to the treatment of cancer and its effects inevitably end up causing several harmful and dangerous conditions for the patient, because of this there is a demand for an effective and specific alternative against these diseases that would generate less adverse effects. To lessen these damaging conditions, some specific targets are suggested, opening doors for new therapies and diagnosis methods.

As there are aberrant structures and the appearing of glycoconjugates with unusual structures in glycans found in cancer cells, the detection of these glycoconjugates is easily accomplished by applying lectins (HASHIM; JAYAPALAN; LEE, 2017). Plant lectins have also been used as tools for tumor recognition considering their capacity to differentiate benign and malignant tumors based on the degree of glycosylation (PIHÍKOVÁ; KASÁK; TKAC, 2015). As an example, *Lens culinaris* (LCA) lectin is useful in discriminating hepatocellular carcinoma from benign liver diseases and is also advantageous in the diagnosis of some hepatoma metastasis conditions (ALSALLOOM, 2016). The development of new lectin microarrays helps in the determination of glycan structures and provides a useful platform for high reducibility analysis of biological samples by providing a new dimension for cancer diagnosis and prognosis.

Besides recognizing cancer cell glycans, several lectins also demonstrate antiproliferative activity directly, this effect is usually caused by binding to glycans in the cell membrane eliciting processes such as agglutination or aggregation concomitantly or not with cytotoxicity by mechanisms such as apoptosis, autophagy and/or necrosis (DE MEJÍA; PRISECARU, 2005). Besides what was previously said, lectins also can induce immunomodulatory activities through interaction with the glycans present on the surface of immune cells, which activates the release of cytokines that exert a tumoricidal effect by altering the production of cytokines (SOUZA et al., 2013). Similarly, new strategies have been

established in preclinical and clinical investigations to develop a combinatorial treatment that consists of immunotherapy together with other anticancer therapies. Plant lectins have also been used extensively for specific targeting in drug delivery mechanisms. For example, therapies for bladder cancer with wheat germ lectin as an agent to deliver doxorubicin directly to the tumor (APFELTHALER et al., 2018) in addition to lectins immobilized on nanostructured structures for drug delivery.

In addition to being used for drug delivery, studies have shown that lectins themselves can be applied as anticancer agents because of their potent antiproliferative effects, and the ability to induce apoptosis and autophagy in several tumor lines (CATANZARO et al., 2019). The effect of these lectins is related to their ability to bind to glycosylated proteins in the membrane of cancer cells and it is well known that glycoconjugates have important functions in several biological processes with the development and maintenance of cancer being one of them.

Studies have shown the antiproliferative effect of lectins such as the lectins of *Canavalia ensiformis* (ConA), *Canavalia bonariensis* (CaBo), *Dioclea violacea* (DVL) and *Dioclea lasiocarpa* (DLL) against glioblastoma cells. All these lectins induced a significant reduction in cell viability. Some of them affected the mitochondrial membrane potential, cell migration and induced morphological changes in the cells. Colorimetric analysis indicated cell death by autophagy, apoptosis and necrosis induced by mitochondrial pathway and metalloproteinase modulation (CAVADA et al., 2018; NASCIMENTO et al., 2019; PRATT; ROY; ANNABI, 2012). Lectins were also tested against cancer cell lines of ovary, lung, prostate, breast, skin, among others and, in practically all cases, some level of cell death induction was usually observed by the induction of some programmed cell death pathway (CAVADA et al., 2018; DE MEJÍA; PRISECARU, 2005; GONDIM et al., 2017).

1.8 Nanotechnology and $CaCO_3$ particles

Nanotechnology is defined as any nanoscale technology that has application in the real world. Nanotechnology encompasses the production and application of physical, chemical and biological systems at scales ranging from individual to several atoms or molecules in nanometric dimensions, as well as the integration of the resulting nanostructures into larger systems. Nanotechnology is likely to have a profound impact on our economy and society in the early 21st century, comparable to that of semiconductor technology, information technology and cellular/molecular biology. Nanotechnology science and technology promises

advances in areas such as materials and manufacturing, nanoelectronics, medicine and health, energy, biotechnology, information technology and national security. Nanotechnology is widely considered to be the next Industrial Revolution (JEEVANANDAM et al., 2018; RAI; RAI 2015).

Nanoscale resources are built mainly from their elementary constituents (KHAN, IBRAHIM; SAEED; KHAN, 2019). Examples include chemical synthesis, self-assembly from simple reagents in solution, biomolecules such as DNA and proteins used as building blocks to produce nanostructures and quantum dots (BHUSHAN, 2007). The discovery of new materials and processes on a nanometric scale and the development of novel experimental and theoretical techniques create new opportunities for the development of innovative nanosystems and nanostructured materials. The properties of nanomaterials can be dramatically different from those on a larger scale. When the size of a material is small, considerable changes in properties take place. If only one length of a three-dimensional nanostructure is of nanometric dimensions, the structure is referred to as a quantum well; if two sides are a nanometer long, the structure is called a quantum wire. A quantum dot has all three dimensions in the nano range. The term quantum is associated with these three types of nanostructures because changes in properties arise from the mechanical nature of physics in the nanometric domain (OWENS; POOLE, 2008). Materials can be nanostructured for new properties and innovative performance. This field is breaking new ground in science, technology and health.

Within nanotechnology, promising candidates for the development of drug delivery and controlled release systems are porous nanoparticles made of inorganic materials (PATRA et al., 2018). In this context, calcium carbonate ($CaCO_3$) is a promising inorganic biomaterial due to its chemical stability, bioactivity, biocompatibility and potential for application in drug delivery (DIZAJ et al., 2015). $CaCO_3$ exists in three anhydrous polymorphic modifications: vaterite, aragonite and calcite, with rhombohedral, orthorhombic, and hexagonal structures, respectively (ERCAN; ORAL; KAPUSUZ, 2019). Among these polymorphs, vaterite is the most promising due to its tendency to form polycrystalline aggregates with high porosity and surface area resulting in high drug loading capacity and the possibility of slow/controlled release of the therapeutic substance (TRUSHINA et al., 2014). The size and shape of the particles has a high influence on recrystallizations, dissolution properties and stability in solution. In drug delivery applications, the particle shape has been shown to be strongly related to cellular responses, the absorption of particles by cells, the effect on cell viability, apoptosis, adhesion, migration and cytoskeleton formation (JINDAL,

2017; ZHENG; YU, 2016).

Calcium carbonate particles have a wide range of applications as models for the assembly of microparticles from hydroxyapatite (TRUSHINA et al., 2014) or in complex with biomolecules like proteins (ERGUL YILMAZ et al., 2016), in addition to microcapsules and nanocapsules created by the technique known as a layer by layer (JOSHI; SRIVASTAVA, 2009; SUKHORUKOV et al., 2004). For the manufacture of capsules, calcium carbonate particles are widely recognized as being among the most attractive cores. This is mainly due to the simple and low-cost methods available for making them. The structure of the capsule is determined by the size, shape and properties of the template (BISWAS; NAGARAJA; MCSHANE, 2014; LIU et al., 2009) and the porosity of the solid template determines the internal structure of the capsule, the morphology of the shell and the release properties of the final material (LIU et al., 2009; YOW; ROUTH, 2006). Porous particles, such as calcium carbonate, allow for more effective encapsulation of molecules than non-porous particles, such as silica and polystyrene (RENDER et al., 2016; SUKHORUKOV et al., 2004; TROFIMOV et al., 2018).

Various morphologies of calcium carbonate particles in the micrometer size range, including hollow spheres (CAI et al., 2008), hexagons (POUGET et al., 2010), microrings (MAO et al., 2013) among others. Many approaches have been widely used to control the phases and morphology of calcium carbonate particles and water-soluble additives, such as anionic surfactants, have typically been used to modify polymorphs of $CaCO_3$ crystals (KANG et al., 2005). Vaterite particles with different morphologies (such as peanuts, dumbbell type, etc.) were produced with hexagonal slices. In such approaches, copolymers were generally made during manufacture (BOYJOO; PAREEK; LIU, 2014). However, the presence of soluble additives can complicate the final structure and lead to interesting properties, especially with regard to biocompatibility.

The size of the spherical crystals of porous $CaCO_3$ vaterite can be regulated by stirring conditions, mainly stirring time and speed during the direct mixing of precursor salts ($CaCl_2$ and Na_2CO_3) in supersaturation (YAN et al., 2009), this phenomenon was explained by a stirring effect on the formation of nuclei during the heterogeneous process of heterogeneous nucleation. An alternative approach that can be used to control the shape and size of vaterite particles is based on decreasing the rate of solubilization of salts, partially replacing the solvent; in fact, the influence of solvent on the size of spherical particles of vaterite and the mechanism of its formation has been previously reported (KONOPACKA-ŁYSKAWA, 2019). Furthermore, the synthesis of anisotropic particles has recently been

demonstrated (PARAKHONSKIY et al., 2015). Some of the more advanced applications of these particles as well as capsules modeled on these particles are envisaged in the area of drug delivery and controlled release.

Biomolecules and particles can interact in different ways, usually through some type of functionalization. Several types of conjugation strategies have been developed. One of the oldest methods for binding molecules and particles is direct adsorption. Biomolecules can interact with particles in direct ways with different types of particles. However, the interaction of this type is usually weak and non-specific, and because of this, few applications make use of this type of conjugation (CAGLIANI; GATTO; BARDI, 2019) although this characteristic may be desirable as in the case of slow release systems. The covalent coupling provides a specific and stable conjugation of biomolecules with particles. Typically, functional groups on the surface of the particles including carboxylic groups, amides and thiols can make connections with biomolecules through various strategies whose end result is the formation of a stable conjugate between the particle and the biomolecule (AUBIN-TAM, 2013; CAGLIANI; GATTO; BARDI, 2019; MOLAVIPORDANJANI; HOSSEINIMEHR, 2019).

1.9 Controlled release systems

Conventional drug administration methods are developed in a way to release the active agent quickly. In these systems, usually, diluent systems are employed to help in the agent solubilization and, with the fast release, keeping the drug inside a therapeutic range is sometimes difficult (WEN; JUNG; LI, 2015). In conventional methods of administration, the drug concentration rises until complete dissolution and starts to decline shortly after, thus leading to some problems. High concentrations of the drug, above therapeutic range, can be toxic while low concentrations may not be effective and have the additional problem of leading to resistance. One of the ways to minimize these problems is the creation of controlled release systems, usually applying nanostructured systems to encapsulate and modulate the drug release and maintaining its concentration in the therapeutic range for as long as possible (SAVJANI; GAJJAR; SAVJANI, 2012; WEN; JUNG; LI, 2015).

Controlled release systems emerge as a new strategy to drug administration leading to some big advantages such as a better control of the liberation of the drug, reduced times in toxic and subtherapeutic doses, possibility of checking the drug levels in the application site, obtention of high concentrations of the drug on the specific local to be treated, directing of the active principle to specific targets without passing through problematic places

such as the gastrointestinal tract, which can be an hindrance if the drug is not absorbed by this route. Some of these systems can also present the capacity of protecting the active substance from degradation which, in a conventional administration method, would require repeated applications of the substance (HOMAYUN; LIN; CHOI, 2019; TIWARI et al., 2012). One of the main factors to be considered in controlled release systems is the degradation of the encapsulating system, which directly influences the drug release thus making the stability of the system one main factors to be considered to avoid premature degradation. Polymeric systems applied in controlled release systems can be synthesized from several polymers, biodegradable or not. These polymers need to be biocompatible, chemically inert, non-carcinogenic and hypoallergenic. In addition, the material should not be physical or chemically altered by the local tissue or elicit any inflammatory response (KAMALY et al., 2016; LEE; YEO, 2015; YANG; PIERSTORFF, 2012). Different kinds of materials and colloidal systems can and will influence the kinetics, distribution, and the release of the active substance, e.g. drug, vaccine, or therapeutic protein.

The main systems applied for drug delivery and release are those which the drug is found incorporated or adsorbed in the structure of the carrier. The drug can be released in the body mainly by two different mechanisms namely diffusion and erosion (LIECHTY et al., 2010; RAVAL; PARIKH; ENGINEER, 2010). In the diffusion, the active agent diffuses out of the encapsulating agent because of the difference of concentration. In the erosion, the chemical control takes place because of the carrier degradation. A third type of release involves the solvent activation process, in which the polymeric chains are broken by the cleavage of the chemical bonds resulting in drug release. Another strategy is applied when the drug is covalently attached to the polymer and can be released by specific enzymes (LIECHTY et al., 2010).

1.10 Molecular docking applied in lectinology

The arising of new sequencing technologies, several species had their genome sequenced, revealing the knowledge about several proteins even before their purification. The obtention of protein structure and its overall analysis is still very dependent on expensive and laborious methodologies such as X-ray crystallography. The bioinformatics field applied to proteins is a nice complement for these studies because of its capacity to predict some results thus directing and reducing the necessary bench experiments. Bioinformatics applied in protein studies focuses on molecular modeling to predict the three-dimensional structure

usually basing itself on other experimentally solved structures with a high degree of identity, molecular docking to predict the binding between proteins and ligands and molecular dynamics to study the states of the protein in solution, this last technique complements the other two helping in their validation as well as generating lots of data regarding the protein dynamics.

The field of bioinformatics in molecular docking has emerged due to the needs of structural biology and structure-based drug discovery. This technique has been facilitated by the dramatic increase in computational power and the facilitation of access to ligand structures in the literature and databases (PINZI; RASTELLI, 2019). The purpose of molecular docking is to predict molecular recognition, both structurally by finding connection poses and energetically by predicting connection affinity. This technique has a variety of applications including prediction of the binding site location in proteins, location of regions for drugs interactions, binding analysis between lectins and carbohydrates, measurement of the strength of interactions between molecules. In addition, it is possible to verify how structural changes can affect the proteins binding with ligands, through comparative analysis with mutated molecules (FAN; FU; ZHANG, 2019; JAIN et al., 2018).

