Structure-Function Studies and Evaluating the Medical Applications Potential of two Newly Identified Plant Lectins

Thesis Submitted to AcSIR
For the Award of the Degree of
DOCTOR OF PHILOSOPHY
In
BIOLOGICAL SCIENCES

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MAY 2016

Dedicated to

My family* and *Bhaskar

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CERTIFICATE

This is to certify that the work incorporated in this Ph.D thesis work entitled **"Structure-function studies and evaluating the medical applications potential of two newly identified plant lectins"** submitted by **Ms. Ruby Singh** to Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirement for the award of the Degree of **Doctor of Philosophy**, embodies original research work under my guidance. We further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree of diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources have been duly cited and acknowledged.

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I hereby declare that the research work reported in the thesis entitled "**Structure-function studies and evaluating the medical applications potential of two newly identified plant lectins**" is an authentic record of the research carried out by me under the supervision of Dr. C. G. Suresh, Chief Scientist, Biochemical Sciences Division, CSIR- National Chemical Laboratory (NCL), Pune, India. The thesis is submitted by me to the AcSIR for the degree of Doctor of Philosophy.

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Acknowledgement

My tenure as PhD student at CSIR- National Chemical Laboratory has been a great learning experience in all aspects. The present thesis is a result of five years of work where many people consciously or unconsciously have assisted me in numerous ways to make this journey worth travelling. I now take this opportunity to express my sincere gratitude to all of them.

I owe huge gratitude to ***Dr. C. G. Suresh****, my research supervisor for allowing me an opportunity to work under his guidance and for his constant encouragement and support in all the considerate ways. I consider myself very fortunate to have him as my mentor who gave me the freedom to explore the science in my own way; however, whenever I was stuck he was always there to help me out with his valuable advice and suggestions. With me he was also patient during my stressful times, giving both scientific and personal help which enabled me to remain optimistic. He always had scientific temperament, innovative approach for any query, dedication towards his profession and down to earth nature has inspired me the most. I really wish that I could be the same in this profession. I preserve an everlasting gratitude for him.*

I would especially like to thank ***Dr. Sushama M Gaikwad****, for teaching and explaining all the biochemical and biophysical techniques personally. She was also my DAC committee member and has helped me coming out from various experimental problems. Our collaboration with her resulted in good scientific work and it has also extended to friendly terms. I will always be grateful to her for her advices and boosting my morale throughout my research.*

I also take this opportunity to thank ***Dr. Dhiman Sarkar*** *for allowing and guiding me to carry out cancer biology work in his lab. I also thank Laxman Nawale and his other lab mates to help me in conducting cell lines studies. Laxman Nawale has been the most sincere lab colleague and very good friend of mine. I have also learnt many things from him.*

I thank ***Dr. Suresh Kumar Ramasamy*** *for his ideas, suggestions and support. He was always cheerful, friendly and has encouraged me a lot. I sincerely thank* ***Dr. M. V. Badiger*** *and* ***Dr. Monisha*** *for allowing me to use the Fluorescence and CD facility in their respective departments.*

I take the opportunity to thank ***Dr. R. V. Gadre***, ***Dr. Archana Pundle*** *and* ***Dr. Vidya Gupta*** *for being my DAC committee members. They have always provided with their timely and beneficial suggestions.*

I thank all my seniors at NCL, Dr Urvashi Sharma, Dr. Nishant Varshney, Dr. Tulika Jaokar, Dr. Priyabrata Panigrahi (PP bhaiya), Dr. Ranu Sharma, and Manas for their support and useful suggestions over my research queries. Dr. Nishant has always been there to support even after leaving the lab boosting my morale during the completion of the work. I sincerely thank him for his continuous support and his brotherly love. I would also like to thank my juniors Deepak, Manu, Ameya, Vijay, Tejashree, Deepanjan, Yashpal, Shiva and Debjyoti for giving me a wonderful company in lab. Special thanks to Deepak who always helped and given a great company during our conferences and Mandappam trip. I personally appreciate the help and support offered by Vijay and Tejashree (Teju). I also thank the short term trainees who have joined the lab during my tenure, Ajay, Abhishek, Ritesh, Sagar, Archana and Bitika.

I also wish to thank my friends from CSIR-NCL in other groups who have helped me in one or the other way for my research work: Priya, Madhurima, Sonali, Sayli, Sanskruti and Ekta, Pushpa, Neha Kandhelwal and Uma. Priya had helped me in my thesis correction and was always there whenever I needed her advices. I would also like to say special thanks to my NCL-RSM friends who have given me such a wonderful and memorable time during RSM events. I enjoyed those days the most. I thank my friends, Pravin, Ravindre, Late Sagar Tendulkar, Mayur, Chaitanya and Swapnil. They have also helped me in getting few chemicals which were not available at times in my lab. Special thanks to Prajita (de Praji) who always kept helped and kept patience for me whenever I was angry or feeling low. I will always cherish the memories of those days.

Most importantly, none of this would have been possible without the love and patience of my family. They have been the constant source of love, concern, support and strength all these years. I would like to express my heart-felt gratitude to my family. My sisters, ***Sonia*** *and* ***Neha****; and my Best friend,* ***Bhaskar*** *have been my rocks, I don't know how this all could have been possible without their everlasting support. I thank my sisters for encouraging me and expressing confidence in my abilities whenever I was morally down. They have been greatest support during each and every day of my PhD life, especially. It is very hard to describe in words the support given by Bhaskar at every stage of my work; understanding and listening to all my problems, helping me in every possible way he can and making it possible to reach the end successfully. I thank them for their love, affection, prayers, support and encouragement.*

Special indebtedness to my parents for what I have achieved today. They have always been the light that shines my way and the drive for my ever-persistent determination. Without their enduring support and encouragement, it would not have been possible to embark upon this journey in life

Finally, I appreciate the financial support from CSIR that funded parts of the research discussed in this dissertation. I thank the Director, National Chemical Laboratory and the Head, Division of Biochemical Sciences, for allowing me to pursue my Ph.D work at the Division of Biochemical Sciences, NCL. I take the opportunity to thank all the scientists and students of biochemical division, NCL for their help whenever required.

Ruby Singh

ABSTRACT

Lectins belong to a group of proteins that bind free sugar or sugar residues of polysaccharides, glycoproteins, or glycolipids, which exist in free or bound form (as in cell membranes). Plant lectins have been attracting much attention because of their ease of isolation mainly from seeds and their usefulness as reagents for detecting glycoconjugates in solution and on cell surfaces. The recognition of carbohydrate moieties by lectins has important implications to a number of biological processes such as in cell-cell interactions, signal transduction, fertilization, and cancer metastasis and in cell growth and differentiation. A large number of lectins have been reported from many plant species but correlation between structure, conformation, activity and stability has been studied only in few of them.

Conditions such as very high temperatures or presence of chemical denaturants are known to disturb the three-dimensional structures of proteins, perturbing their physical properties and biological activities. Measuring the intrinsic fluorescence of a protein and following structural changes using circular dichroism (CD) and Fourier transform infra red (FTIR) are methods for studying conformational transitions of proteins under denaturing conditions.