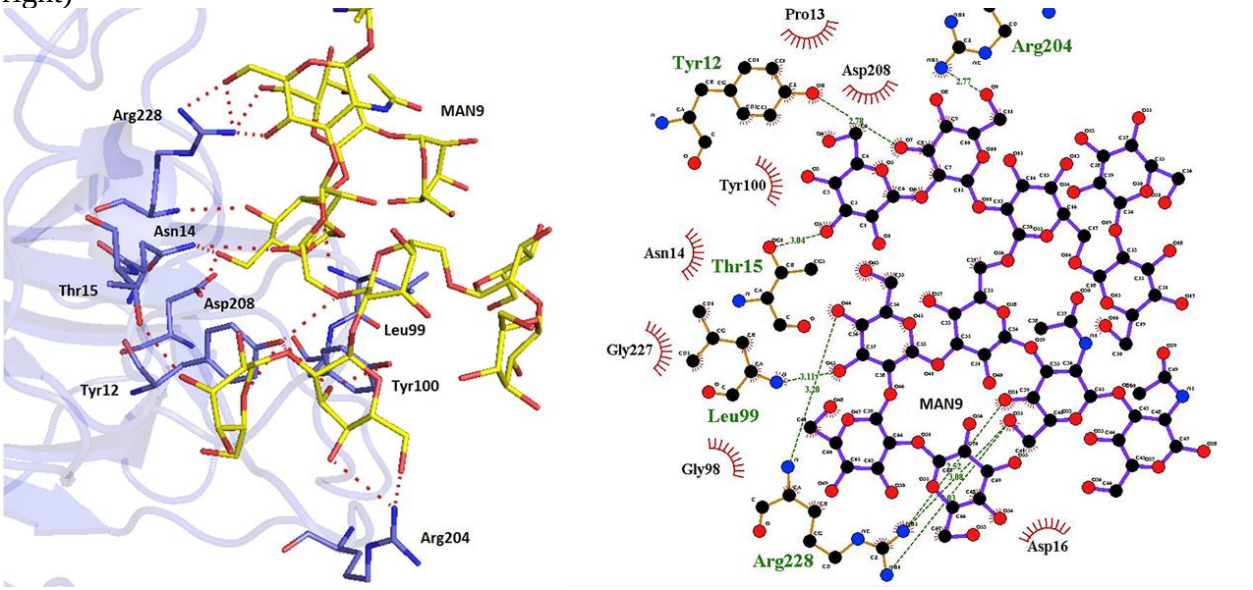
This technique allows the screening of large libraries of compounds, evaluation of the affinity and specificity based on structural and chemical properties, such as size, geometry, charge distribution, polarity and potential for hydrophobic interactions and hydrogen bonds. The purpose of screening banks of possible ligands is to identify compounds that bind to a target protein. In this way, the biochemical reaction that the target protein performs can be altered, allowing the discovery of possible medical and/or biotechnological tools (CHEN et al., 2012; COLEMAN et al., 2013). Currently, access to a database of protein structures, (Protein Data Bank), and large libraries of small commercially accessible molecules, such as ZINC database (IRWIN; SHOICHET, 2005) provide a large number of possible new ligands. Once these ligands are tested in silico they can be purchased or synthesized for in vitro and/or in vivo testing, thus speeding up pharmaceutical and biotechnology research (LORBER; SHOICHET, 2005). This approach of testing a large number of compounds using molecular docking is called virtual screening.

The docking of molecules with possible ligands follows an order. First, the separation of the probable connection sites is made, after that, the overlapping of these regions is made, and by means of statistical analysis and punctuation functions, one tries to obtain the complexes closer to what occurs experimentally. The analysis of the inserts is given by punctuating the formation of each complex. From this score, a comparative analysis is

performed, in which each formed complex is evaluated, and, at the end, they are positioned in a ranking (SCHNEIDMAN-DUHOVNY et al., 2005, 2012).

Molecular docking has been widely applied in the study with lectins in order to seek carbohydrate structures capable of binding lectins of interest. Some examples of application of this technique are: use of it in *Dolichos lablab* lectin for analysis of the interaction between protein and oligomannosides (TEIXEIRA et al., 2014), search for ligands for bacterial lectins (TOPIN et al., 2013) and for the analysis of the interaction between lectins and several glycans (CAVADA et al., 2018; MARQUES et al., 2017), among others. An image of a molecular docking between the lectin of *Dioclea lasiophylla* and a glycan is shown in figure 7.

Figure 7 – Molecular docking between *Dioclea lasiophylla* lectin and a high-mannose glycan. Stick representation of the lectin CRD in complex with the glycan with carbons in blue for the protein and in yellow for the ligand (in the left). 2D Ligplot diagram of the interactions (in the right)



Source: Pinto-Junior and colleagues (2017).

2 OBJECTIVES

2.1 Main objective

This Ph.D. research is part of a much bigger project that focus on the purification, physicochemical and structural characterization, biological activities and bioinformatics studies of multiple lectins. The results of the current project can be accessed in the papers listed in the attachment section. With regard to the present work, the main objective was to build a controlled/slow release system for the lectin of *Dioclea violacea* by applying particles of calcium carbonate and to investigate the effects of the lectin and the derived bioconjugates against cervical cancer cells of HeLa strain.

2.2 Specific objectives

- To purify the lectin from *Dioclea violacea* seeds by applying consolidated techniques of protein chemistry.
- To synthesize micro- and nanoparticles of calcium carbonate.
- To evaluate the capacity of the particles to adsorb DVL as well as the adsorption yield.
- To characterize the adsorption regarding its dependence of time and lectin concentration.
- To compare the recrystallization process of the particles alone and the bioconjugates.
- To evaluate the release of DVL from the particles.
- To characterize the bioconjugates by FITR and electron microscopy.
- To evaluate the cytotoxic potential of DVL and the bioconjugates against HeLa cells.
- To study the interaction between DVL and glycans found in the surface of HeLa cells using *in silico* simulations of molecular docking.
- To investigate the uptake of lectin conjugates by HeLa cells using confocal microscopy.

3 METHODOLOGY

3.1 DVL isolation and purification

The purification of the lectin from *Dioclea violacea seeds* has been performed according to a protocol adapted from Moreira et al. (1996) (MOREIRA et al., 1996). Initially, peeled seeds were ground in a coffee grinder until the obtention of a fine powder. Afterward, the power was resuspended in 150 mM NaCl (1:10 w/v) and kept under constant stirring for 4 h at room temperature for soluble protein extraction. The suspension was then centrifuged at 10.000 *g* for 20 min, the pellet was discarded, the supernatant filtered in a paper filter (Whatman TM), collected, and designated as crude extract. Following, this extract was applied in an affinity chromatography in Sephadex(R)-G50 matrix previously equilibrated with 150 mM NaCl containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$.Non-retained proteins (P1) were eluted with the same solution and the retained fraction (P2) was eluted with 100 mM glucose in 150 mM NaCl. Fractions of 1.5 mL were collected with 2 mL/min flow and all fractions were monitored by spectrophotometry at 280 nm wavelength. Retained fractions were pooled, dialyzed against distilled water and freeze-dried. Hemagglutination tests were applied to determine the lectin activity during the purification steps. This test was performed in 96-well plates in which 50 mM Tris-HCl pH 7.6 + 150 mM NaCl was added in each well followed by the sample addition in the first well, the solution was then serially diluted and a suspension of rabbit erythrocytes at 3% in 150 mM NaCl was added to each well. Plates were incubated at 37 ºC for 30 min followed by 30 min of incubation at room temperature after which the agglutination was macroscopically observed. This assay was performed in triplicate and buffer-only was applied as control.

3.2 Particle Synthesis

Spherical particles of $CaCO_3$ were obtained as a result of colloidal aggregation reaction due to the rapid mixture of $CaCl_2$ and Na_2CO_3 (PARAKHONSKIY et al., 2014). For the preparation of big particles of $CaCO_3$ (MPs), previously prepared solutions of $CaCl_2$ and Na_2CO_3, both at 330 mM concentration were mixed in equal proportions and kept under agitation on a magnetic stirrer (500 rpm) at room temperature for 1 h. After which, the particles were separated from the suspension by centrifugation at 3200 *g* for 3 min, washed with 100% ethanol and dried in an oven at 50 °C overnight.

For the preparation of smaller particles (NPs), $CaCl_2$ and Na_2CO_3, both at 330 mM were mixed in equal proportions followed by the addition ethylene glycol was added in the reaction to a final concentration of 83%. The suspension was kept under constant stirring (500 rpm). The same way as the bigger particles, the smaller particles were separated from the suspension by centrifugation at 3200 *g* for 10 min, washed with ethanol 100% and dried in an oven at 50 °C.

3.3 Bioconjugation

Initially, solutions of DVL at the concentrations 1 mg/mL, 0.5 mg/mL and 0.1 mg/mL were prepared by solubilizing the freeze-dried lectin in 50 mM Tris-HCl pH 7.6 + 150 mM NaCl. To verify the adsorption of DVL on MPs and NPs, 1 mL of each solution was mixed with 10 mg of MPs and NPs and the suspension was kept under constant stirring on an orbital shaker for 1 h. Following, the solid was separated from the liquid by centrifugation at 5000 *g* for 5 min. The amount of lectin adsorbed in each condition was calculated by spectrophotometry at 280 nm wavelength in which the supernatant was measured, the absorbance applied in a previously prepared standard curve and the concentration was estimated and removed from the initial value to determine the amount of lectin in the particles. The concentration with higher adsorption without a big waste of lectin was chosen for the next steps of the characterization. These experiments were performed in triplicate.

To check the activity of the lectin after the conjugation, hemagglutination assays were employed by using rabbit erythrocytes

3.4 Bioconjugate characterization

3.4.1 Adsorption over time

The dependence of the time for the adsorption yield was checked as follows: 1 mL of a freshly prepared solution of DVL in 50 mM Tris-HCl pH 7.6 + 150 mM NaCl at 1 mg/mL was mixed with 10 mg of MPs and NPs and kept under constant stirring for different times (5 min, 30 min, 1 h, 2 h and 3h) at room temperature. After each time point, the suspension was centrifuged at 5000 *g* for 5 min, the supernatant was removed and the concentration of lectin was measured by spectrophotometry at 280 nm, in order to determine the amount of lectin in the particles after each time point. The optimum time with higher

adsorption was chosen for the next steps. Each time point was tested three times.

3.4.2 Recrystallization

The time required for the particles to recrystallize in a calcite phase was calculated for non-conjugated and DVL-conjugated particles to verify if the conjugates are compatible with the biological activities to be performed as well as to verify if the lectin can affect the recrystallization time. For this, MPs-DVL and NPs-DVL were freshly prepared by mixing 1 mL of a 1 mg/mL solution of DVL with 10 mg of MPs and NPs and stirring the suspension in an orbital shaker until maximum adsorption yield. After the removal of the supernatant, both MPs-DVL and NPs-DVL conjugates and MPs-only and NPs-only were mixed with 1 mL of 50 mM Tris-HCl pH 7.6 + 150 mM NaCl and kept under stirring for several days. At regular intervals, a drop of the suspension was removed and observed by optical microscopy (100x magnification) with a 100x/1.4-0.7-oil immersion objective. The number of particles in vaterite and calcite were then manually counted. A minimum of 100 particles have been counted each time. The results were expressed as a percentage of calcite particles. This experiment was performed in triplicate.

3.4.3 DVL release assays

For the construction of a controlled/slow release system it is necessary to verify the release of DVL from the particles over time. To check the time for the release of DVL from MPs and NPs, conjugates were prepared by mixing 1 mL of a 1 mg/mL solution of DVL with 10 mg of MPs and NPs and stirring the suspension in an orbital shaker for 1 h after which the particles were separated from the solution by centrifugation at 5000 *g* for 5 min. Conjugates were then mixed with 1 mL of 50 mM Tris-HCl pH 7.6 + 150 mM NaCl and stirred in orbital shaker and, after regular intervals (5 min, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days and 10 days) the supernatant was removed by centrifugation and the amount of lectin was measured by spectrophotometry at 280 nm and the concentration of released lectin was calculated. The solution was returned to the tube containing the particles and the stirring was continued until the last point. MPs and NPs-only with 50 mM Tris-HCl pH 7.6 + 150 mM NaCl were applied as controls. This experiment was performed in triplicate.

3.4.4 Fourier-transform infrared spectroscopy (FTIR)

The molecular structure of MPs and NPs with and without DVL was examined by Fourier-transform infrared spectroscopy. Fresh conjugates were prepared by mixing 1 mL of a 1 mg/mL solution of DVL with 10 mg of MPs and NPs and stirring the suspension in an orbital shaker for 1 h after which the particles were separated from the solution by centrifugation at 5000 g for 5 min. The particles were then dried in an oven at 50 ºC and one spectrum per sample was collected in the 400 and 1500 cm^{-1} spectral range with a resolution of 2 cm^{-1} and an average of 25 scans.

3.4.5 Electron microscopy

The particles morphology was determined by electron microscopy in a field-emission environmental scanning electron microscope with 5000x to 50000x magnification.

3.5 Cell assays

3.5.1 Cell culture

The human HeLa cervical cancer cell line (LANDRY et al., 2013) was maintained in Dulbecco-modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). The cells were incubated at 37 ° C in a humidified atmosphere of 95% O_2 and CO_2 5% until 80-90% confluence, the point which they were applied in the subsequent tests.

3.5.2 Viability assays

DVL and the bioconjugates were solubilized/suspended in sodium phosphate buffer saline pH 7.4 at the initial concentration of 1 mg/mL of lectin. For the tests, 96 wells plates were seeded with DMEM medium supplemented with 10% fetal serum (FBS), followed by the lectin/bioconjugate in different DVL concentrations ranging from 6.25 µg/mL (0.062 µM) to 100 µg/mL (1 µM) or vehicle (control) followed by the addition of cells to a final number of 10,000 cells/well. Following, the plates were incubated for 24 h, 48 h and 72 h at 37 ºC in a humidified atmosphere of 95% O_2 and 5% CO_2. After the treatment period, the PrestoBlue™ Cell Viability reagent (ThermoFisher Scientific) was added and the plates were

incubated for 2 h at 37 °C protected from the light for the determination of the cell viability. Fluorescence was measured on equipment Infinite 200 PRO (Tecan) with excitation at 560 nm and emission at 590 nm. The number of cells in each well was determined by comparing the fluorescence value with a previously prepared standard curve. Three biological replicates were made each one with 3 technical repetitions. The effect of the treatment of cell morphology was assessed by optical microscopy in a widefield microscope (40x magnification).

3.5.3 Uptake of bioconjugates in cells

The visualization of the uptake in bioconjugates in HeLa cells was performed using confocal microscopy. For the labelling of DVL with rhodamine, a 4 mg/mL solution of lectin was prepared in sodium bicarbonate buffer while 1 mg/mL tetramethylrhodamine (TRITC) was prepared in DMSO. TRITC solution was added to the lectin in a ratio of 50 µL TRITC per mL of lectin solution.

For protein-particle adsorption, 10 milligrams of MPs and NPs were incubated each with 1 mL DVL-TRITC for 30 min under constant stirring. Afterwards, the particles were centrifuged for 3 min at 3,000 *g* and washed twice with PBS (Sigma-Aldrich) to remove the excess of DVL-TRITC. The control was prepared with BSA-TRITC. Before confocal experiments, particles were counted using a hemocytometer and diluted in DMEM medium to reach a final concentration of 10 particles per cell.

The assays were performed as follows: Hela cells were cultivated as explained in section 3.5.1, seeded in glass bottom cell culture dishes (35,000 cell/dish) and incubated overnight at 37 ºC in atmosphere of 95% O_2 and 5% CO_2 for cell adhesion. Afterwards, cell culture medium was discarded, cells were washed with PBS and the conjugates DVL-TRITC-particles were added to dishes and incubated for 48h at 37 ºC. Cells were stained with calcein (Thermofisher) for 45 min at 37 ºC and washed twice with PBS before analyses under confocal microscope.

Confocal images were obtained using a Nikon Instruments A1 Confocal Laser Inverted Microscope. NIS-Elements C Software (Laboratory Imaging) was used for data collection and image analysis. Emissions/excitation wavelengths were 525/488 for calcein and 595/560 for TRITC.

3.6 Bioinformatics - Molecular docking

To verify the potential ability of DVL to interact with *N*-glycans present on the HeLa cell membrane, the molecular docking technique was applied. Initially, the structure of the glycans present in the cells was obtained from the literature (HORVAT et al., 2013) and designed with the help of the Carbohydrate Builder server (http://glycam.org) (KIRSCHNER et al., 2008) and all the generated structures were submitted to energy minimization with a module built in the server. Molecular docking simulations were performed using the GOLD v. 5.5 (CDCC, Cambridge, England), a program that tests various conformations of ligands, allowing partial flexibility of the receptor residues (JONES et al., 1997). The docking region has been defined as the center being the carbohydrate-recognition domain of DVL as well as all atoms within a radius of 11 Å from it. The parameters used for docking were: size population of 100, selection pressure of 1.1, number of operations of 10,000, number of islands 5, niche size 2, crossing frequency 95, a total of 20 poses for each ligand and Chemscore scoring function (ELDRIDGE et al., 1997). The best poses were chosen by combining the fitting score, penalties in the geometry ligand, hydrogen bonds and hydrophobic interactions (DE ÁVILA et al., 2017; HEBERLÉ; AZEVEDO, 2011; HECK et al., 2017; XAVIER et al., 2016). Figures were generated by PyMol software (Schrodinger).