In the present work, two plant lectins have been isolated and purified. Both the lectins were characterized using various biochemical and biophysical techniques. Due to its specificity to N-acetyl glucosamine oligomers we have also evaluated its biomedical applications like anti-neoplastic activity. Both the lectins possesses chito-oligosaccharide specificity: one is purified from the Ashgourd Fruit , *Bh*L (*Benincasa hispida* lectin) belonging to Cucurbitaceae family and another one purified from the seeds of the Datura, *Di*L9 (*Datura innoxia* lectin) of Solanaceae family.

The work has been divided into three working parts.

I Biochemical and biophysical characterization of Benincasa hispida lectin (BhL)

II Structural-functional characterization of a cytokinin binding Datura innoxia lectin (DiL9)

III Medical application potentials of chito-specific agglutins (BhL and DiL9). (A)Anti biofilm activity. (B) Antineoplastic and anti angiogenic activity.

I. Biochemical and biophysical characterization of *Benincasa hispida* lectin

Benincasa hispida (Family: Cucurbitaceae) is commonly known as Ashgourd. Fruits of this plant have many medicinal uses. Traditionally it is used as a laxative, diuretic, tonic, aphrodisiac, cardiotonic, urinary calculi, blood disease, epilepsy, and also in cases of jaundice, dyspepsia, fever, and menstrual disorders. The methanolic extract of the fruit is reported to possess antiulcer, anti-inflammatory, antihistaminic, and antidepressant activities. In addition to the above peculiarities, several bioactive proteins have been identified in this plant, for example, osmotin like protein, a member of pathogenesis related proteins and a serine proteinase inhibitor. There are very few reports available about the presence of lectins. Among the Cucurbitaceae family a few lectins isolated from the phloem exudate of *Cucurbita maxima, Cucumis sativus* and *Cucumis melo,* found to be specific for chitooligosaccharides. Hence, we decided to study presence of lectins from this Cucurbitaceae species.

The lectin from *Benincasa hispida* was extracted and purified using 2step chromatography: a) chitin-affinity column and b) Gel filtration column (S200). The lectin was found to be a homodimer of 34 kDa with subunit molecular mass of 17 kDa. From hemagglutination inhibition activity, it was observed that the lectin, *Bh*L is specific to N-acetyl glucosamine oligomer, i.e Chitotriose and higher chito-sachharides. Crystallization trials were unsuccessful. However, other biophysical experiments provided insights on structural folding and

stability of the lectin. It was found to be stable at extreme pH condition, at high temperature and in the presence of 50 % (v/v) organic solvents and chemical denaturants maintaining its structure and activity. Chemical modification studies implied the involvement of tryptophan in sugar binding site, since in the presence of 1mM NBS there was complete loss of hemagglutination activity. The intrinsic fluorescence of native *Bh*L gave λ_{max} of 349 nm indicating that the tryptophans are exposed to the polar environment. From NBS titration studies it was found that in native form the lectin has 2 trp residues exposed and decomposition analysis revealed Class II or III of tryptophan conformers. Fluorimetric quenching analysis also revealed electropositive environment near tryptophan residues. The calculation of secondary structure elements by CONTINLL program from CDPro showed α-helix: 3 %, β-sheet: 41.7 % turns: 21 % and random coil: 33 %. Thus *Bh*L is a β protein containing several turns and unordered elements. Ligand binding studies showed higher affinity for chitotriose as compared to any disaccharide. Interestingly, it also showed distinct hydrophobic binding site for adenine indicating its physiological role in plants acting as a plant growth regulators.

II Structural-functional characterization of a cytokinin binding *Datura innoxia* lectin (*Di*L9)

Datura innoxia, is a further member of the thorn apple family (Solanaceae), are native to Central and South America. The main purpose for cultivating Datura was the production of scopolamine and other toxic alkaloids. It's been known for years about the toxicity of all the parts of the plant, possessing hallucinogenic, hypnotic and narcotic effects but still used as traditional medicines. The correct dosage and method of preparation of Datura extract can be used for various purposes such as pain killer, reducing fevers, diarrhea and skin diseases. From previous reports it's known that many isolectins are present in *Datura innoxia* seeds, rich in hydroxyproline, glycine and cysteine. The main lectin interacts with N-acetyl glucosamine (GlcNAc) oligomers and specifically binds to chitin and fetuin. The thermal stability is similar to

that reported for the other Solanaceae lectins. Very less information is available regarding its structure- function activity.

In this report, the lectin from Datura innoxia seeds was isolated and purified using ion exchange and gel filtration chromatography. From SDS-PAGE and MALDI-TOF/TOF analysis the lectin found to be monomer of 9 kDa. It was found to be a glycoprotein with 4% neutral sugar content and showed specificity towards chito-sugars. Chemical modification studies revealed that tryptophan and tyrosine residues are involved in sugar binding site, since on modification of these residues loss in hemagglutinating activity was observed. Similar to *Bh*L, *Di*L9 also showed structural and functional stability at extreme pH range, high temperature and in the presence of chemical denatutants or organic solvents. Interestingly, the lectin also showed resisitance to proteolytic digestion retaining its full activity. But there was complete loss of activity and structure when the lectin was treated with β-mercaptoethanol implying presence of disulphide bridges plays an important role in maintaining the native conformation of the lectin. We have also evaluated the antimicrobial potential of the lectin against various pathogens using agar disc diffusion method. *Di*L9 exhibited significant antibacterial activity against *E. faecalis* and *B. cereus* MIC of 150 and 250 µg/ml, respectively. No significant activity was found against pathogenic fungal strains. From these findings, we would like to suggest that *Di*L9 has potential antibacterial activity though less of antifungal activity against specific fungi only.

Fluorescence studies showed emission maxima at 354 nm (λ_{max}), as three trps are exposed to the solvent while the denatured lectin gave red shift of 3 nm, implying that the buried single trp was exposed on denaturation. PFAST decomposition analysis of the steady state fluorescence spectrum revealed Class III (100%) tryptophan conformer implying fully exposed in the solvent with highly mobile water. From solute quenching studies, it was accounted the tryptophan microenvironment is electropositive in nature. The spectroscopic studies like CD and FTIR indicated that the lectin comprises both α-helix and β-sheets. DLS studies showed polydisperse

nature of the lectin, which means that the distribution of the lectin in the solution is of different sizes. This could be the reason for the crystallization trials being unsuccessful.

III Medical application potentials of chito-specific agglutins (BhL and DiL9).

(A)Anti biofilm activity.

It's been well known fact that bacteria can exist as planktonic, the lifestyle in which single cells exist in suspension, and as biofilms also, which are surface attached bacterial communities embedded in a self produced matrix. Most of the antibiotics and the methods for antimicrobial work have been developed for planktonic bacteria. However, the majority of the bacteria in natural habitats live as biofilms. Biofilms develop dauntingly fast high resistance towards conventional antibacterial treatments and thus, there is a great need to meet the demands of effective anti -biofilm therapy. Previous studies have demonstrated that bacteria inside biofilms can be up to 1000 timesmore resistant to antibiotics than free-living bacteria. Taken together, these features make infections caused by biofilm-producing pathogens difficult to treat; thus, biofilms are an area of concern in human and animal infections.

In this thesis work it was attempted to fill the void of antibiofilm screening of different pathogenic organisms by using crystal violet assays that can evaluate the effect that screened compounds have on the total biomass, viability and the extracellular polysaccharide (EPS) layer of the biofilms. Our experiments concluded that both the lectins have broad range of anti-biofilm activity. The assay showed that these lectins could inhibit biofilm formation even though there is no inhibition to bacterial growth.