4 RESULTS AND DISCUSSION

4.1 DVL purification

The lectin from *Dioclea violacea* seeds was purified by affinity chromatography on a dextran-affinity matrix. The retained protein was eluted with 100 mM D-glucose. The hemagglutination activity of each fraction after chromatography was determined using agglutination assays with rabbit erythrocytes, and hemagglutination was observed solely on the fraction retained on the matrix, thus indicating that DVL was completely separated from the other proteins in the crude extract.

4.2 Particle synthesis

In the present work, two different sizes of $CaCO_3$ particles have been obtained. Bigger particles (MPs) presented an average diameter of 4 μm and have been obtained by mixing equimolar amounts of $CaCl_2$ and Na_2CO_3 while the synthesis of smaller particles (NPs), with about 700 nm of diameter, included ethylene glycol in the reaction which favored the synthesis of smaller particles as explained below.

$CaCO_3$ vaterite particles were obtained using a rapid mixture of calcium chloride and sodium carbonate salts. These particles emerge due to the processes of nucleation, growth and aggregation of calcium carbonate (PARAKHONSKIY et al., 2014). The particle size during this process is inversely proportional to the reaction time, but this process is negatively affected by the formation of particles in the calcite phase with a non-porous structure in the order of micrometers. Polycrystalline vaterite crystals have higher free energy than calcite particles. This ends up naturally inducing the transition from vaterite phases to a more stable and less energetic calcite phase.

When particles in metastable phases such as vaterite are placed in aqueous media, they naturally recrystallize in the form of calcite particles, which have much lower solubility. The addition of ethylene glycol in the solution decreases the solubility of both phases, reducing the transformation energy and the recrystallization rate. The reduced solubility by ethylene glycol, in combination with the high carbonate activity resulting from the high alkalinity, favors the precipitation of calcium carbonate particles with smaller sizes.

4.3 Bioconjugation

The adsorption of DVL in MPs and NPs is shown in tables 2 and 3. In MPs, DVL adsorbed more at a concentration of 1 mg/mL, with approximately 0.35 mg of lectin adsorbed on 10 mg of particles, while at concentrations of 0.5 mg/mL and 0.1 mg/mL DVL, the amount of lectin adsorbed was 0.3 and 0.085 mg in 10 mg of particles respectively (Table 2).

For NPs, at the concentration of 1 mg/mL, the amount of lectin adsorbed was approximately 0.4 mg in 10 mL of nanoparticles while at concentrations of 0.5 and 0.1 mg/mL, the amounts of 0.32 and 0.062 mg were adsorbed on the same amount of particles (Table 3).

Table 2 – Effect of the lectin concentration on the adsorption on MPs. Presented data is the average of three experiments.

Initial concentration of DVL (mg/mL)	**Average amount of DVL adsorbed in the particles (mg/mL)**
1.0	0.35 ± 0.025
0.5	0.3 ± 0.02
0.1	0.085 ± 0.005

Source: Elaborated by the author.

Table 3 – Effect of the lectin concentration on the adsorption on NPs. This experiment was performed in triplicate

Initial concentration of DVL (mg/mL)	**Average amount of DVL adsorbed in the particles (mg/mL)**
1.0	0.40 ± 0.03
0.5	0.32 ± 0.024
0.1	0.062 ± 0.004

Source: Elaborated by the author.

It is observed that, in both cases, lectin at a concentration of 1 mg/mL adsorbed the largest amount of lectin to the particles, although a considerable amount of lectin still remained in the supernatant, which shows that a saturation point has been reached. Also, it is clear that a higher concentration of lectin increases adsorption due to a larger amount of protein in the reaction medium, until a maximum level is reached. In neither case 100% adsorption was achieved, even at very low concentrations. The NPs showed a larger capacity to adsorb DVL, which can be explained by its higher surface area. The ability of smaller particles to conjugate with larger amounts of biomolecules has previously been reported and is a phenomenon known in nanotechnology (KOHANE, 2007). The protocol that allowed to adsorb the highest amount of lectin to the particles was applied in subsequent tests. All bioconjugates were able to agglutinate rabbit erythrocytes indicating that the adsorption has not denatured the lectin.

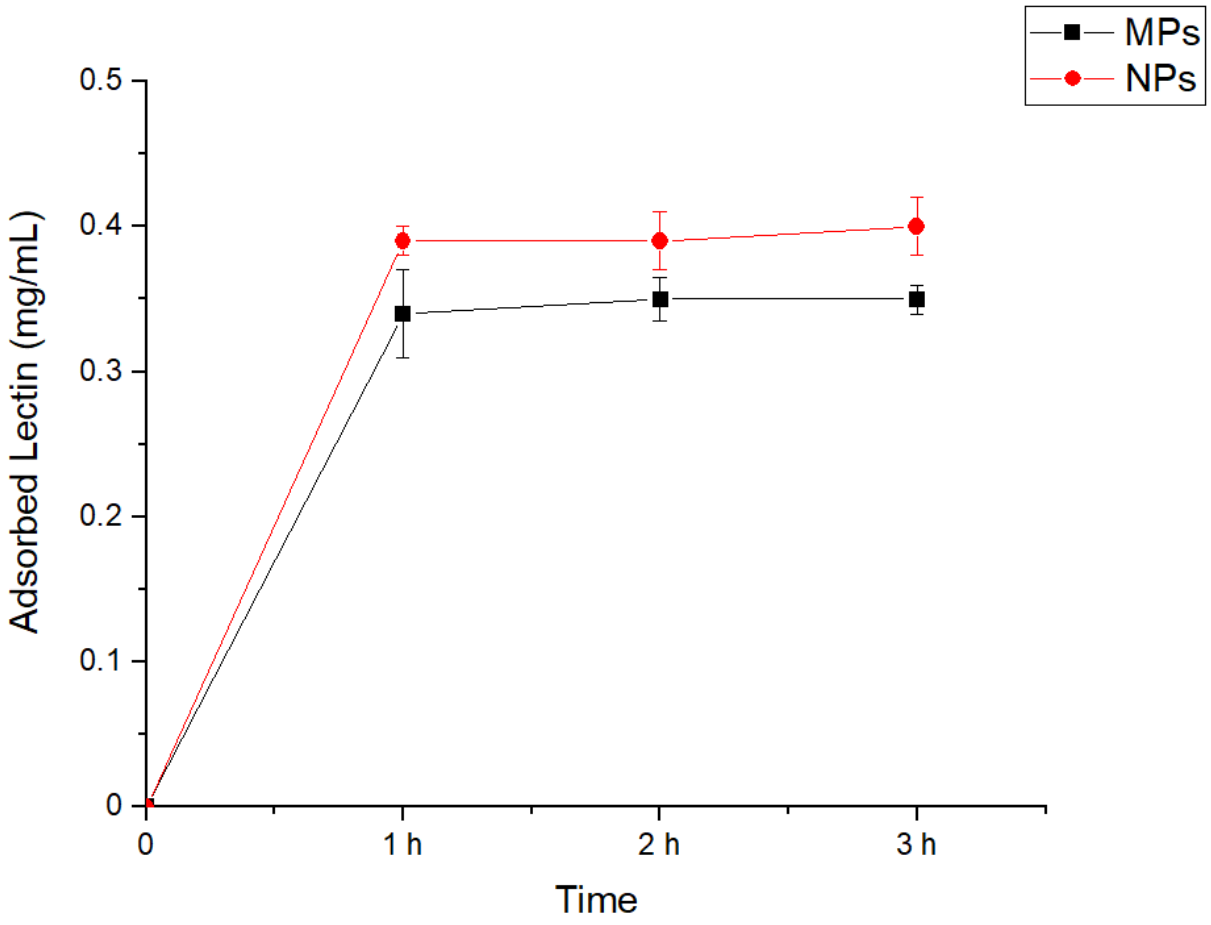
In general, proteins and biomolecules can interact directly with different types of particles. Particles, even without additional functionalization, are able to interact with molecules, even in the interior of cells (AURÍA-SORO et al., 2019; BEHZADI et al., 2017). This interaction is usually weak and non-specific and has limited use, but it is still important for the generation of controlled release systems. Previous works clearly showed that (nano)particles usually adsorb proteins on their surface forming what is called a corona around the particle. In addition, lectins have been used in combination with nanoparticles for a series of applications including oral immunotherapy, biosensing, detection of tumors and drug targeting (DEVI; BASIL-ROSE, 2018; DIESNER et al., 2012; NGUYEN; LEE, 2017;

SIMÃO et al., 2020; TERÄVÄ et al., 2019).

4.4 Time-dependence

The time required for lectin adsorption to the particles was verified in order to optimize the subsequent experiments and verify the best condition. The results are shown in figure 8. It can be seen that a large part of the adsorption process occurs relatively quickly. In fact, after 5 minutes, about 0.3 mg (or 85%) of the total adsorbed DVL had interacted with the MPs and about 0.25 mg (or 62%) interacted with the NPs. Adsorption appears to occur faster in the MPs compared to NPs. The results obtained were interesting from the application point of view considering that the process is fast and has an acceptable yield.

Figure 8 – DVL adsorption on MPs and NPs over time (0h to 3h). Error bars correspond to the standard deviation of three separate experiments

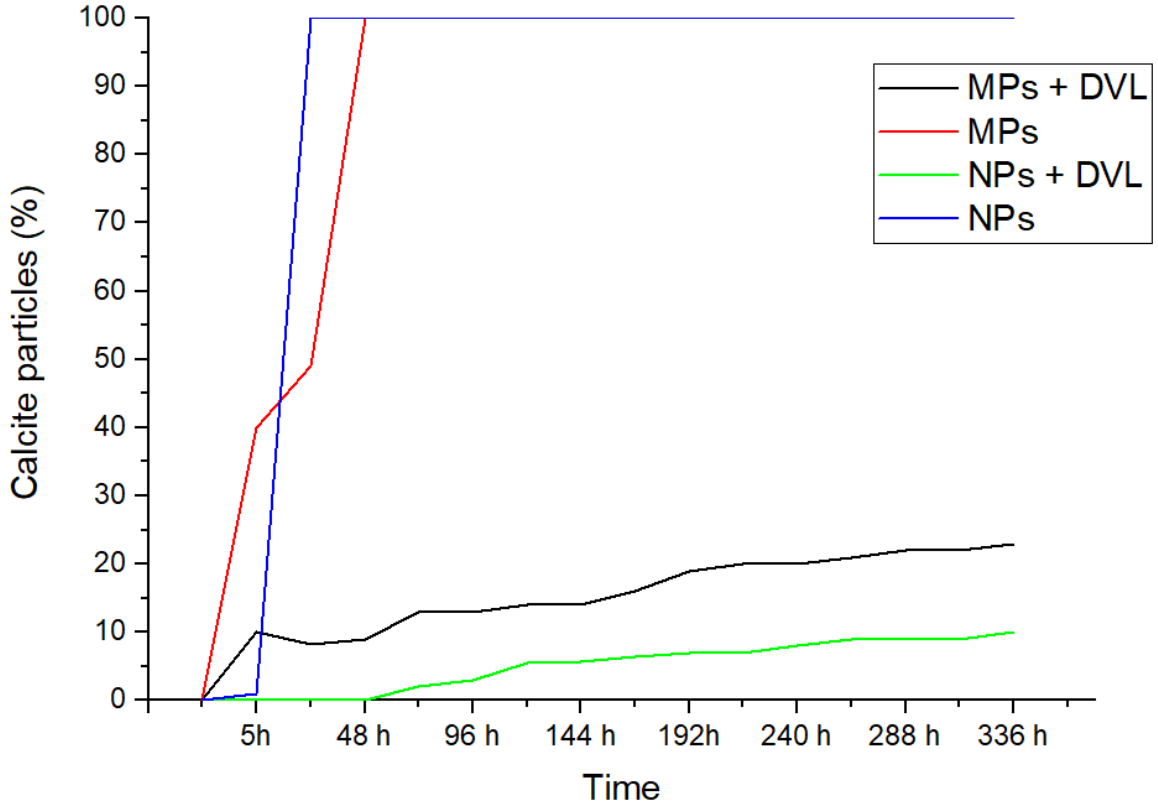


Source: Elaborated by the author.

4.5 Vaterite/calcite recrystallization

As previously stated, when particles of calcium carbonate in metastable phases such as vaterite are placed in aqueous media, they naturally recrystallize in the form of calcite particles which have much lower solubility. In the present work, the transition from vaterite to calcite particles and bioconjugates was verified in order to identify whether the lectin adsorption affects the natural transition profile of the particles (Figure 9). As a result, it was observed that the adsorption stabilizes the particles quite strongly. Both MPs and NPs without the presence of lectin recrystallized in relatively short times, 48h for MPs and 24h for NPs. As can be seen in figure 9, a highly significant delay in the recrystallization process was observed in the conjugates in comparison to the unconjugated particles, after 336 h (or 14 days), only 23% of the MPs-DVL were in the form of calcite while only 10% of the NPs-DVL were in this form. For the unconjugated particles, MPs recrystallized completely after about 48 h while the same phenomena occurred with the NPs after about 5 h. It appears that the presence of DVL results in stabilization of the particles, even after many days, which is desirable for their application. Stabilization of particles by proteins is something that usually take place since their presence can considerably reduce particles aggregation, a factor responsible for an increase in the particle size, which can lead to shorter precipitation times because the aggregation reaches a point at which the particles become too big and heavy, thus disrupting the colloid and precipitating.

Figure 9 – Percentage of calcite particles over time. MP s+ DVL (in black), MPs-only (in red), NPs + DVL (in green) and NPs-only (in blue). These experiments were performed in triplicate and a hundred particles were counted at each time-point

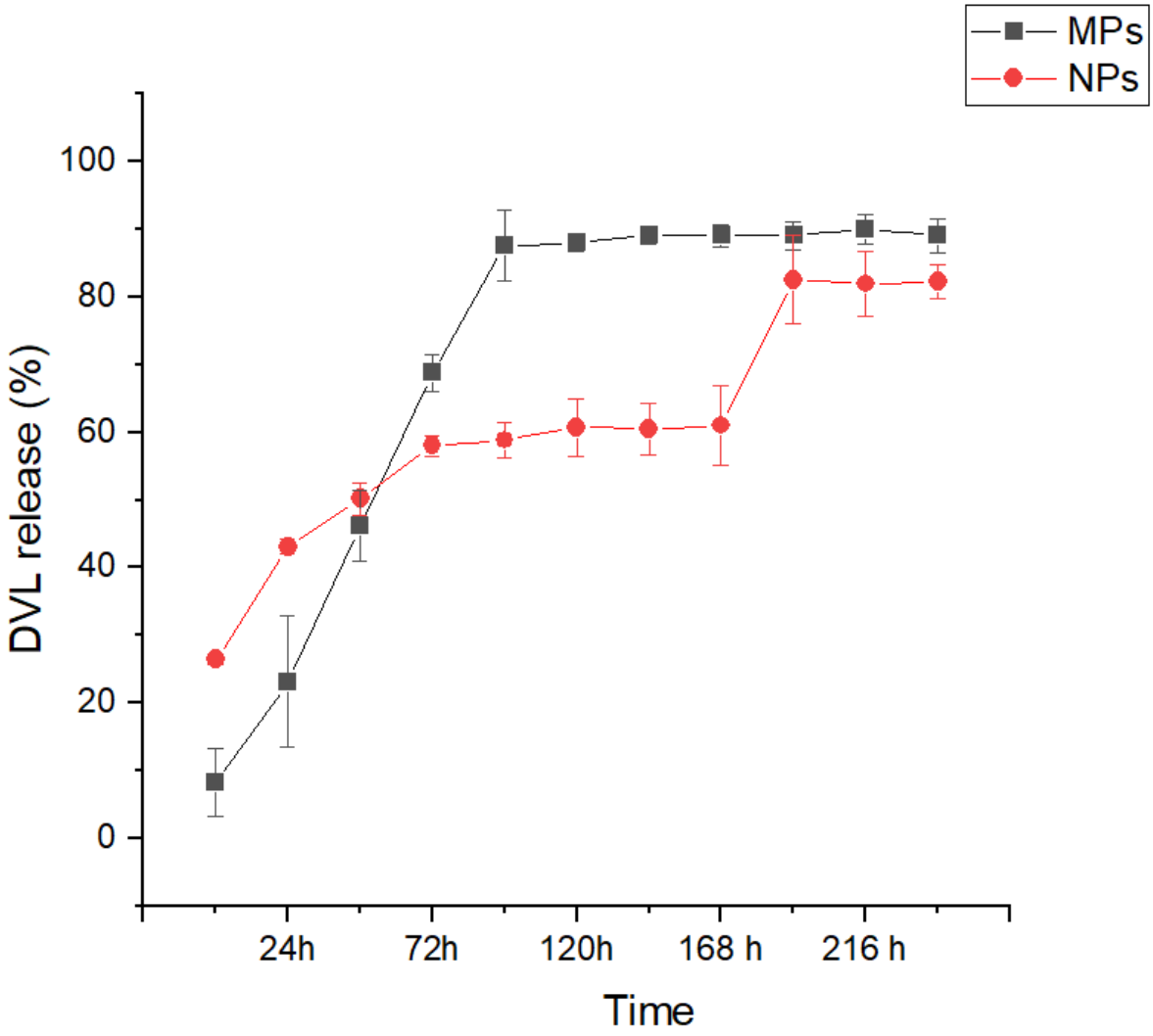


Source: Elaborated by the author.

4.6 DVL release from the particles

The release profile of DVL from MPs and NPs in 25 mM Tris-HCl pH 7.5 with 150 mM NaCl at room temperature showed an initial release followed by a slower release as shown in figure 10. The initial release was higher for NPs, with approximately 27% release, while MPs released about 8% DVL within 5 minutes after contact of the bioconjugates with the buffer. For MPs bioconjugates, the release occurred in a time-dependent manner with a maximum release after 96 hours with more than 90% of DVL released from the particles. For NPs, the release of DVL occurred more slowly reaching a maximum after approximately 8 days, with a release of approximately 82% DVL. It was also observed that for NPs, the increase in release occurred in a time dependent manner up to 4 days, then remained practically stationary from 4 days to 7 days with a drastic increase in the release on the 8th day.

Figure 10 – Graph showing DVL release from MPs and NPs over time. Data correspond to the average between three experiments with error bars representing to the standard deviation



Source: Elaborated by the author.

The lectin is released from the particles as a result of several factors, the main ones being: the diffusion of proteins from the particles and the natural degradation of the particle in addition to the gradual change of the calcium carbonate particles of a porous spherical shape to a non-porous cubic shape. The cumulative effect of these factors results in the observed release profile, formed by an initial release followed by a more constant release over time. The initial release can be explained by the diffusion of the lectin molecules coupled to the surface of the particles. The slow release profile can be explained by the slow diffusion of lectin through the pores of the particles. The increase in particle size decreases the time needed for maximum release of DVL which can be explained by a difference in internal organization compared to NPs which would affect diffusion and slow it down (MUKHERJEE et al., 2008). The release time of DVL is compatible with cell assays, which increases the

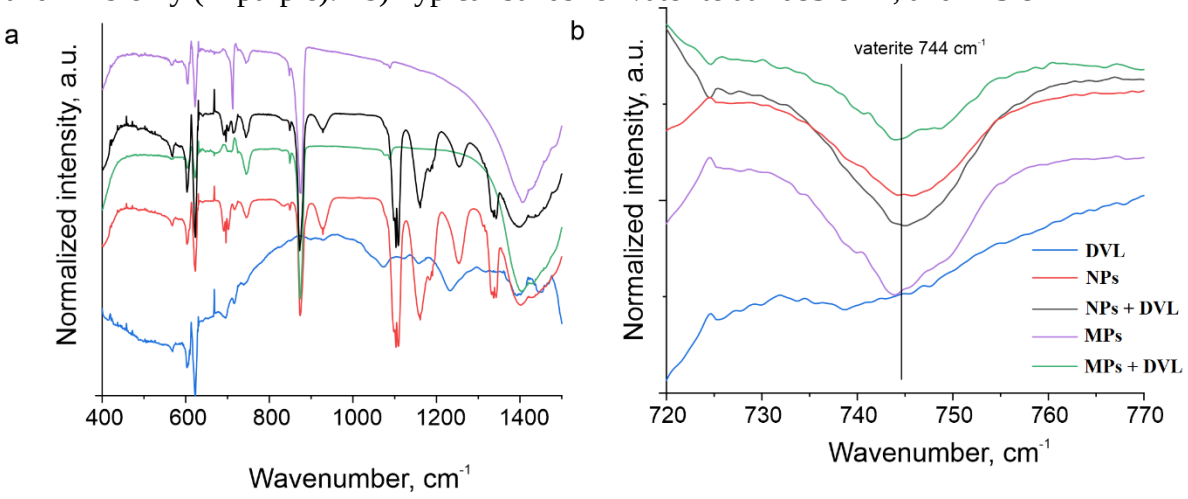
chances of applying these bioconjugates in the construction of prototypes of slow release systems.

4.7 FTIR and electron microscopy

FTIR spectra of conjugated and unconjugated NPs and MPs are displayed in comparison with DVL (Figure 11). To analyze the phase of the calcium carbonate, the absorption bands for planar CO_3^{2-} ion have been investigated. There are four vibrational modes in the free CO_3^{2-} ion, namely the symmetric stretching at 1080 cm^{-1} (ν1), the out-of-plane bending at 870 cm^{-1} (ν2), the asymmetric stretch 1400 cm^{-1} (ν3), and the split in-plane bending vibrations at 700 cm^{-1} (ν4). The spectra with bands centered at 712 cm^{-1} (ν4) and 873 cm^{-1} (ν2) reveal the presence of calcite, while bands centered at 744 cm^{-1} (ν4), 878 cm^{-1} (ν2) and 1085 cm^{-1} (ν1) are characteristic of vaterite and, there is a broad band at 1400 (ν3) which indicates an asymmetric stretch of the carbonate ion. Figure 11B clearly shows a v4 band at 744 cm^{-1} which confirms that the particles exist in a vaterite phase and the loading of DVL doesn't influence the phase of the carbonate. Due to the relatively small number of adsorbed molecules of DVL with respect to the mass of the inorganic matrix the impact of the organic molecules doesn't appear in the spectrum.

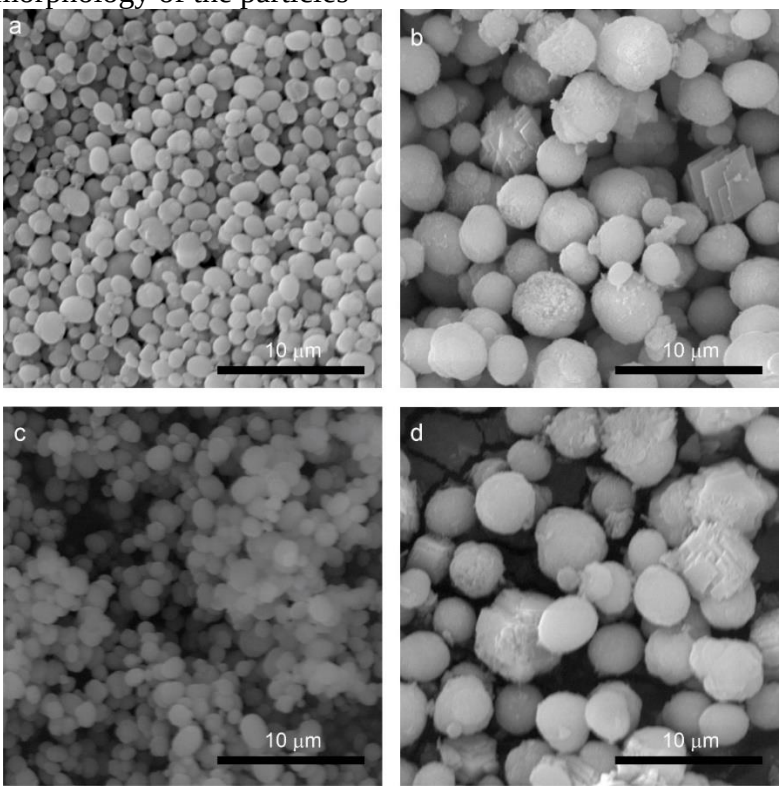
Scanning electron microscopy was employed to closely visualize the shape of the particles and bioconjugates. Not much difference could be observed indicating that the adsorption of DVL did not affect the shape of the particles (Figure 12)

Figure 11 – FTIR spectra of the lectin, the particles and the bioconjugates. a) Detailed spectra of 720-770 cm^{-1} of DVL (in blue) NPS-DVL (in black) NPs-only (in red), MPs-DVL (in green) and MPs-only (in purple). b) Typical bands for vaterite at 1085 cm^{-1}, and 745 cm^{-1}



Source: Elaborated by the author.

Figure 12 – Scanning electron microscopy of the vaterite particles and bioconjugates showing their spherical morphology. a) NPs-only, b) MPs-only, c) NPs-DVL and d) MPs-DVL. DVL does not appear to change the general morphology of the particles



Source: Elaborated by the author.

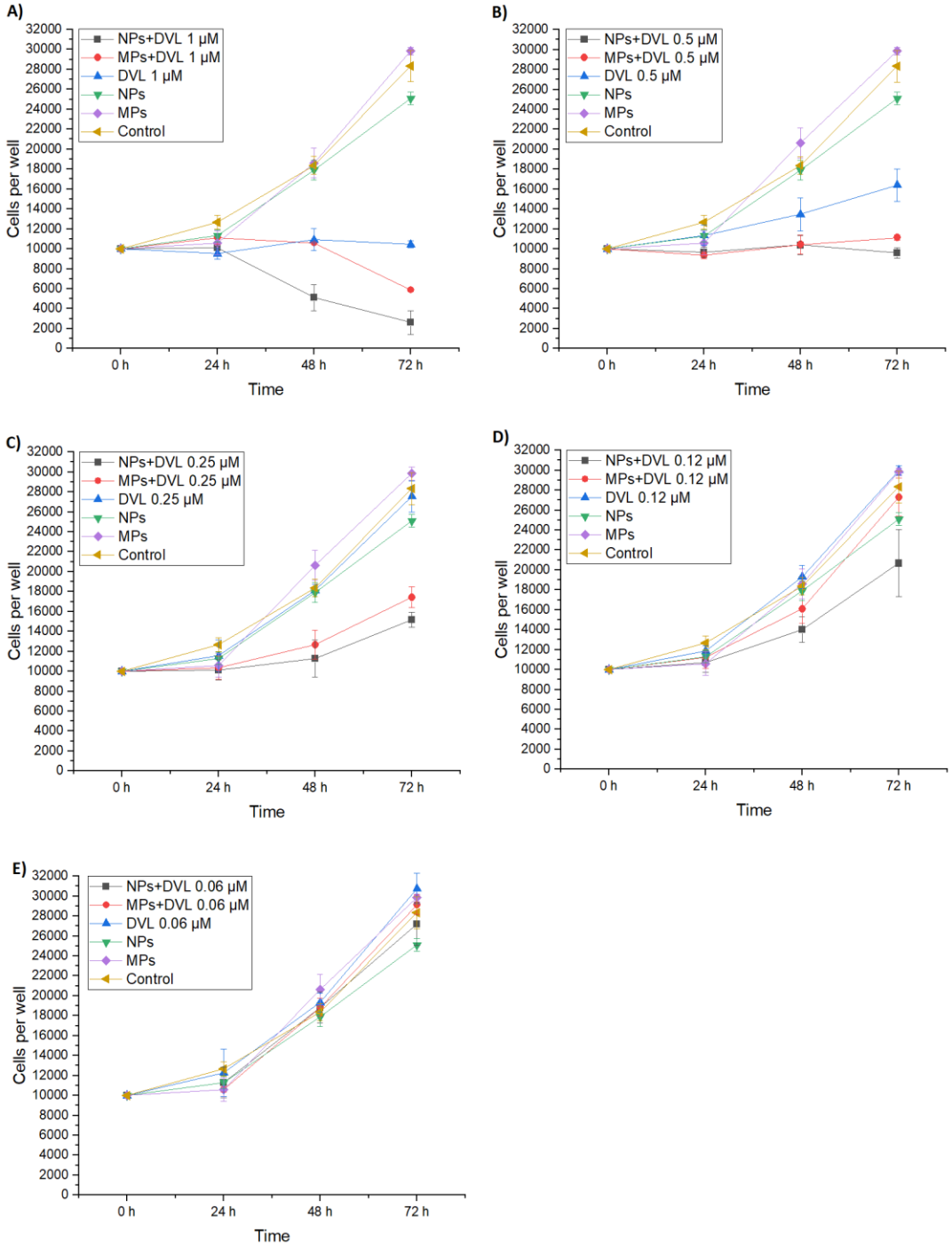
4.8 Cell toxicity assays and bioconjugate uptake in cells

The effect of DVL and the bioconjugates was evaluated by analyzing the viability and morphology of HeLa cells. Results show that DVL and the bioconjugates affected the viability of the cells in comparison to the controls, mostly in 48h and 72h time points (Figure 13). In the highest concentration (1 μM) tested, unconjugated DVL presented a biostatic effect in which the cell number did not go below the initial value of 10,000, but the cell division was much slower than in the control. For the bioconjugates, MPs-DVL and NPs-DVL, a severe effect in the cell viability was observed after 48h and 72h, with cell counts going as low as 2,500 cells, a decrease of roughly 75% in viability, considering the initial number of 10,000 cells. The cytotoxic effect of the conjugates presented a dose-dependency and, in all concentrations, the NPs-DVL conjugate presented the highest cytotoxic effect in comparison with unconjugated-DVL, MPs-DVL and the controls. In the presence of DVL at 1 μM and 0.5 μM, the cell morphology changed to a spherical shape, indicative of the loss of adhesion, also cell clumps can be observed which suggest a lectin-induced agglutination (Figure 14). Regarding the controls, the MPs-only control does not appear to affect the cells and the NPs-only effect is quite minor in comparison to the bioconjugates and unconjugated DVL.