(B) Antineoplastic and anti angiogenic activity.

For decades, many cancer researches have been going on around the world to identify the causes and to design and develop new strategies for its prevention, diagnosis, treatment and cure. For many years' researches has focused the main target for anticancer drugs to DNA, but due to

its reoccurrence, the molecular targeted therapy with molecules having specificity and selectivity to a particular cancer should be the strategy for new anticancer therapy. Molecules like lectins do have such selectivity and specificity for its oligosaccharide. Depending on its specificity, lectins can recognize tumor associated glycans and therefore can differentiate malignant cells from normal cells based on the degree of glycosylation associated with metastasis. Suitable and economical approach is to induce apoptosis in cancer cells by using non-immunogenic molecules like lectins. These proteins can induce activation of caspases leading to apoptosis in cancer cells or by anti angiogenesis.

In the present study, the two purified lectins was undertaken to investigate the anticancer properties. Firstly, the cytotoxicity (MTT) assay was performed to see its effect on both normal and cancer cell lines. Both the lectins inhibited the viability of cancer cell lines in a dose-dependent manner, where *Bh*L was much more active in causing toxicity to cancer cells. Lectins showed no significant cytotoxicity towards HUVECs and L929 cells (<30% inhibition), implying its non-toxicity towards normal cells. Both the lectins had higher inhibitory effect on human pancreatic cancer cells, thus PANC-1 cells was used for further experimental studies. *Bh*L and *Di*L9 caused growth inhibition by inducing apoptosis of PANC-1cells, which was confirmed by observing cell cycle arrest at G_0/G_1 phase, disruption of mitochondrial transmembrane potential, increase in intracellular calcium release and the apoptotic signal was amplified by activation of caspases executed cell death. Also, these lectins induced disruption of tubule formation by endothelial cells displaying its anti-angiogenic activity. Therefore, we would like to conclude that these lectins belong to the class of molecules with antitumor that specifically binds to a cancer associated glycan, has potential to be developed as an alternative to other conventional anticancer drugs.

ABBREVIATIONS

Abbreviation	Long form
ANS	8-anilino-1-napthalene sulfonic acid
ACN	Acetonitrile
*Bh*L	*Benincasa hispida* lectin
BAP	6- Benzylaminopurine
CD	Circular dichroism
CsCl	Cesium chloride
CV	Crystal violet
DAPI	4', 6-Diamidino-2-phenylindole
DI	Deionised
*Di*L9	*Datura innoxia* lectin (MW 9kDa)
DLS	Dynamic Light Scattering
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetra-acetate
FITC	Fluorescein isothiocyanate
FTIR	Fourier Transform infrared
GDn-HCl	Guanidine hydrochloride
GDn-SCN	Guanidine-thiocyanate
GalNAc	N-acetyl galactosamine
GlcNAc	N-acetyl glucosamine
HCS	High content screening
iPA	Indole-3-propionic acid

KI	Potassium iodide
LCMS	Laser-scanning confocal microscope
MALDI-TOF	Matrix-assisted laser-desorption ionization-time of flight
MRE	Mean residual ellipticity
MTT	,4, 5-dimethylthiazol-2-yl-2, 5- diphenyltetrazolium bromide
NAI	N-acetylimidazole
NBS	N-bromosuccinimide
PBS	Phosphate buffer saline
PI	Propium iodide
RMSD	Root mean square deviation
NRMSD	Normalized root mean square deviation
SDS	Sodium dodecyl sulphate
WRK	Woodward's Reagent K
HUVECs	Human umbilical vein endothelial cells
DMEM	Dulbecco’s Modified Eagle Medium
EMEM	Eagle's Minimum Essential Medium

Chapter 1

Introduction and review of literature on lectins

Glycan moiety differentially expressed on living cell surfaces play important role in biological functions such as bacterial, viral and parasitic infections, fertilization, growth and differentiation and cancer metastasis. Macromolecules like *lectins* are proteins of non-immune origin that can recognize and bind reversibly to complex saccharide moiety attached to proteins or lipids (Sharon, 2008). Lectin molecules are highly specific and bind glycan arrays in a non-catalytic manner (Lis & Sharon, 1998). The "lectinology", the detailed study of lectins began with the documentation of the agglutinating activities of ricin as reported by Herrmann Stillmark (Stillmark, 1888).

However, "agglutinin" was coined to include all molecules possessing property of cell clumping or erythrocytes agglutination. By 1950, the word "lectin" was used for any molecule that identified and distinguished the blood groups on the basis of differentially expressed glycan moiety on cell membrane (Boyd & Shapleigh, 1954). The modern era of lectins started with the efforts of Sharon and Lis in 1972 by purifying many plant lectins (Sharon & Lis, 1972). Owing to their high specificity lectin molecules were widely used in histopathology to differentiate between normal and pathological cells.

1.1. Distributions of lectins

Lectins are a heterogeneous family of proteins found in the nature (Goldstein & Poretz, 1986b). Lectins are ubiquitous with presence in microorganisms to the higher plants and animals. Since they were first identified and best characterized in plants, they were referred to as phytoagglutinins. Later on, many other lectins were discovered and characterized in other life forms like animals, bacteria, fungi and viruses (Lis & Sharon, 1998, Loris *et al.,* 1998) mainly present on cell membrane or in the cell cytoplasm.

Marine bioresources such as marine cyanobacteria, algae, invertebrate animals, and fishes also possess a great diversity of specific and potent bioactive molecules including lectins (Ogawa *et al.*, 2011).

i) Bacterial lectins:

Bacterial species, mainly enterobacteria and *Salmonella* species are capable of producing submicroscopic hair-like elongated appendages, known as fimbriae (hairs) or pili (threads), which can also interact with glycan moiety of the glycoprotein /and glycolipid receptors present on host cells surface (Sharon, 1987). The best characterized surface lectins are the mannose-specific type-1 fimbriae, the galabiose-specific P fimbriae, and the *N*-acetylglucosamine-binding F-17 fimbriae produced by different strains of *Escherichia coli.* For macrophages and ploymorphonuclear leukocytes, these surface lectins acts as recognition molecules aiding in lectinophagocytosis (happens in the absence of opsonins). The carbohydrate recognition domain (CRD) is present at the tip of the fimbriae as minor subunit, for instance, FimH is present in type-1 fimbriae and PapG in P fimbriae (Esko & Sharon, 2009).

Their main function is to initiate the infection by adhering to the host cells. For example, the fimbriated strains of *Escherichia coli* and *Klebsiella pneumonia* causes infection by attaching to the epithelial lining of the urinary and gastrointestinal tracts (Sharon, 1987) **(Fig. 1.1)**. These fimbriated strains express multiple numbers of surface lectins having variable CRD, due to which they can bind to broad range of susceptible tissues. Although the interactions between surface lectins and host receptors are of low affinity the presence of clusters leads to higher avidity in causing infections.