Previous studies reported the antiproliferative effect of several ConA-like lectins against cancer cells, with most of these lectins activating programmed cell death pathways such as apoptosis and autophagy. It is suggested that these effects are related to the interaction of lectins with specific glycosylated targets. DVL was reported to present high cytotoxicity to both rat and human glioma lines, as published by (NASCIMENTO et al., 2018) and (NASCIMENTO et al., 2019). In both cases, DVL reduced the cell viability by about 60% in both cases while changing the cell morphology from a polygonal to a spherical shape, and severely reducing the cell migration. Cell death induced by DVL is shown to be mostly related to autophagy induction, corroborating with was observed by (PRATT; ROY; ANNABI, 2012), (OSTERNE et al., 2017; CAVADA et al., 2018) on other ConA-like lectins. In addition, DVL does not present toxicity for non-malign astrocytes thus suggesting a directed effect on malign cells, an effect related to the differential glycosylation on their surface (NASCIMENTO et al., 2019). There are few reports in the literature with details on the controlled release of a lectin for improved cytotoxicity against cancer cells, most works report the use of lectins aiming for a drug delivery application and/or tumor detection (BRUSCHI, 2019; DEVI; BASIL-ROSE, 2018; TERÄVÄ et al., 2019). The local increase in concentration and the protection of the lectin from denaturation may be responsible for the superior effect in

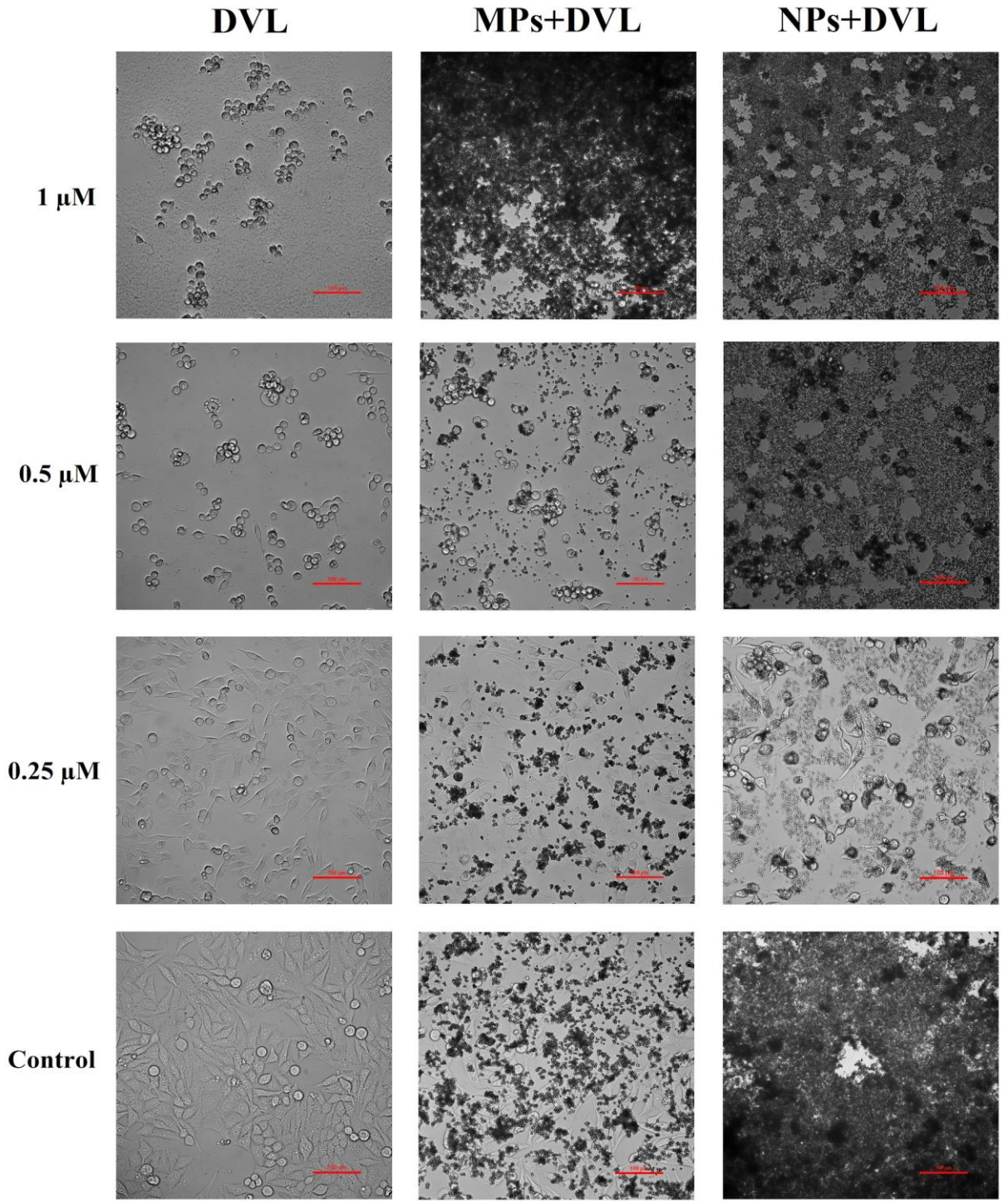
the MPs and NPs bioconjugates. In addition, preliminary data about the uptake of bioconjugates in HeLa cells was obtained using confocal microscopy (Figure 15). Fluorescence data collected at the 48h time point indicate that both MPs-DVL and NPs-DVL are internalized by the cells. This internalization can explain the differences observed when comparing the unconjugated lectin with the bioconjugates. The internalization of $CaCO_3$ particles in HeLa cells was previously observed and is dependent on size, shape, chemistry, aggregation state, stability and stiffness (PARAKHONSKIY et al., 2015). The uptake data still need to be completed to give a good insight about the bioconjugates effect on the cells.

Figure 13 – PrestoBlue cell viability assay with HeLa cells after 24 h, 48 h and 72 h of incubation with DVL, MPs-DVL, NPs-DVL, MPs-only and NPs-only. The amount of bioconjugates applied in these experiments was defined based on the concentration of DVL A) DVL at 1 µM, B) DVL at 0.5 µM, C) DVL at 0.25 µM, D) DVL at 0.12 µM and E) DVL at 0.06 µM. Control, MPs-only and NPs-only are repeated in each curve. This data is the average of three biological replicates with three technical repetitions each.



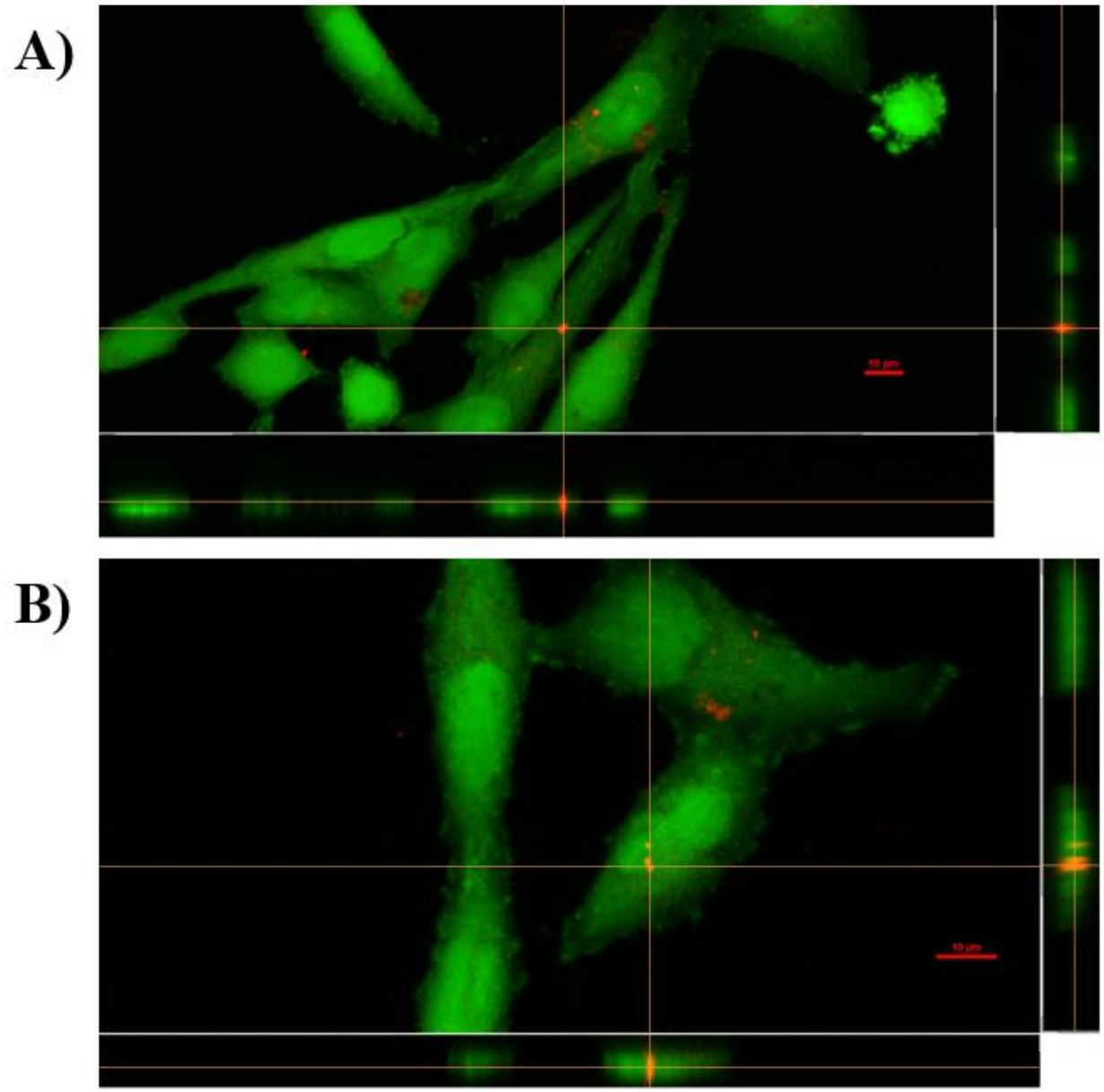
Source: Elaborated by the author.

Figure 14 – Representative images of HeLa cells in the presence of DVL, MPs-DVL and NPs-DVL, MPs-only, NPs-only and the cells-only control 48h after the treatment. The red bar corresponds to 100 µM



Source: Elaborated by the author.

Figure 15 – Confocal images of HeLa cells after 48h incubation with A) MPs-DVL and B) NPs-DVL. Cells were stained with calcein (green) and DVL was tagged with with tetramethylrhodamine-isothiocyanate (TRITC). In the images it is possible to see that both particles can be found inside the cells

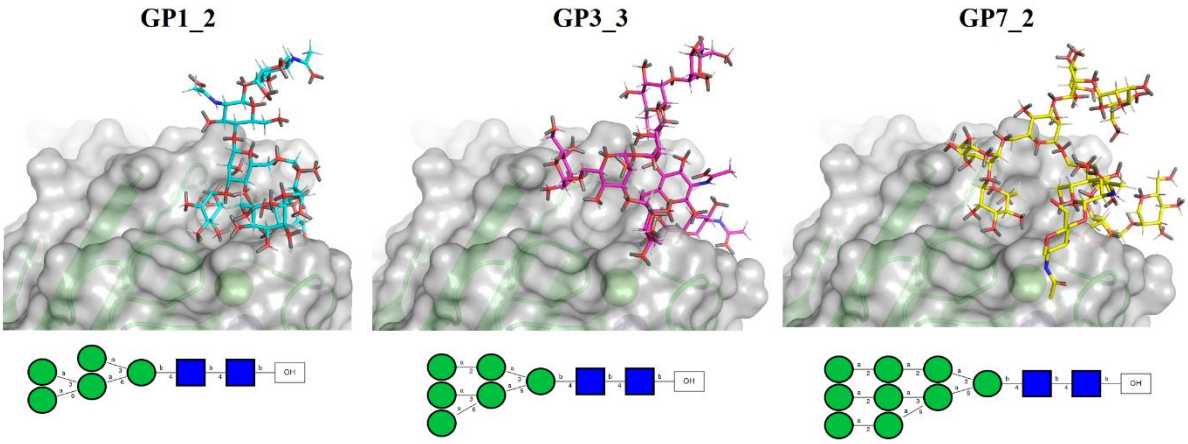


Source: Elaborated by the author.

4.9 Molecular docking

The present results with molecular docking suggest that DVL can interact favorably with the high-prevalent high-mannose glycans on HeLa cells. GP1_2 (Score: -56) is a Man5 glycan, GP3_3 (Score: -58,12) is a Man6 glycan and GP7_2 (Score: -61) is a Man9 glycan. Docking scores were considerably higher than the crystallography ligand X-man that presented a score of -41, thus indicating a very favorable binding. DVL also interacted well with other glycans of HeLa, albeit with lower scores, but manual validations suggest that interactions with high-mannose glycans are the most probable as expected for a mannose-specific lectin (Figure 16).

Figure 16 – Best poses of the molecular docking between DVL and three of the major *N*-glycans found on the glycome of HeLa cells. DVL is represented as cartoon (in green) with surface (in gray). All ligands are presented as sticks with carbons in blue (GP1_2), in pink (GP3_3) and in yellow (GP7_2). Poses were selected according to docking score and geometric parameters



Source: Elaborated by the author

A large proportion of the glycans on the HeLa surface is of the high-mannose type making the binding of DVL with them quite probable (HORVAT et al., 2013). The molecular target for DVL on the cell surface has not been identified yet but it is likely that this target presents some high-mannose glycosylation on its surface. The lectin-glycoconjugate interaction is likely to be a first step to explain the antiproliferative activity. The effect elicited by lectins is almost invariably related to their carbohydrate-binding capacity to a glycosylated target (MARQUES et al., 2017; PRATT; ROY; ANNABI, 2012)

5 CONCLUSIONS

With this work, one can conclude that is possible to construct a system for controlled/slow release for the *Dioclea violacea* lectin by employing $CaCO_3$ particles to be used against HeLa cells. The construction of the systems is simple and fast, with the system NPs-DVL presenting the highest cytotoxic effect against cancer cells by reducing its viability as much as 75%. This effect is likely to be related to the capacity of DVL to bind glycoconjugates on HeLa cells and potentialized by the internalization of the conjugates by the cells, as suggested by molecular docking and confocal microscopy respectively. As a final conclusion, these systems and methodologies are very promising in several areas in which lectins can be applied, mostly on cancer research, diagnostics and therapeutics, but further work is needed to evaluate the safety of the particles and conjugates on normal cells as well as to address the mechanisms behind the effect induced by DVL and the conjugates aiming to optimize their applications. Other lectins can also be tested aiming for an even better effect.

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APPENDIX A – SUMMARY

Recent data indicate that the mortality induced by cancer is significantly increasing in the last years, being considered a serious public health concern. Cancer cells present several alterations in their glycosylation profiles in comparison to the normal counterparts. In this context, lectins, proteins capable of specific and reversible binding to carbohydrates, are important due to their glycocode decoding properties and elicitation of several biological activities. Recent studies indicate the capacity of ConA-like lectins to induce death in tumoral cells with a great degree of selectivity and efficiency.

One of the most efficient ways of using these lectins is in conjunction with nanotechnology. One promising material to apply are calcium carbonate particles, a cheap, non-toxic material that allows encapsulation of biomolecules in their porous structure which enables a plethora of applications. This Ph.D. research is part of a much bigger project with focus on the purification, physicochemical and structural characterization, biological activities and bioinformatics studies of multiple lectins. The present work aims to build a controlled/slow release system by applying two sizes of $CaCO_3$ particles to adsorb the lectin of *Dioclea violacea* (DVL), investigate the effect of the lectin and the conjugates against cervical cancer cells (HeLa strain) and evaluate the lectin binding with common HeLa glycans via bioinformatics simulations.