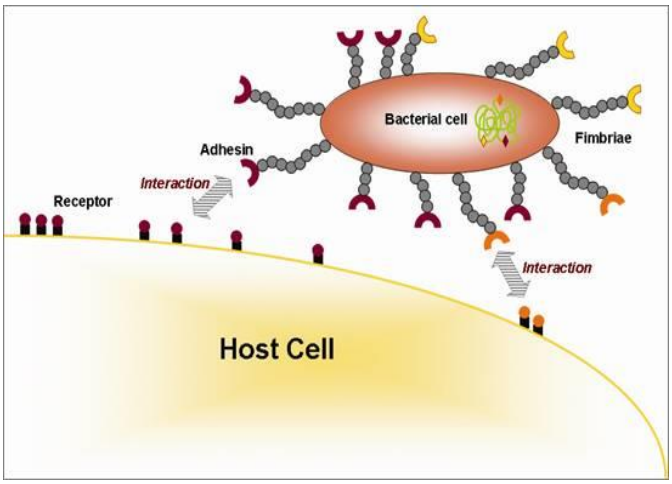


Figure 1.1 Schematic diagrams representing bacterial adhesion to a host cell by using fimbriae. The host cell receptor specific binding unit is termed the adhesin which lies at the tip of the fimbria *(Adopted from (Antão, 2010)).*

ii) Fungal lectins:

Fungi, including mushrooms, express high levels of lectins as storage proteins, similar to plant lectins, having potential role in defence (Singh *et al.*, 2010). Fungal lectins have been reported from mycelium (Candy *et al.*, 2003), fruiting bodies (Thakur *et al.*, 2007, Wang & Ng, 2003), basidiomes (Guillot & G, 1997) and conidia (Tronchin *et al.*, 2002). Pathogenic fungus like *Macrophomina phaseolina* is reported to secrete extracellular lectins (Bhowal *et al.*, 2005). Mushroom lectins have attracted attention because of its role in symbiotic association formed with other organisms as observed in mycorrhizas, mycelia aggregation and in mating (Swamy *et al.*, 2004, Varrot *et al.*, 2013).

The lectin expression level in mushroom depends on the age, location and season. For example, lectin is highly expressed in mature *Laccaria laccata* whereas in case of *Amanita muscaria*, *Tricholomopsis rutilans* and *Lactarius rufusits* it is highly expressed in young mushrooms (Rouf *et al.*, 2011) . Discoidin I (DiscI) and discoidin II (DiscII) are two developmentally regulated *N*-acetyl galactosamine (GalNAc) specific lectins expressed by slime mold called *Dictyostelium discoideum* (Mathieu *et al.*, 2010).

iii) Viral lectins:

The best studied example of viral lectins is the influenza virus hemagglutinin, which binds to sialic acid moiety present on erythrocytes. As already mentioned, the low affinity is overcome by hemagglutinin oligomerizing to form trimers. The influenza hemagglutinin is also responsible for the production of neutralizing antibodies in our bodies, but due to antigenic changes it escapes detection and causes new viral outbreaks (Esko & Sharon, 2009). The complete structural knowledge of the sialic acid-hemagglutinin interaction provides insights into designing antiviral drugs to block the attachment of hemagglutinin to the host cells.

iv) Parasitic lectins:

The best studied protozoan lectin is from pathogenic amoeba *Entamoeba histolytica*, which is responsible for causing human dysentry and disruption and invasion of the colonic mucosa. Gal (galactose/*N*-acetylgalactosamine)-lectin is the best characterized one that activates dendritic cells and drives Th1 responses in vitro and in vivo (Ivory & Chadee, 2007). The other lectins include Jacob, chitinase, and Jessies 2 and 3 which are expressed in the secretory vesicles of transfected amebae (Van Dellen *et al.*, 2002). Similar lectins are also identified from *Hartmannella vermiformis* which is reported to act as a potential receptor for attachment and invasion by the legionnaires' disease causing bacterium (Venkataraman *et al.*, 1997). In case of *Plasmodium falciparum* (malaria), the merozoites interact with sialic acid present on host's RBCs, thus, invading the cells and producing more of merozoites (Esko & Sharon, 2009).

v) Animal lectins:

They belong to eight structurally different families, classified into intracellular and extracellular lectins. The intracellular lectin families are *calnexin family, M-type, L-type and P-type* which are responsible for functioning in trafficking, sorting and targeting through secretory pathway. On the other hand, the extracellular lectin families comprises of *C-type, R-type, siglecs* and *galectins* which are secreted into the extracellular matrix or body fluids. These extracellular lectins are also involved in cell adhesion, migration, cell signalling and pathogenic interactions. Another group which play important role in innate immunity, includes *F-box lectins, ficolins, chitinase-like lectins, F-type lectins* and *intelectins*. **Table 1.1** summarizes the types of animal lectins with their localization and function.

Table.1.1 Classification of Animal lectins (Adopted from *http://www.imperial.ac.uk/research/animallectins/ctld/lectins.html*)

Lectin family	Typical saccharide ligands	Subcellular location	Examples of functions
Calnexin	Glc_1Man_9	ER	Protein sorting in the endoplasmic reticulum.
M-type lectins	Man_8	ER	ER-associated degradation of glycoproteins.
L-type lectins	Various	ER, ERGIC, Golgi	Protein sorting in the endoplasmic reticulum.
P-type lectins	Man 6-phosphate, others	Secretory pathway	Protein sorting post-Golgi, glycoprotein trafficking, ER-associated degradation of glycoproteins, enzyme targeting.
C-type lectins	Various	Cell membrane, extracellular	Cell adhesion (selectins), glycoprotein clearance, innate immunity (collectins).

Galectins	β-Galactosides	Cytoplasm, extracellular	Glycan crosslinking in the extracellular matrix.
I-type lectins (siglecs)	Sialic acid	Cell membrane	Cell adhesion.
R-type lectins	Various	Golgi, Cell membrane	Enzyme targeting, glycoprotein hormone turnover.
F-box lectins	$GlcNAc_2$	Cytoplasm	Degradation of misfolded glycoproteins.
Ficolins	GlcNAc, GalNAc	Cell membrane, extracellular	Innate immunity.
Chitinase-like lectins	Chito-oligosaccharides	Extracellular	Collagen metabolism (YKL-40).
F-type lectins	Fuc-terminating oligosaccharides	Extracellular	Innate immunity.
Intelectins	Gal, galactofuranose, pentoses	Extracellular/cell membrane	Innate immunity. Fertilization and embryogenesis.

vi) Plant lectins:

Plant lectins differ with respect to molecular structure, biochemical properties, and carbohydrate-binding specificity, making them a large group of heterogenous family (Damme *et al.*, 1998). Plant lectins are distributed both in seeds as well as vegetative tissues which are developmentally regulated and also show seasonal variations in their expression (Peumans & Van Damme, 1995).

Lectin localization in the plant

Etzler in 1986, had conducted a short survey on the difference in occurrence and concentration of lectins present in seeds or vegetative tissues revealing drastic differences in the location and relative profusion of individual lectins (Etzler, 1986).

a) Seeds:

In 1972, Howard had reported that mainly plant lectins are present in seeds (cotyledons) which appear during the seed maturation at later stages (Howard *et al.*,

1972). In some cases, lectins are reported to be present in the embryos and on seed coats (Pueppke & Bauer, 1978). Usually seed lectins constitute upto 5 % of the total protein content with exceptions to legume lectins representing 50 % of the total seed protein (e.g. *Phaseolus species*). These seed lectins act as storage proteins displaying anti-predation and anti-pathogenesis against many microbes, insects or herbivores (Brewin & Kardailsky, 1997). The level of lectins or other storage protein decreases from seed cotyledon tissue after germination since they are metabolized for energy (Pusztai, 1991).