Section 1 introduces the subject of glycans and glycosylation, lectins with focus on ConA-like lectins, and the properties and activities of DVL. In addition, the subjects of cancer, nanotechnology with focus on $CaCO_3$ particles and molecular docking simulations are introduced.

Section 2 presents the general and specific objectives of the research.

Section 3 addresses the materials and methodology applied for the purification of DVL, particle synthesis, DVL adsorption and characterization of conjugates, the cell assays with HeLa cells and the molecular docking.

Section 4 reports on the results and discussion of the research work. Briefly, the adsorption of DVL on two types of particles was successful with parameters such as adsorption yield, DVL release and recrystallization being compatible with the application. The lectin and the conjugates presented antiproliferative effects while also inducing agglutination and changes in cell morphology. *In silico* experiments corroborate with the results by suggesting a favorable binding with common glycans of HeLa cells. Finally, confocal microscopy with labelled conjugates shows the internalization of the conjugates in HeLa cells.

Section 5 formulates the conclusion of the work and some suggestions for future research.

APPENDIX B – SAMENVATTING

Uit recente gegevens blijkt dat de sterfte als gevolg van kanker de laatste jaren aanzienlijk toeneemt, dit is een ernstig probleem voor de volksgezondheid. Kankercellen vertonen verschillende veranderingen in hun glycosyleringsprofielen in vergelijking met de normale cellen. In deze context zijn lectines, eiwitten die in staat zijn tot specifieke herkenning en omkeerbare binding met koolhydraten, belangrijk vanwege hun glycocode-decoderingseigenschappen en het uitlokken van verschillende biologische activiteiten. Recente studies wijzen op het vermogen van ConA-achtige lectines om met een grote mate van selectiviteit en efficiëntie de dood in tumorcellen te induceren.

Een van de meest efficiënte manieren om deze lectines te gebruiken, is in combinatie met nanotechnologie. Een veelbelovend materiaal om te gebruiken zijn calciumcarbonaatdeeltjes, een goedkoop, niet-giftig materiaal dat de inkapseling van biomoleculen in hun poreuze structuur mogelijk maakt, wat een overvloed aan toepassingen mogelijk maakt. Dit doctoraatsonderzoek maakt deel uit van een veel groter project dat zich richt op de zuivering, fysicochemische en structurele karakterisering, biologische activiteiten en bio-informatica-analyse van meerdere lectines. Het huidige werk heeft tot doel een systeem met gecontroleerde / langzame afgifte op te zetten door CaCO3-deeltjes van twee groottes toe te passen om het lectine van *Dioclea violacea* (DVL) te adsorberen, het effect van het lectine en de conjugaten tegen baarmoederhalskankercellen (HeLa cellen) te onderzoeken en de lectinebinding te evalueren met de glycanen op het celoppervlak van de HeLa cellen via bioinformatica-simulaties.

Sectie 1 introduceert de onderwerpen glycanen en glycosylering, lectines met de nadruk op ConA-achtige lectines, en de eigenschappen en activiteiten van DVL. Daarnaast worden de onderwerpen kanker, nanotechnologie met focus op $CaCO_3$-deeltjes en moleculaire docking-simulaties geïntroduceerd.

Hoofdstuk 2 presenteert de algemene en specifieke doelstellingen van het onderzoek.

Hoofdstuk 3 beschrijft de gebruikte materialen en methodologie voor de zuivering van DVL, deeltjessynthese, DVL-adsorptie en karakterisering van conjugaten, de celassays met HeLa-cellen en de moleculaire docking.

Hoofdstuk 4 rapporteert over de resultaten en bespreking van het onderzoekswerk. Kort samengevat was de adsorptie van DVL op twee soorten deeltjes succesvol, waarbij parameters zoals adsorptieopbrengst, DVL-afgifte en herkristallisatie compatibel waren met

de toepassing. Het lectine en de conjugaten vertoonden antiproliferatieve effecten en veroorzaakten ook agglutinatie en veranderingen in celmorfologie. In silico-experimenten bevestigen de resultaten door een gunstige binding te suggereren met gewone glycanen van HeLa-cellen. Tot slot toont confocale microscopie met gelabelde conjugaten aan dat de conjugaten in HeLa-cellen worden opgenomen.

Hoofdstuk 5 formuleert de conclusie van het werk en enkele suggesties voor toekomstig onderzoek.

APPENDIX C – *Curriculum vitae*

Curriculum Vitae: **Vinicius Jose da Silva Osterne** (born: 19/03/1992)
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- **Last updated 06/04/2020**

Area of Specialization: Biotechnology (Plant Proteins; Structural Biology);

Education:

Grad.	Biotechnology	Federal University of Ceara	(Fortaleza – CE, Brazil) 2013
M.Sc.	Natural Resources Biotechnology	Federal University of Ceara	(Fortaleza - CE, Brazil) 2015.

Current: Ph.D. student in Biotechnology of Natural Resources - Federal University of Ceara. Fortaleza- Ceará, Brazil since 2016. Supervisors: Kyria Santiago Nascimento and Benildo Sousa Cavada.
Joint Ph.D. in Applied Biological Sciences: Cell and Gene Biotechnology - Ghent University in Gent - Belgium. Supervisor: Els Van Damme

Summary of Publications and Presentations

Full papers published in international journals	32
Scientific events	17
Abstracts presented in events	09
Technical productions	14
Patents	3

Full papers published:

1. Cavada BS, **Osterne VJS**, Oliveira MV, Pinto-Junior VR, Silva MTL, Bari AU, et al. Reviewing *Mimosoideae* lectins: A group of under explored legume lectins. Int J Biol Macromol. 2020; 154: 159–165.

2. Dubiel M, De Coninck T, **Osterne VJS**, Verbeke I, Van Damme D, Smagghe G, et al. The ArathEULS3 Lectin Ends up in Stress Granules and Can Follow an Unconventional Route for Secretion. Int J Mol Sci. 2020; 21. doi:10.3390/ijms21051659

3. Cavada BS, **Osterne VJS**, Pinto-Junior VR, Souza LAG, Lossio CF, Silva MTL, et al. Molecular dynamics and binding energy analysis of *Vatairea guianensis* lectin: a new tool for cancer studies. J Mol Model. 2020; 26: 22.

4. Nascimento KS, Silva MTL, Oliveira MV, Lossio CF, Pinto-Junior VR, **Osterne VJS**, et al. Dalbergieae lectins: A review of lectins from species of a primitive *Papilionoideae* (leguminous) tribe. Int J Biol Macromol. 2020; 144: 509–526.

5. Cavada BS, Bari AU, Pinto-Junior VR, Lossio CF, Silva MTL, Souza LAG, Oliveira MV, Souza-Filho CHD, Correia SEG, Vital APMS, Lima LD, **Osterne VJS**, Nascimento KS. Purification and partial characterization of a new lectin from *Parkia panurensis* Benth. ex H.C. Hopkins seeds (*Leguminosae* family; *Mimosoideae* subfamily) and evaluation of its biological effects. International Journal of Biological Macromolecules. 2020. pp. 845–855. doi:10.1016/j.ijbiomac.2019.10.102

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8. Cavada BS, **Osterne VJS**, Pinto-Junior VR, Nascimento KS. ConBr, the Lectin from *Canavalia brasiliensis* Mart. Seeds: Forty Years of Research. Curr Protein Pept Sci. 2019; 20: 600–613.

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ATTACHMENT A – PAPER 1

International Journal of Biological Macromolecules 154 (2020) 159–165

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Review

Reviewing Mimosoideae lectins: A group of under explored legume lectins

Benildo Sousa Cavada [a,*], Vinicius Jose Silva Osterne [a], Messias Vital Oliveira [a], Vanir Reis Pinto-Junior [b], Mayara Torquato Lima Silva [c], Alfa Umaro Bari [a], Lara Dias Lima [a], Claudia Figueiredo Lossio [a], Kyria Santiago Nascimento [a,*]

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ARTICLE INFO

Article history:
Received 13 February 2020
Received in revised form 2 March 2020
Accepted 13 March 2020
Available online 14 March 2020

Keywords:
Lectins
Mimosoideae
Leguminosae

ABSTRACT

Lectins are proteins capable of specific and reversible binding to mono- and/or oligosaccharides, and within this group, Legume lectins are the most studied. However, most of these studies focus on the Papilionoideae subfamily, with Caesalpinioideae and Mimosoideae lectins being significantly less explored in the literature. The Mimosoideae subfamily consists of at least 79 genera and 3275 species, but, to date, only about 14 lectins have been purified, a fact which shows the lack of studies for this group. Based on their purification protocols, as well as physicochemical and structural properties, Mimosoideae lectins are very heterogeneous. Despite the few studies, a wide variety of biological activities have been tested, including, for example, inflammatory, anticancer, antibacterial, and antifungal. In this context, the present review aims to summarize the available data regarding the purification, physicochemical and structural properties, as well as biological activities, of lectins extracted from plants of the Mimosoideae subfamily in order to bring more insight to researchers interested in further exploring the potential of these molecules.

ATTACHMENT B – PAPER 2

International Journal of *Molecular Sciences*

MDPI

Article

The ArathEULS3 Lectin Ends up in Stress Granules and Can Follow an Unconventional Route for Secretion

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Received: 29 January 2020; Accepted: 26 February 2020; Published: 28 February 2020

check for updates

Abstract: Stress granules are cytoplasmic compartments, which serve as mRNA storage units during stress, therefore regulating translation. The *Arabidopsis thaliana* lectin *ArathEULS3* has been widely described as a stress inducible gene. This study aimed to examine in detail the localization of ArathEULS3 lectin in normal and stressed cells. Colocalization experiments revealed that the

ATTACHMENT C – PAPER 3

Journal of Molecular Modeling (2020) 26: 22
https://doi.org/10.1007/s00894-019-4281-3

ORIGINAL PAPER

Molecular dynamics and binding energy analysis of *Vatairea guianensis* lectin: a new tool for cancer studies

Benildo Sousa Cavada[1] · Vinicius Jose Silva Osterne[1] · Vanir Reis Pinto-Junior[2] · Luiz Augusto Gomez Souza[3] · Claudia Figueiredo Lossio[1] · Mayara Torquato Lima Silva[4] · Corneville Correia-Neto[1] · Messias Vital Oliveira[1] · Jorge Luis Almeida Correia[1] · Antonio Hadson Bastos Neco[1] · Jorge Luiz Coelho Domingos[2] · Wandemberg Paiva Ferreira[2] · Gil Aquino Farias[2] · Kyria Santiago Nascimento[1]

Received: 30 May 2019 / Accepted: 19 December 2019 / Published online: 7 January 2020

Abstract
The Tn antigen is an epitope containing *N*-acetyl-D-galactosamine present in the extracellular matrix of some carcinoma cells in humans, and it is often used as a biomarker. Lectins are proteins capable of binding to carbohydrates and can be used as a molecular tool to recognize antigens and to differentiate cancer cells from normal cells. In this context, the present work aimed to characterize the interaction of *Vatairea guianensis* seed lectin with *N*-acetyl-D-galactosamine and the Tn antigen by molecular dynamics and molecular mechanics/Poisson–Boltzmann solvent-accessible surface area analysis. This study revealed new interacting residues not previously identified in static analysis of the three-dimensional structures of *Vatairea* lectins, as well as the configuration taken by the carbohydrate recognition domain, as it interacts with each ligand. During the molecular dynamics simulations, *Vatairea guianensis* lectin was able to bind stably to Tn antigen, which, as seen previously for other lectins, enables its use in cancer research, diagnosis, and therapy. This work further demonstrates the efficiency of bioinformatics in lectinology.

Keywords Tn antigen · Cancer · *Vatairea guianensis* · Lectin · Molecular dynamics

ATTACHMENT D – PAPER 4

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Review

Dalbergieae lectins: A review of lectins from species of a primitive Papilionoideae (leguminous) tribe

Kyria Santiago Nascimento [a,*], Mayara Torquato Lima Silva [b], Messias Vital Oliveira [a], Claudia Figueiredo Lossio [a], Vanir Reis Pinto-Junior [c], Vinicius Jose Silva Osterne [a], Benildo Sousa Cavada [a,*]

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ARTICLE INFO

Article history:
Received 6 September 2019
Received in revised form 6 November 2019
Accepted 14 December 2019
Available online 17 December 2019

Keywords:
Lectins
Leguminosae
Dalbergieae tribe

ABSTRACT

Lectins are (glyco)proteins capable of reversibly binding to specific carbohydrates, thus having various functions and applications. Plant lectins are the best studied, and the *Leguminoseae* family is highlighted in a number of published works, especially species of the Papilionoideae subfamily. Dalbergieae is one of the tribes in this subfamily comprising 49 genera and over 1300 species. From this tribe, about 26 lectins were studied, among which we can highlight the *Arachis hypogaea* lectin, widely used in cancer studies. Dalbergieae lectins demonstrate various carbohydrate specificities and biological activities including anti-inflammatory, vasorelaxant, nociceptive, antibacterial, antiviral among others. Structurally, these lectins are quite similar in their three-dimensional folding but present significant differences in oligomerization patterns and in the conservation of carbohydrate-recognition domain. Despite the existence of structural data from some lectins, only sparse literature has reported on this tribe's diversity, not to mention the range of biological effects, determined through specific assays. Therefore, this work will review the most important studies on Dalbergieae lectins and their potential biomedical applications.

ATTACHMENT E – PAPER 5

International Journal of Biological Macromolecules 145 (2020) 845–855

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Purification and partial characterization of a new lectin from *Parkia panurensis* Benth. ex H.C. Hopkins seeds (*Leguminosae* family; *Mimosoideae* subfamily) and evaluation of its biological effects

Benildo Sousa Cavada [a,*], Alfa Umaro Bari [a], Vanir Reis Pinto-Junior [a], Claudia Figueiredo Lossio [a], Mayara Torquato Lima Silva [b], Luiz Augusto Gomes Souza [c], Messias Vital Oliveira [a], Claudio Henrique Dahne Souza-Filho [a], Sarah Elizabeth Gomes Correia [a], Ana Paula Moreira Sousa Vital [a], Lara Dias Lima [a], Vinicius Jose Silva Osterne [a], Kyria Santiago Nascimento [a,*]

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ARTICLE INFO

Article history:
Received 24 September 2019
Received in revised form 8 October 2019
Accepted 10 October 2019
Available online 15 November 2019

ABSTRACT

Lectins are proteins that have as one of their main characteristics recognizing and reversibly binding to carbohydrates. In this work, it was possible to purify and characterize a lectin from *Parkia panurensis* (*Leguminosae* family; *Mimosoideae* subfamily) seeds by a combination of the techniques: protein precipitation, along with affinity and then ion exchange chromatography using the Sepharose-mannose and diethylaminoethyl matrices, respectively. The pure lectin, called PpaL, has affinity by D-mannose, D-

ATTACHMENT F – PAPER 6

International Journal of Biological Macromolecules 134 (2019) 901–911

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Review

One century of ConA and 40 years of ConBr research: A structural review

Benildo Sousa Cavada [a,*], Vinicius Jose Silva Osterne [a], Claudia Figueiredo Lossio [a], Vanir Reis Pinto-Junior [a], Messias Vital Oliveira [a], Mayara Torquato Lima Silva [a], Rodrigo Bainy Leal [b], Kyria Santiago Nascimento [a,*]

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ARTICLE INFO

Article history:
Received 22 March 2019
Received in revised form 16 May 2019
Accepted 16 May 2019
Available online 18 May 2019

Keywords:
Lectin
Canavalia
Structure

ABSTRACT

Lectins are proteins that can bind specifically and reversibly to carbohydrates. This capacity gives lectins multiple biological roles and biotechnological applications. Although lectins can be found in all organisms, plant lectins, especially legume lectins, are undoubtedly the most thoroughly studied. Among legume lectins, the lectin from *Canavalia ensiformis* (ConA) and *Canavalia brasiliensis* (ConBr), both from Diocleinae subtribe, are two of the most well-known lectins. It has been 100 years since the first report of ConA and 40 years since the first report of ConBr, making 2019 an important year for lectinology. Structural data of these lectins in combination with biological activity tests clearly indicate that even a small shift in amino acid sequence can affect the tertiary and quaternary structures, consequently affecting the biological activity of these proteins. It is in this context that the present paper aims to review the structural data of ConA and ConBr, focusing on the primary structure, crystallography, tertiary and quaternary structures of these lectins, as well as their binding sites. This paper also expands the structural data by employing molecular dynamics to evaluate carbohydrate-binding properties and structural stability. It is anticipated that these data will increase knowledge about the structure-function relationships of these proteins.