Secreted phytoagglutinins are synthesized as propeptides on membrane bound polyribosomes with a secretory signal sequence. These propeptides are processed with the removal of signal peptide in the endoplasmic reticulum lumen and then transported to Golgi apparatus for N-linked glycosylation. Glycosylation is reported to mediate the lectin targeting to its final destination (Pusztai, 1991) or facilitating in proper folding by calnexins and calreticulins prior to its export (Banerjee *et al.*, 2007). Lectins act as defensive proteins against invading microbes or insects by agglutinating the pathogenic cells (Chrispeels & Raikhel, 1991).

b) Vegetative tissues:

Lectins are expressed in vegetative tissues like stem, bark, leaves, flowers, bulb, roots, ovarian, phloem sap and sometimes present in the nectar also (Peumans & Van Damme, 1995). These vegetative lectins may occur simultaneously in different tissues of the same plant with difference in expression levels, for instance Solanaceae lectins occurring in stems, tubers, fruits, seeds, leaves etc (Kilpatrick, 1980). The snowdrop and daffodil lectins are abundantly present in the bulbs (Van Damme & Peumans, 1990). The ground elderberry lectin is restricted to the rhizome only (Peumans *et al.*, 1985) and tulip

lectins are abundantly present in the bulb but are undetectable in stems and leaves (Van Damme & Peumans, 1989). These vegetative lectins share similarities with seed lectins but predominantly present in roots, nodules and aerial tissues. For example, BLEC4 protein from the *Pisum sativum* shows 37 % and 52 % sequence identity with PSL and *Lotus tetragonolobus* seed lectin, respectively (Mandaci & Dobres, 1993). The well characterized bark lectins of *Robinia pseudoacacia* are a mixture of five isolectins and have sequence similarity with its seed lectins (Van Damme, Barre, Rouge *et al.*, 1995, Van Damme, Barre, Smeets *et al.*, 1995). Similarly, *Sophora japonica* bark lectins also show sequence similarity to seed lectins (Herman *et al.*, 1988).

These plant lectins bind to exogenous carbohydrate ligands to display either symbiotic or defensive roles, whereas animal/microbial lectins bind to endogenous ligands for their function. Till date, the actual biochemical function of plant lectin-ligand binding is not fully elucidated. Vegetative lectins are less characterized compared to seed lectins (Etzler, 1985). Various approaches and studies have been undertaken to study the expression of crucial vegetative lectins in various plant parts which may aid in outlining the biological function of these lectins.

With the advancements in the field of genomics and mRNA expressions profiling a large number of genes with lectin or lectin-like domains have been discovered. Microarray data analysis showed that lectins are expressed in a multitude of tissues having unique or overlapping expression patterns (Schmid *et al.*, 2005, Sato *et al.*, 2008, Benedito *et al.*, 2008). Elementary expression of vegetative lectins can vary from organ to organ in plants, and these expression level changes in response to attack by microbes or any other environmental stimuli. For instance, lectin from the *Medicago truncatula*

shows high levels of protein expression in roots just prior to rhizobium inoculation which is $1/6^{th}$ of expression observed in the seeds (Benedito *et al.*, 2008).

More than 50 % of the three-dimensional structures of lectins and their complexes with sugars deposited in Protein Data Bank *(http://www.rcsb.org/pdb/home)* are from plants, the remaining ones are from animals, bacteria, fungi and viruses. However, direct evidence for the biological role played by plant lectins is not yet elucidated. In contrast, functions of animal, microbial and viral lectins have been well characterized. Best way to study protein-carbohydrate interactions is to determine the crystal structure lectin-carbohydrate complexes. The biophysical methods such as fluorescence spectrometry, Circular Dichroism (CD), Isothermal Titration Calorimetry (ITC), Surface Plasmon Resonance (SPR) etc. are also employed to study lectins.

1.2. Detection of lectins

Lectins have unique ability to agglutinate cells which is absent in many other proteins like glycosidases, glycosyltransferases, antibodies which can also bind to carbohydrate moiety. In the laboratory, hemagglutination using rabbit's RBC's is routinely used to detect the presence of lectins in any biological source (Burger, 1974). The erythrocytes can be used directly or after treating with trypsin, pronase or neuraminidase (Sharon & Lis, 1972). Many other techniques have been developed to detect the presence of lectins like affinity electrophoresis (Horejsi & Kocourek, 1974), enzyme multiplied immunoassay technique (Ghosh *et al.*, 1979).

Recently, interactions between lectins and carbohydrates have been investigated electronically using field-effect transistor (FET) devices made up of chemically converted graphene (CCG) and single-walled carbon nanotubes (SWNTs). Here, the

glyco-conjugates are made either pyrene or porphyrin-based and were noncovalently attached on the surface of CCG-FET and SWNT-FET devices. Later, these devices are treated with different concentrations of specific (PA-IL, PA-IIL, and Con A) and non-specific lectins. The carbon nanostructure/glycoconjugate interactions were compared and rationalized using computer-aided models **(Fig.1.2)**. These devices resulted in optimal lectin detection and with lectin titration data of SWNT- and CCG-based biosensors, the lectin dissociation constants (K_d) can also be calculated (Chen *et al.*, 2012).

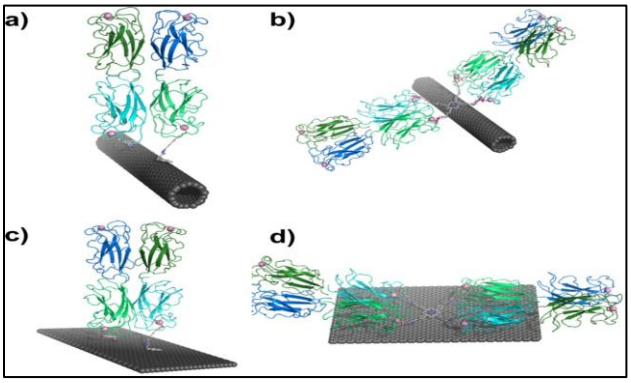


Figure 1.2 Computer-aided models of (a) SWNT/**1a**/PA-IL interaction, (b) SWNT/**2a**/PA-IL interaction, (c) CCG/ **1a**/PA-IL interaction, and (d) CCG/ **2a**/PA-IL interaction (Adopted from (Chen *et al.*, 2012)).

1.3 Molecular Structure of Lectins

- ***Carbohydrate binding***

Depending on the saccharide specificity, the lectins are categorized into various groups: mannose/glucose-, (L)-fucose-, sialic acid-binding, GlcNAc/glucosamine and GalNAc/galactosamine (Goldstein & Poretz, 1986b). These sugars are typically present on eukaryotic cell surfaces. There is another category of lectins which bind solely to oligosaccharides like *Datura stramonium* and *Phaseolus vulgaris* called as lectins having "complex sugar specificity". The interactions between a lectin and its specific carbohydrate are via a network of hydrogen bonding (from H_2O molecules),

hydrophobic, electrostatic and sometimes weak van der Waals interactions (Lis & Sharon, 1998). Hydrogen bonds are usually formed between the amino groups of amino acid residues and hydroxyl groups present in sugars (Sharon & Lis, 2003). The steric disposition of the carbohydrate hydroxyl groups, like methyl group of *N*-acetyl amino sugars can interact with aromatic residues present in lectins (Lis & Sharon, 1998) showing the presence of hydrophobic interactions. The lectin affinity (or dissociation constants, K_d) for monosaccharides ranges from 0.1-1.0 mM, whereas for oligosaccharides (or complex sugars) the K_d value ranges in micro molar concentration. This is because, these oligos also interact with secondary sites in addition to primary sites present on lectin surface, enhancing the affinity termed as subsite multi-valency. However, when more than one subunit is involved in carbohydrate binding then the increase in affinity is termed as subunit multi-valency, leading to K_d values in the nanomolar ranges.