ATTACHMENT G – PAPER 7

International Journal of Biological Macromolecules 134 (2019) 660–672

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Lectin from *Dioclea violacea* induces autophagy in U87 glioma cells

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ARTICLE INFO

Article history:
Received 24 January 2019
Received in revised form 5 April 2019
Accepted 30 April 2019
Available online 1 May 2019

Keywords:
Autophagy
Dioclea violacea
mTOR

ABSTRACT

The antitumor activity of DVL, a lectin purified from *Dioclea violacea* seeds, on the U87 human glioma cell line was evaluated and compared with *Canavalia ensiformis* lectin (ConA). Treatment with DVL (10–100 μg/mL; 24–96 h) induced alterations in cell morphology, decreased cell numbers and clonogenic survival in a time- and concentration-dependent manner. DVL caused significant decreases in cell viability and impaired cell migration. Mechanistically, DVL treatment (12 h) disrupted mitochondrial electrochemical gradient, without ROS accumulation or caspase activation. In the absence of apoptosis, DVL (30–100 μg/mL), instead, induced autophagy, as detected by acridine orange staining and cleavage of LC3I. Inhibition of autophagy with 3-Methyladenine (3-MA) and Chloroquine partially abrogated DVL, but not ConA, cytotoxicity. The modulation of signaling pathways that orchestrate autophagic and cell survival processes were analyzed. DVL (30–100 μg/mL) decreased Akt,

ATTACHMENT H – PAPER 8

600

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Current Protein and Peptide Science, 2019, *20*, 600-613

REVIEW ARTICLE

ConBr, the Lectin from *Canavalia brasiliensis* Mart. Seeds: Forty Years of Research

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ARTICLE HISTORY

Received: October 16, 2018
Revised: November 30, 2018
Accepted: December 29, 2018

DOI:
10.2174/1389203720666190104123210

Abstract: Lectins are defined as proteins or glycoproteins capable of specific and reversible binding to carbohydrates. Inside this group of proteins, the most well-studied lectins belong to the Leguminosae family, and inside this family, the Diocleinae subtribe includes the most characterized lectin Concanavalin A (ConA), as well as ConBr, the lectin from *Canavalia brasiliensis*, the subject of this review. Since 1979, several studies have been published in the literature regarding this lectin, from its isolation and characterization to its several biological activities. This year, 2019, will mark 40 years since researchers have begun to study ConBr and 100 years since the discovery of ConA, making 2019 a momentous year for lectinology. Owing to the abundance of studies involving ConBr, this review will focus on ConBr's purification, physicochemical properties, functional and structural analyses, biological activities and biotechnological applications. This will give researchers a broad glimpse into the potential of this lectin, as well as it characteristics, as we look ahead to its expanding applications in glycomics and biotechnology.

Keywords: *Canavalia brasiliensis*, lectin, ConBr, biological activities, properties, lectinology.

ATTACHMENT I – PAPER 9

International Journal of *Molecular Sciences*

MDPI

Review

ConA-Like Lectins: High Similarity Proteins as Models to Study Structure/Biological Activities Relationships

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Received: 28 November 2018; Accepted: 19 December 2018; Published: 21 December 2018

check for updates

Abstract: Lectins are a widely studied group of proteins capable of specific and reversible binding to carbohydrates. Undoubtedly, the best characterized are those extracted from plants of the Leguminosae family. Inside this group of proteins, those from the Diocleinae subtribe have attracted attention, in particular Concanavalin A (ConA), the best-studied lectin of the group. Diocleinae lectins, also called ConA-like lectins, present a high similarity of sequence and three-dimensional structure and are known to present inflammatory, vasoactive, antibiotic, immunomodulatory and antitumor activities, among others. This high similarity of lectins inside the ConA-like group makes it possible to use them to study structure/biological activity relationships by the variability of both carbohydrate specificity and biological activities results. It is in this context the following review aims to summarize the most recent data on the biochemical and structural properties, as well as biological activities, of ConA-like lectins and the use of these lectins as models to study structure/biological

ATTACHMENT J – PAPER 10

Biochimie 158 (2019) 34–42

Contents lists available at ScienceDirect

Biochimie

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

Research paper

Dioclea violacea lectin ameliorates inflammation in the temporomandibular joint of rats by suppressing intercellular adhesion molecule-1 expression☆

Juliana T. Clemente-Napimoga [a], Maria A.S.M. Silva [a], Sylvia N.C. Peres [b], Alexandre H.P. Lopes [c], Claudia F. Lossio [d], Messias V. Oliveira [d], Vinicius J.S. Osterne [d], Kyria S. Nascimento [d], Henrique B. Abdalla [e], Juliana M. Teixeira [a], Benildo S. Cavada [d, **], Marcelo H. Napimoga [f, *]

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ARTICLE INFO

Article history:
Received 4 August 2018
Accepted 13 December 2018
Available online 14 December 2018

ABSTRACT

Inflammation of temporomandibular joint (TMJ) tissues are the most common cause of pain conditions associated with temporomandibular disorders (TMDs). After a tissue and/or neural damage, the inflammatory response is characterized by plasma extravasation and leukocytes infiltration in the TMJ tissues, which in turn, release inflammatory cytokines cascades responsible for inflammatory pain.

ATTACHMENT K – PAPER 11

Journal of Molecular Modeling (2018) 24: 251
https://doi.org/10.1007/s00894-018-3800-y

ORIGINAL PAPER

Homology modeling, molecular docking, and dynamics of two α-methyl-D-mannoside-specific lectins from *Arachis* genus

Kyria Santiago Nascimento[1] · David Alencar Araripe[1] · Vanir Reis Pinto-Junior[1] · Vinicius Jose Silva Osterne[1] · Francisco William Viana Martins[1] · Antonio Hadson Bastos Neco[1] · Gil Aquino Farias[2] · Benildo Sousa Cavada[1]

Received: 20 October 2017 / Accepted: 16 August 2018 / Published online: 25 August 2018

Abstract
The *Arachis* genus belongs to the Dalbergieae tribe, a group of plants that show promising potential novel lectins. Three lectins of the well-known *Arachis hypogaea* have already been purified, while lectins from related species are still unknown. Genomes of two closely related species, *Arachis duranensis* and *Arachis ipaensis*, were recently sequenced. Therefore, this study aimed to establish the three-dimensional structure of *Arachis duranensis* lectin (ADL) and *Arachis ipaensis* lectin (AIL) by homology modeling, test their activity against mannosides, and perform molecular dynamics (MD) simulations on these two proteins, both unligated and interacting with mannose or α-methyl-D-mannoside. The fold obtained for the molecular models agrees with data obtained from previous leguminous lectins, showing a conserved jelly roll motif. Docking scores indicate that these lectins have different theoretical binding energy with monosaccharides, disaccharides, and high-mannose glycans. MD simulations revealed that these proteins are α-methyl-D-mannoside-specific, having less stable interactions with mannose. This study thus serves as a guide for further research on lectins of the *Arachis* genus.

Keywords *Arachis* lectins · Molecular dynamics · Homology modeling

ATTACHMENT L – PAPER 12

International Journal of Biological Macromolecules 120 (2018) 566–577

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Anti-glioma properties of DVL, a lectin purified from *Dioclea violacea*

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ARTICLE INFO

Article history:
Received 30 May 2018
Received in revised form 17 July 2018
Accepted 21 August 2018
Available online 23 August 2018

Keywords:

ABSTRACT

Plant lectins have been studied owing to their structural properties and biological effects that include agglutinating activity, antidepressant-like effect and antitumor property. The results from this work showed the effects of the lectin extracted from the *Dioclea violacea* plant (DVL) on the C6 rat glioma cell line. DVL treatment was able to induce caspase-3 activation, apoptotic cell death and cellular membrane damage. Furthermore, DVL decreased mitochondrial membrane potential and increased the number of acidic vesicles and cleavage of LC3, indicating activation of autophagic processes. DVL also significantly inhibited cell migration. Compared to ConA, a well-studied lectin extracted from *Canavalia ensiformes* seeds, some effects of DVL were more potent, including de-

ATTACHMENT M – PAPER 13

International Journal of Biological Macromolecules 117 (2018) 124–133

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Structural analysis, molecular docking and molecular dynamics of an edematogenic lectin from *Centrolobium microchaete* seeds

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ARTICLE INFO

Article history:
Received 27 March 2018
Received in revised form 11 May 2018
Accepted 23 May 2018
Available online 24 May 2018

Keywords:
Dalbergieae

ABSTRACT

Lectins represent a class of proteins or glycoproteins capable of reversibly binding to carbohydrates. Seed lectins from the Dalbergieae tribe (Leguminosae) have structural variability, carbohydrate specificity, and biological effects, such as inflammation, vasorelaxation and cancer antigen binding. To comprehensively address these factors, the present work aimed to establish and characterize the three-dimensional structure of *Centrolobium microchaete* lectin (CML) by homology modeling, investigate protein-carbohydrate interactions and evaluate its inflammatory effect on mice. Molecular docking was performed to analyze interactions of the lectin with monosaccharides, disaccharides and *N*-glycans. Two dimannosides, methyl mannose-1,3-α-D-mannose

ATTACHMENT N – PAPER 14

International Journal of Biological Macromolecules 114 (2018) 64–76

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Crystal structure of DlyL, a mannose-specific lectin from *Dioclea lasiophylla* Mart. Ex Benth seeds that display cytotoxic effects against C6 glioma cells

Rodrigo Bainy Leal [a,*], Vanir Reis Pinto-Junior [b], Vinicius Jose Silva Osterne [b], Ingrid Alessandra Victoria Wolin [a], Ana Paula Machado Nascimento [a], Antonio Hadson Bastos Neco [b], David Alencar Araripe [b], Priscilla Gomes Welter [a], Corneville Correia Neto [b], Jorge Luis Almeida Correia [b], Cintia Renata Costa Rocha [c], Kyria Santiago Nascimento [b], Benildo Sousa Cavada [b,*]

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ARTICLE INFO

Article history:
Received 31 October 2017
Received in revised form 28 February 2018
Accepted 16 March 2018
Available online 17 March 2018

Keywords:

ABSTRACT

Lectins are a class of carbohydrate-binding proteins or glycoproteins with diverse specificities and functions. The determination and characterization of the three-dimensional structures of these proteins are keys to understanding their biological effects. Recent studies have explored the anticancer potential of *Diocleinae* lectins (from *Leguminoseae* family), evaluating their antiproliferative effect and their ability to induce glioma cell death *via* apoptosis and autophagy. In this work, the three-dimensional structure of *Dioclea lasiophylla* seed lectin (DlyL) complexed with Xman (5-bromo-6-chloro-3-indolyl-α-D-mannopyranoside) was determined by X-ray crystallography. Moreover, interactions with relevant *N*-glycans were evaluated by molecular docking. DlyL presented

ATTACHMENT O – PAPER 15

International Journal of Biochemistry and Cell Biology 92 (2017) 79–89

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

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

Research paper

Structural analysis of *Dioclea lasiocarpa* lectin: A C6 cells apoptosis-inducing protein☆

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ARTICLE INFO

Keywords:
Dioclea lasiocarpa lectin
DLL
Crystal structure
Molecular docking
Glioma

ABSTRACT

Lectins are multidomain proteins that specifically recognize various carbohydrates. The structural characterization of these molecules is crucial in understanding their function and activity in systems and organisms. Most cancer cells exhibit changes in glycosylation patterns, and lectins may be able to recognize these changes. In this work, *Dioclea lasiocarpa* seed lectin (DLL) was structurally characterized. The lectin presented a high degree of similarity with other lectins isolated from legumes, presenting a jelly roll motif and a metal-binding site stabilizing the carbohydrate-recognition domain. DLL demonstrated differential interactions with carbohydrates, depending on type of glycosidic linkage present in ligands. As observed by the reduction of cell viability in C6

ATTACHMENT P – PAPER 16

International Journal of Biological Macromolecules 107 (2018) 236–246

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Structural studies and nociceptive activity of a native lectin from *Platypodium elegans* seeds (nPELa)

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ARTICLE INFO

Article history:
Received 28 July 2017
Received in revised form 29 August 2017
Accepted 30 August 2017
Available online 1 September 2017

Keywords:
nPELa
Crystal structure
Nociception

ABSTRACT

A native lectin (nPELa), purified from seeds of the species *Platypodium elegans*, Dalbergieae tribe, was crystallized and structurally characterized by X-ray diffraction crystallography and bioinformatics tools. The obtained crystals diffracted to 1.6 Å resolution, and nPELa structure were solved through molecular substitution. In addition, nPELa has a metal binding site and a conserved carbohydrate recognition domain (CRD) similar to other Dalbergieae tribe lectins, such as PAL (*Pterocarpus angolensis*) and CTL (*Centrolobium tomentosum*). Molecular docking analysis indicated high affinity of this lectin for different mannosides, mainly trimannosides, formed by α-1,3 or α-1,6 glycosidic bond, as evidenced by the obtained scores. In addition, molecular dynamics simulations were performed to demonstrate the structural behavior of nPELa in aqueous solution. In solution, nPELa was highly stable, and structural modifications in its carbohydrate recognition site allowed interaction between the lectin and the different ligands. Different

ATTACHMENT Q – PAPER 17

International Journal of Biological Macromolecules 106 (2018) 369–378

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Canavalia bonariensis lectin: Molecular bases of glycoconjugates interaction and antiglioma potential

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ARTICLE INFO

Article history:
Received 28 June 2017
Received in revised form 19 July 2017
Accepted 3 August 2017
Available online 10 August 2017

ABSTRACT

CaBo is a mannose/glucose-specific lectin purified from seeds of *Canavalia bonariensis*. In the present work, we report the CaBo crystal structure determined to atomic resolution in the presence of X-man, a specific ligand. Similar to the structural characteristics of other legume lectins, CaBo presented the jellyroll motif, a metal binding site occupied by calcium and manganese ions close to the carbohydrate-recognition domain (CRD). *In vitro* test of CaBo cytotoxicity against glioma cells demonstrated its ability to decrease