- ***Non Carbohydrate binding***

Hydrophobic binding site - Some lectins, especially legume lectins show affinity towards hydrophobic ligands like adenine and adenine-derived plant hormones, i.e. cytokinins. The exact function is unknown but seems to be involved in plant physiological role like regulation of hormones or growth (Roberts & Goldstein, 1983). Such lectin examples includes *Phaseolus lunatus* with binding affinity of $K_a = 8.3 \times 10^4$ M^{-1} (Roberts & Goldstein, 1983) and *Dolichos biflorus* lectin (Gegg *et al.*, 1992) with $K_a = 7.31 \times 10^5$ M^{-1} and winged bean agglutinin with $K_a = 1.5 \times 10^4$ M^{-1} (Puri & Surolia, 1994) having highest affinity for adenine.

Few lectins have binding site for nonpolar ligands like porphyrins. Lectin-porphyrin interactions are observed in lectins like Jacalin (Komath *et al.,* 2000), *Momordica charantia* lectin (Sultan *et al.,* 2004) and *Trichosanthes cucumerina* seed lectin (Kenoth *et al.*, 2001) with association constant (K_a) in the range of 2.4 x 10^3 M^{-1} to 1.3 x 10^5 M^{-1} at room temperature. Currently, porphyrins are used as photosensitizers in Photo dynamic therapy (PDT) for targeted therapy for cancer. There are certain hydrophobic dyes namely, 2,6-toluidinylnaphthalenesulfonic acid (TNS) and 1,8-anilinonaphthalenesulfonic acid (ANS) used to detect the presence of partially unfolded states in protein, by binding to exposed hydrophobic clusters of unfolded proteins. The function is not yet known but these ligands binds to protein at cofactor or hormone binding region. Examples of lectin known to bind TNS and ANS are lectin from jack beans, concanavalin A (Yang *et al.*, 1974)**,** and ricin (Houston, 1980).

1.4 Physicochemical properties of plant lectin

1.4.1 Composition

Plant lectins share no common structural features. Generally legume lectins are rich in acidic and hydroxyl amino acids with low sulfur-containing amino acids whereas Solanaceae lectins from potato, datura and tomato are rich in cysteine and hydroxyproline content (Petrescu *et al.*, 1993, Lamport, 1969). The content of cysteine residues results in more number of disulphide bond formation conferring higher stability against heat, hydrolytic enzymes and denaturants like urea (Nagata & Burger, 1972, Rice & Etzler, 1974). Most of the lectins are glycoproteins, for example Solanaceae lectins have 40-50 % sugar content (Petrescu *et al.*, 1993). Lectins are made up of identical (homogenous) or non-identical (heterogenous) subunits depending upon the activity. For instance, the anti-

B lectin from *Bandeiraea simplicifolia* consists of two subunits, one subunit is specific for GalNAc, whereas the specificity of the other is subunit is confined to galactose (Goldstein & Hayes, 1978).

1.4.2. Metal ion requirements

Few lectins are reported to have Mn^{2+} or Ca^{2+} binding to maintain their activity as seen in the case of Concanavalin A (Brewer *et al.,* 1983) and lima bean lectin (Nissen & Magnuson, 1986). Using transition metal ions, Mn^{2+} can be replaced as shown for Con A. Similarly, without losing lectin activity Ca^{2+} in Con A could be replaced by Cd^{2+}, but not by Ba^{2+}(Agrawal & Goldstein, 1968, Shoham *et al.*, 1973). Con A has been characterized in detail for metal binding sites using x-ray crystallography. There are two metal ions sites designated as S1 (for $Mn^{2+)}$ and S2 (for Ca^{2+}). Only when both these sites are occupied, the lectin does bind to sugars. From these observations, it was conferred that these metal ions provides a high degree of structural stability to Con A against thermal inactivation and proteolytic hydrolysis (Thomasson & Doyle, 1975). There are other examples of lectins which bind more than 2 metal ions, for instance the *D. biflorus* seed lectin binds $Ca^{2+,}$ Mg^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+} detected by atomic absorption spectrophotometry (Borrebaeck *et al.,* 1981).

1.5 Classification of lectins

In the last few decades, a rapid progress in plant lectin biochemistry has been seen. Around the world, new lectins have been isolated and characterized revealing interesting properties. Many lectins have also been cloned, crystallographic structures determined at atomic resolution and their biosynthetic pathway has been elucidated. Few lectins have been engineered/ mutated, expressed in different bacteria and eukaryotic

cells to determine their function and carbohydrate binding specificity. Genetic engineering has allowed us to use a number of lectins transfected into food crops to provide resistance against insects/pests. In the light of the discoveries and data obtained for lectins, simple classification will be inadequate. Therefore, they can be classified in different ways according to:

(i) Overall structure: Depending upon the number and activity of subunits, plant lectins are classified into 4 distinct groups as tabulated in **Table 1.2**.

Table 1.2 Classification of lectins based on carbohydrate binding domains (CBDs)

Sl.No	Name	Carbohydrate binding domain (CBD)	Features/properties	Examples
1	**Merolectins**	Single CBD	Small protein, incapable of agglutinating the cells.	Hevein: chitin binding protein from the rubber tree latex (Van Parijs *et al.*, 1991), mannose binding proteins from orchid (Van Damme *et al.*, 1998a, Van Damme *et al.*, 1998b) and class I chitinases (Collinge *et al.*, 1993).
2	**Hololectins**	2 or more same CBD	Binds homogenous sugars. Capable of cell agglutinating and/or precipitating glycoconjugates due to multivalency.	Includes majority of plant lectins (Van Damme *et al.*, 1998a)
3	**Chimerolectins**	Fusion proteins: 2 different CBDs are present.	CBD and unrelated catalytic domain, both domain functions independently.	Type 2 ribosome inactivating proteins (RIPs) ricin and abrin. Made up of a toxic A chain with N glycosidase activity and a carbohydrate binding B chain (Barbieri *et al.*, 1993).
4	**Superlectins**	Fusion proteins. 2 CBDs	A type of chimerolectin with 2 structurally different CBDs having specificities to unrelated sugar.	Tulip lectin (TxLCI), with an N-terminal mannose-binding domain tandemly arrayed with an unrelated GalNAc-binding domain (Van Damme, Brike *et al.*, 1996).

(ii) Carbohydrate-specificities:

Depending on the type of sugar interactions, the lectins could be specific either to mannose/glucose, Gal/GalNAc, GlcNAc, sialic acid, (-L-) fucose or complex glycan groups (Van Damme *et al.*, 1998a). Mainly these sugars are present on the eukaryotic cell surfaces. Few lectins exhibit specificity for other sugars as well, for instance, a lectin called human serum amyloid P component (SAP) binds to 4,6-cyclic pyruvate acetal of galactose (Emsley *et al.*, 1994) only found in algal polysaccharides of marine sponges, but absent in bacteria or higher plants.