ATTACHMENT R – PAPER 18

Archives of Biochemistry and Biophysics 630 (2017) 27–37

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Crystal structure of *Pisum arvense* seed lectin (PAL) and characterization of its interaction with carbohydrates by molecular docking and dynamics

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ARTICLE INFO

Article history:
Received 25 May 2017
Received in revised form
19 July 2017
Accepted 23 July 2017

ABSTRACT

The *Pisum arvense* lectin (PAL), a legume protein belonging to the Vicieae tribe, is capable of specific recognition of mannose, glucose and its derivatives without altering its structure. In this work, the three-dimensional structure of PAL was determined by X-ray crystallography and studied in detail by a combination of molecular docking and molecular dynamics (MD). Crystals belonging to monoclinic space

ATTACHMENT S – PAPER 19

Biochimie 140 (2017) 58–65

Contents lists available at ScienceDirect

Biochimie

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

Research paper

Contribution of the carbohydrate-binding ability of *Vatairea guianensis* lectin to induce edematogenic activity

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ARTICLE INFO

Article history:
Received 23 March 2017
Accepted 9 June 2017
Available online 16 June 2017

Keywords:
Vatairea guianensis

ABSTRACT

Vatairea guianensis lectin (VGL), Dalbergiae tribe, is a N-acetyl-galactosamine (GalNAc)/Galactose (Gal) lectin previously purified and characterized. In this work, we report its structural features, obtained from bioinformatics tools, and its inflammatory effect, obtained from a rat paw edema model. The VGL model was obtained by homology with the lectin of *Vatairea macrocarpa* (VML) as template, and we used it to demonstrate the common characteristics of legume lectins, such as the jellyroll motif and presence of a metal-binding site in the vicinity of the carbohydrate-recognition domain (CRD). Protein-ligand docking

ATTACHMENT T – PAPER 20

International Journal of Biological Macromolecules 102 (2017) 323–330

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Partial characterization and immobilization in CNBr-activated Sepharose of a native lectin from *Platypodium elegans* seeds (PELa) and comparative study of edematogenic effect with the recombinant form

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ARTICLE INFO

Article history:
Received 22 November 2016
Received in revised form 30 March 2017
Accepted 31 March 2017
Available online 2 April 2017

ABSTRACT

The lectin from *Platypodium elegans* seeds (PELa) was purified by affinity chromatography in a mannose-agarose column. The lectin agglutinated rabbit erythrocytes and the agglutinating effect was inhibited by previous incubation with the glycoprotein fetuin, along with *N*-acetyl-D-glucosamine, D-mannose and its derivatives. The lectin maintained complete activity in temperatures ranging from 40 to 60 °C and pH values ranging from 9 to 10. As a glycoprotein, PELa has a carbohydrate content of 2.2%, and its activ

ATTACHMENT U – PAPER 21

Biochimie 135 (2017) 126–136

Contents lists available at ScienceDirect

Biochimie

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

Research paper

Molecular modeling, docking and dynamics simulations of the *Dioclea lasiophylla* Mart. Ex Benth seed lectin: An edematogenic and hypernociceptive protein

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ARTICLE INFO

Article history:
Received 10 January 2017
Accepted 9 February 2017
Available online 11 February 2017

ABSTRACT

Lectins are proteins, or glycoproteins, capable of reversibly binding to specific mono- or oligosaccharides via a noncatalytic domain. The Diocleinae subtribe presents lectins with high structural similarity, but different effects based on biological activity assays. This variability results from small structural differences. Therefore, in this context, the present study aimed to perform a structural analysis of the lectin from *Dioclea lasiophylla* Mart. ex Benth seeds (DlyL) and evaluate its inflammatory effect. To accomplish

ATTACHMENT V – PAPER 22

International Journal of Biological Macromolecules 94 (2017) 271–282

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Structural characterization of a lectin from *Canavalia virosa* seeds with inflammatory and cytotoxic activities

Vinicius Jose Silva Osterne[a], Jose Caetano Silva-Filho[b], Mayara Queiroz Santiago[a], Vanir Reis Pinto-Junior[a], Alysson Chaves Almeida[a], Adolph Annderson Gonçalves Costa Barreto[a], Ingrid Alessandra Victoria Wolin[c], Ana Paula Machado Nascimento[c], Renata Morais Ferreira Amorim[d], Bruno Anderson Matias Rocha[a], Plinio Delatorre[b], Celso Shiniti Nagano[a], Rodrigo Bainy Leal[c], Ana Maria Sampaio Assreuy[d], Kyria Santiago Nascimento[a], Benildo Sousa Cavada[a,*]

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ARTICLE INFO

Article history:
Received 26 August 2016
Received in revised form 8 October 2016
Accepted 9 October 2016

ABSTRACT

A lectin from *Canavalia virosa*, Diocleinae subtribe, was purified by affinity chromatography with Sephadex G-50 matrix and named ConV. The primary structure of ConV was obtained by mass spectrometry and crystals were obtained by the vapor diffusion method at 293 K and belonged to orthorhombic

ATTACHMENT W – PAPER 23

International Journal of Biological Macromolecules 98 (2017) 12–23

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Structural studies of a vasorelaxant lectin from *Dioclea reflexa* Hook seeds: Crystal structure, molecular docking and dynamics

Vanir Reis Pinto-Junior[a], Vinicius José Silva Osterne[a], Mayara Queiroz Santiago[a], Jorge Luis Almeida Correia[a], Francisco Nascimento Pereira-Junior[b], Rodrigo Bainy Leal[c], Maria Gonçalves Pereira[d], Larissa Silva Chicas[a], Celso Shiniti Nagano[a], Bruno Anderson Matias Rocha[a], José Caetano Silva-Filho[a], Wandemberg Paiva Ferreira[a], Cíntia Renata Costa Rocha[e], Kyria Santiago Nascimento[a], Ana Maria Sampaio Assreuy[d,*], Benildo Sousa Cavada[a,*]

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ARTICLE INFO

Article history:
Received 5 December 2016
Received in revised form 16 January 2017
Accepted 20 January 2017

ABSTRACT

The three-dimensional structure of *Dioclea reflexa* seed lectin (DrfL) was studied in detail by a combination of X-ray crystallography, molecular docking and molecular dynamics. DrfL was purified by affinity chromatography using Sephadex G-50 matrix. Its primary structure was obtained by mass spectrometry, and crystals belonging to orthorhombic space group $P2_12_12_1$ were grown by the vapor diffusion method

ATTACHMENT X – PAPER 24

International Journal of Biological Macromolecules 92 (2016) 194–201

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Lectins from *Parkia biglobosa* and *Parkia platycephala*: A comparative study of structure and biological effects

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ARTICLE INFO

Article history:
Received 11 March 2016
Received in revised form 7 July 2016

ABSTRACT

The relation structure–activity of the Mimosoideae lectins of *Parkia platycephala* (PPL) and *Parkia biglobosa* (PBL) was analyzed in this study. PBL was solved by X-ray crystallography at a resolution of 2.1 Å, and the crystal structure belonged to the $C222_1$ space group. Structural organization and binding sites were also

ATTACHMENT Y – PAPER 25

Archives of Biochemistry and Biophysics 596 (2016) 73–83

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Structural analysis of *Centrolobium tomentosum* seed lectin with inflammatory activity

Alysson Chaves Almeida [a], Vinicius Jose da Silva Osterne [a], Mayara Queiroz Santiago [a], Vanir Reis Pinto-Junior [a], Jose Caetano Silva-Filho [b], Claudia Figueiredo Lossio [a], Francisco Lucas Faustino Nascimento [c], Ricardo Patricio Honorato Almeida [d], Claudener Souza Teixeira [d], Rodrigo Bainy Leal [e], Plinio Delatorre [b], Bruno Anderson Matias Rocha [d], Ana Maria Sampaio Assreuy [c], Kyria Santiago Nascimento [a], Benildo Sousa Cavada [a, *]

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ARTICLE INFO

Article history:
Received 7 January 2016
Received in revised form

ABSTRACT

A glycosylated lectin (CTL) with specificity for mannose and glucose has been detected and purified from seeds of *Centrolobium tomentosum*, a legume plant from *Dalbergieae* tribe. It was isolated by mannose-

ATTACHMENT Z – PAPER 26

Archives of Biochemistry and Biophysics 588 (2015) 33–40

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

A novel vasorelaxant lectin purified from seeds of *Clathrotropis nitida*: partial characterization and immobilization in chitosan beads

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ARTICLE INFO

Article history:

ABSTRACT

A novel lectin from seeds of *Clathrotropis nitida* (CNA) was purified and characterized. CNA is a glyco-

ATTACHMENT AA – PAPER 27

Research article

Journal of Molecular Recognition

Received: 1 July 2015, Revised: 1 September 2015, Accepted: 5 September 2015, Published online in Wiley Online Library: 14 October 2015

(wileyonlinelibrary.com) DOI: 10.1002/jmr.2512

Purification and molecular characterization of a novel mannose-specific lectin from *Dioclea reflexa* hook seeds with inflammatory activity

Vanir R. Pinto-Junior[a], Jorge L. A. Correia[a], Ronniery I. Pereira[a], Francisco N. Pereira-Junior[a], Mayara Q. Santiago[a], Vinicius J. S. Osterne[a], Juliana C. Madeira[c], João B. Cajazeiras[a], Celso S. Nagano[b], Plinio Delatorre[d], Ana M. S. Assreuy[c], Kyria S. Nascimento[a] and Benildo S. Cavada[a]*

A novel lectin present in *Dioclea reflexa* seeds (DrfL) was discovered and described in this study. DrfL was purified in a single step by affinity chromatography in a Sephadex G-50 column. The lectin strongly agglutinated rabbit erythrocytes and was inhibited by α-methyl-D-mannoside, D-mannose, and D-glucose. The hemagglutinating activity of DrfL is optimum at pH 5.0–7.0, stable up to 50 °C, and dependent on divalent cations. Similar to other lectins of the subtribe Diocleinae, the analysis by mass spectrometry indicated that DrfL has three chains (α, β, and γ) with masses of 25 562, 12 874, and 12 706 Da, respectively, with no disulfide bonds or glycosylation. DrfL showed inflammatory activity in the paw edema model and exhibited low cytotoxicity against *Artemia* sp.

Keywords: lectin; Dioclea reflexa; ESI mass spectrometry; inflammatory activity

ATTACHMENT AB – PAPER 28

Process Biochemistry 49 (2014) 529–534

Contents lists available at ScienceDirect

Process Biochemistry

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

Mannose-specific legume lectin from the seeds of *Dolichos lablab* (FRIL) stimulates inflammatory and hypernociceptive processes in mice

Claudener Souza Teixeira[a,b], Ana Maria Sampaio Assreuy[c], Vinícius José da Silva Osterne[a], Renata Morais Ferreira Amorim[c], Luiz André Cavalcante Brizeno[c], Henri Debray[d], Celso Shiniti Nagano[e], Plinio Delatorre[f], Alexandre Holanda Sampaio[a,e], Bruno Anderson Matias Rocha[a], Benildo Sousa Cavada[a,*]

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ARTICLE INFO

Article history:
Received 21 October 2013

ABSTRACT

Lectins are proteins that specifically bind to carbohydrates and form complexes with molecules and biological structures containing saccharides. The FRIL (Flt3 receptor interacting lectin) is a dimeric two-chain

ATTACHMENT AC – PAPER 29

Research Article

Journal of Molecular Recognition

Received: 18 September 2013, Revised: 05 November 2013, Accepted: 08 November 2013, Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jmr.2340

Purification, characterization and partial sequence of a pro-inflammatory lectin from seeds of *Canavalia oxyphylla* Standl. & L. O. Williams

Mayara Q. Santiago[a], Cintia C. F. Leitão[a], Francisco N. Pereira-Junior[a], Vanir R. Pinto-Junior[a], Vinicius J. S. Osterne[a], Claudia F. Lossio[a], João B. Cajazeiras[a], Helton C. Silva[a], Francisco V. S. Arruda[c], Livia P. Pereira[d], Ana M. S. Assreuy[d], Kyria S. Nascimento[a], Celso S. Nagano[b] and Benildo S. Cavada[a]*

Recent studies have shown that lectins are promising tools for use in various biotechnological processes, as well as studies of various pathological mechanisms, isolation, and characterization of glycoconjugates and understanding the mechanisms underlying pathological mechanisms conditions, including the inflammatory response. This study aimed to purify, characterize physicochemically, and predict the biological activity of *Canavalia oxyphylla* lectin (CoxyL) *in vitro* and *in vivo*. CoxyL was purified by a single-step affinity chromatography in Sephadex® G-50 column. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that the pure lectin consists of a major band of 30 kDa (α-chain) and two minor components (β-chain and γ-chain) of 16 and 13 kDa, respectively. These data were further confirmed by electrospray ionization mass spectrometry, suggesting that CoxyL is a typical ConA-like lectin.

ATTACHMENT AD – PAPER 30

Appl Biochem Biotechnol (2014) 172:3342–3353
DOI 10.1007/s12010-014-0751-3

Purification, Partial Characterization, and CNBr-Sepharose Immobilization of a Vasorelaxant Glucose/Mannose Lectin from *Canavalia virosa* Seeds

Vinicius J. S. Osterne · Mayara Q. Santiago · Vanir R. Pinto-Junior · João B. Cajazeiras · Jorge L. A. Correia · Cintia C. F. Leitão · Rômulo F. Carneiro · Francisco N. Pereira-Junior · Mayron A. Vasconcelos · Bruno A. M. Rocha · Ana Maria S. Assreuy · Pedro Henrique S. F. Bringel · Celso S. Nagano · Kyria S. Nascimento · Benildo S. Cavada

Received: 23 October 2013 / Accepted: 20 January 2014 /
Published online: 13 February 2014

ATTACHMENT AE – PAPER 31

Archives of Biochemistry and Biophysics 543 (2014) 31–39

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Vasorelaxant activity of *Canavalia grandiflora* seed lectin: A structural analysis

Ito Liberato Barroso-Neto [a], Rafael Conceição Simões [a], Bruno Anderson Matias Rocha [a], Maria Julia Barbosa Bezerra [a], Francisco Nascimento Pereira-Junior [a], Vinicius José Silva Osterne [a], Kyria Santiago Nascimento [a], Celso Shiniti Nagano [a,b], Plinio Delatorre [c], Maria Gonçalves Pereira [d], Alana Freitas Pires [d], Alexandre Holanda Sampaio [a,b], Ana Maria Sampaio Assreuy [d], Benildo Sousa Cavada [a,*]

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ARTICLE INFO

Article history:
Received 30 October 2013
and in revised form 2 December 2013
Available online 17 December 2013

ABSTRACT

Lectins are comprised of a large family of proteins capable of the specific and reversible recognition of carbohydrates. Legume lectins, the most studied plant lectins, show high structural similarity, but with modifications that imply a variation in the intensity of some biological activities. In this work, the primary and tertiary structures of *Canavalia grandiflora* (ConGF) were determined. ConGF, a lectin isolated from *C. grandiflora* seeds, is able to induce relaxant activity in rat aortic rings. The complete

ATTACHMENT AF – PAPER 32

Molecules **2013**, *18*, 10857-10869; doi:10.3390/molecules180910857

OPEN ACCESS

molecules

ISSN 1420-3049

www.mdpi.com/journal/molecules

Article

Purification, Partial Characterization and Immobilization of a Mannose-Specific Lectin from Seeds of *Dioclea lasiophylla* Mart.

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