The lectin affinity for monsaccharides is usually weak ranging in millimolar concentration (Goldstein *et al.*, 1986). These plant lectins are highly specific i.e, galactose specific lectin will not react with glucose or mannose or vice versa. There are other lectins that bind galactose and interact with higher affinity to GalNAc, e.g. soybean agglutinin (SBA), whereas *Erythrina corallodendron* lectin (ECorL) binds both sugars with equal affinity (http://www.uniprot.org/uniprot/P16404). Thus, they are classified into the same specificity group. Occasionally, few lectins also binds to structurally unrelated sugars but having similar topographical appearance, as seen in the case of wheat germ agglutinin (WGA) which binds both GlcNAcand sialic acid.

The monosaccharides specific lectins often exhibit 1000-fold higher affinity for di-, tri-, and tetrasaccharides; also show different affinities for various other oligosaccharides. Considering the lectin function, oligosaccharides act as natural ligands. Due to different shape or freedom of rotation around the glycosidic bonds of individual monosaccharide constituents in the oligosaccharides; different oligosaccharides with similar topology can bind to the same lectin (Lis & Sharon, 1998). For example, in the

Lewis blood group system, out of the 5 phenotypes, Le^a - Le^x and Le^b - Le^y show similarity in their determinant structure, making possible recognition by the same lectin, as shown in **Fig. 1.3.**

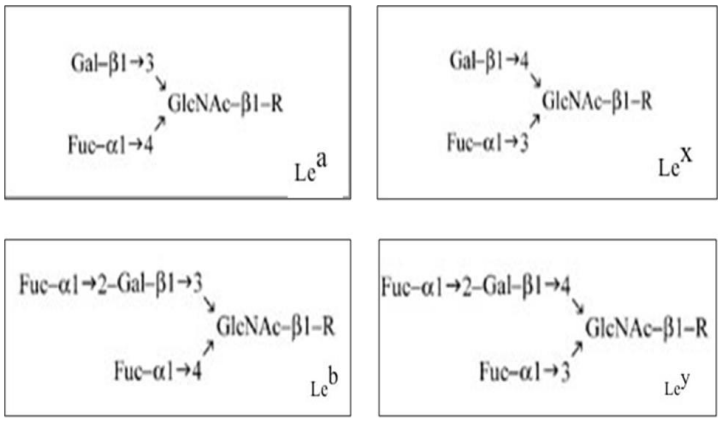


Figure 1.3. Comparison of determinant structure present in the Lewis blood group system. *(Adopted from NCBI: Blood Group Antigen Gene Mutation Database)*

On the other hand, same oligosaccharide can be recognized by different lectins by making integrations at different regions of its surface (as shown in **Fig.1.4**), this leads to conformational heterogeneity since glycosidic linkages are flexible.

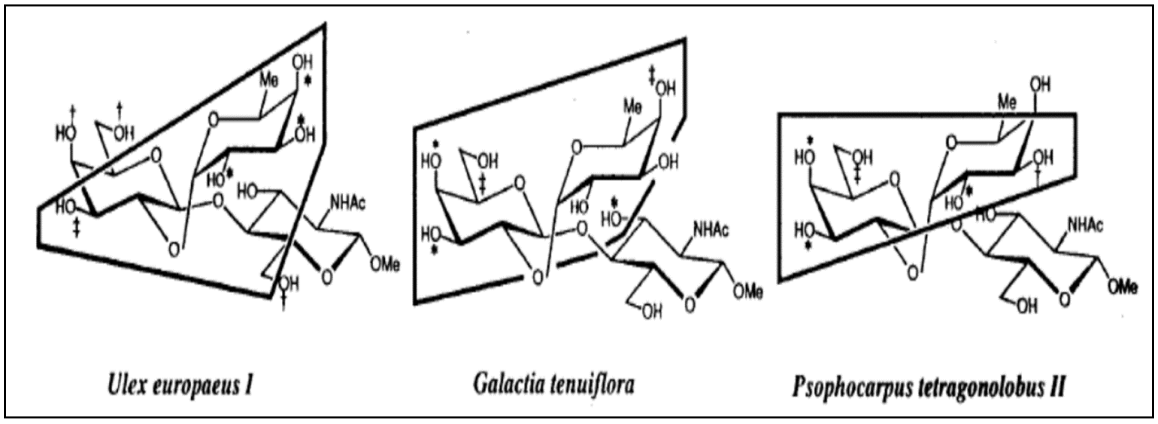


Figure 1.4. Three different lectins are involved in interacting with the eight hydroxyl groups of the H-type trisaccharide Me-β-Fuc(α1-2)Gal(β1-4)GlcNAc in complex formation *(Adopted from (Du et al., 1994).*

(iii) Structural classification and evolution

The lectins are again classified into seven different families based on biochemical, structural and molecular characteristics (Van Damme *et al.*, 1998a). They are grouped into large and small families. The large families include the legume lectins, the chitin-binding lectins containing hevein domains, the type-II ribosome-inactivating proteins (RIPs) and the monocot mannose-binding lectins. On the other hand, the amaranthins, the Cucurbitaceae phloem lectins and the Jacalin-related lectins belong to small families. These families are described in brief.

- **Legume lectins**

The lectins belonging to this family are the best characterized ones. Majority of the legume lectins are isolated and characterized form the mature seeds that account for 10 % of the soluble seed proteins. The first legume lectin to be reported was from *Canavalia ensiformis* (Jackbean) (Edelman *et al.*, 1972, Hardman & Ainsworth, 1972). Soybean seed lectin was the first to be sequenced (Vodkin, 1983). These lectins are dimers or tetramers, among them few are glycosylated like soybean agglutinin and PHA (Van Damme *et al.*, 1998). The metal binding sites important for sugar recognition are conserved in all legume lectins; they mainly bind Mn^{2+} and Ca^{2+}. Legume lectins share the structural "jelly roll motif" commonly found in viral coat proteins. It comprises of curved 7 stranded β-sheets which forms the front face, a flat 6-stranded back sheet and short 5 stranded β-sheets present at the top holding the two larger β-sheets together. These β-sheets are again interconnected by four loops (A, B, C and D) at the top front forming carbohydrate binding region. (Young & Oomen, 1992, Sharma & Surolia, 1997). Loop D serves as the preliminary carbohydrate binding determinant, having 4-7 gaps

resulting in the broader sugar specificity of the legume lectins (Sharma & Surolia, 1997). A few examples of lectins with diverse specificity for sugars reported in legume family are tabulated in **Table 1.3.**

Table 1.3 List of leguminous plant lectins with their sugar specificity and Structure PDB ID

Sl.No.	Legume lectin examples	Specificity	Structure/ PDB ID
1.	Concavalin A	Glucose/mannose	2CNA
2.	*Pisum sativum* lectin		1OFS/2LTN
3.	*Lathyrus ochrus* isolectin I		1LOA
4.	Lentil lectin		2LAL
5.	*Erythrina corrallodendron* lectin (ECorL)	Gal/GalNAc	3N36/1SFY
6.	Peanut agglutinin (PNA)		2DV9
7.	Soybean agglutinin (SBA)		1SBE
8.	Winged bean agglutinins (WBA I and II)		2DTY
9.	*Dolichos biflorus* lectin (DBL)		3UJQ/3UK9
10.	*Ulex europeaus I*	Fucose	1JXN
11.	*Cicer arietinum* Leguminoseae	complex	4OEI/3V6N/3S18

- ***Chitin binding lectins (Hevein domains)***

Next to legume lectins it is the chitin binding lectins that are widespread among the plant families such as Gramineae, Urticaceae, Solanaceae, Papaveraceae, Euphorbiaceae, Phytolaccaceae (Raikhel *et al.*, 1993) and Viscaceae (Peumans *et al.*, 1996). These lectins possess "hevein" domain, named after a small 43 amino acid protein, having high content of cysteine and glycine, isolated from the rubber tree (*Hevea*

brasiliensis) latex (Waljuno *et al.*, 1975). There are other chitin binding lectins, from structurally unrelated families, i.e they do not have hevein domain, reported from Cucurbitaceae families (Van Damme *et al.*, 1998b). Chitin binding lectins also fall under merolectins, hololectins as well as chimerolectins classification, found in seeds as well as vegetative tissues. The 3D structure of the hevein domain contains 2 short stretches of α-helices and three strands of β-sheet. The entire structure is stabilized by 4 disulphide bonds.

These lectins are built up of tandemly arrayed hevein domain repeats among various plant species and do not have any regular secondary structure, for example *Urtica dioica* (Peumans *et al.*, 1984) lectin has 2 hevein domains whereas dimeric Wheat Germ Agglutinin (WGA) and other Graminae lectins possess 4 hevein domains (Raikhel *et al.*, 1993). Wright et al suggested that the repeated chitin-binding domains in a lectin were evolved by gene duplication of an ancestral disulfide-rich domain (Wright *et al.*, 1991). Interestingly, all lectins that belong to this category show specificity towards only GlcNAc and GalNAc–oligomers, conserved during evolution implying importance of their function by binding to chitin- or (GlcNAc)-containing glycoconjugates. Other examples of lectins of this category include, lectin isolated form *Datura stramonium*, *Lycopersicon esculentum, Solanum tuberosum* and *Urtica dioica.*

- ***Type-II ribosome-inactivating proteins (RIPs)***

Ribosome-inactivating proteins (RIP) possess a catalytically active domain that inactivate the ribosomes of host cells by adenine excision from the large rRNA (Barbieri *et al.*, 1993). There are three types of RIPs known so far. Type I RIPs are made up of single chain polypeptide of MW 30 kDa with enzymatic polynucleotide adenosine glycosidase (PAG) activity. Type II RIPs are made up of two separate chains linked via

disulphide bonds. The A chain has similar activity as that of type I RIP and has RNA N-glycosidase activity, whereas B chain has carbohydrate binding domain. The type III RIPs are synthesized as a single chain (30 kDa). Post-translational modifications convert the protein into enzymatically active form with removal of a short internal repeat. Till now, type III RIP has been identified in maize and barley only (Mehta & Boston, 1998). Among these three RIPs, the best studied one is type II RIP found in several plant families like Euphorbiaceae, Fabaceae, Viscaceae, Passifloraceae, Ranunculaceae, Lauraceae, Sambucaceae, Cucurbitaceae and Iridaceae.

The first example studied in detail was monomeric type II RIP ricin, isolated from the seeds of *Ricinus communis* and abrin from the seeds of *Abrus precatorius*. The crystal structure of many type II RIPs including ricin (Rutenber *et al.,* 1991), abrin-A (Tahirov *et al.,* 1995), amaranthin (Transue *et al.,* 1997) and Mistletoe lectin I (Sweeney *et al.,* 1998) have been reported. The tertiary structure of the ricin molecule exhibited β-trefoil fold (Murzin *et al.,* 1992). Similar structure was observed in the case of abrin, a heterodimer of 34 kDa and 32 kDa subunits joined together by single disulphide bridge (Tahirov *et al.,* 1995, Hegde *et al.,* 1991, Wu *et al.,* 2001).

The A chain of ricin has regular secondary structure whereas the B chain has coiled coil structures connected by turns and loops responsible for carbohydrate binding. Four disulphide bonds are present that help in stabilizing the B chain fold. Unlike chitin binding lectins, the type II RIPs differ from each other with respect to their sugar specificities even though they share similar structural fold. For example, ricin and abrin are specific for galactose or GalNAc, whereas, SNA-I shows affinity for NeuAca(2,6)Gal/GalNAc (Van Damme, Barre *et al.,* 1996).

Since the chain A has glycosidase activity, it confers RIPs with defense against different pathogenic organisms. For instance, cinnamomin, a type-II RIP found in the seeds of the camphor tree (*Cinnamomum camphora*) exhibits cytotoxicity against the bollworm (*Helicoverpa armigera*) and the common mosquito (*Culex pipienspallens*) (Zhou *et al.*, 2000). Similarly, when maize RIP ectopically expressed in tobacco plants showed insecticidal activity towards the larvae of the cigarette beetle (*Lasioderma serricorne*) and the corn earworm (*H. zea*) (Dowd *et al.*, 2003).

- ***Monocot mannose binding lectins***

These lectins belong to an extended superfamily of structurally and evolutionarily related proteins which are strictly specific to mannose, exclusively present in monocotyledonous plants. These lectins are isolated and characterized extensively from different monocot families namely, Alliaceae, Amaryllidaceae, Araceae, Bromeliaceae, Liliaceae, and Orchidaceae (Van Damme *et al.*, 1998). Lectins are mainly found in various vegetative tissues like flower, nectar, roots, rhizomes, bulbs, ovaries and leaves (Peumans & Van Damme, 1995), rarely in seeds also. Monocot mannose binding lectins show binding towards only D-mannose but can differ to some degrees in their affinities for oligomannosides; this differentiates them from the Glc/Man/Gal specific lectins from dicotyledonous legume family (Goldstein & Poretz, 1986a) and the C-type mannose binding animal lectins. The first structurally characterized monocot mannose binding lectin was from snowdrop (*Galanthus nivalis*) bulbs belonging to Amaryllidaceae family (Van Damme *et al.*, 1987), showing a β-prism II fold, a characteristic of this family. In β-prism II fold, there are 3 four-stranded β-sheets perpendicular along the three-fold axis which resulted in the formation of a 12-stranded β-barrel structure connected by loops.

Hence, the structure has 3 mannose binding sites formed by the three bundles of β-sheet. Two such monomers aligned to form dimers through hydrogen bond contacts and further associates to form tetramers mainly through hydrophobic interactions. Thus, overall a tetrameric GNA molecule has 12 mannose binding sites.

The other examples of tetrameric mannose binding lectins are from daffodil (*Narcissus pseudonarcissus*) (Sauerborn *et al.,* 1999) and bluebell (*Scilla campanulata*) (Wood *et al.,* 1999). These tetrameric lectins can also recognize mannose containing glycoproteins, this specificity may have originated from the oligomerization of the lectin subunits. Thus, GNA, daffodil and bluebell lectins can recognized and bind to surface glycoproteins present on HIV, Gp 120 with high affinity, as compared to the dimeric garlic lectin which does not recognize Gp 120 (Vijayan & Chandra, 1999).

- ***Amaranthin lectins***

The lectins belonging to amaranthin family was first isolated from the *Amaranthus caudatus* seeds, hence the name was deduced. It is a homodimeric lectin of MW 33 kDa having GalNAc specificity. It also showed specificity towards T-antigen disaccharide, an α- linked glycosides (Rinderle *et al.,* 1989, Rinderle *et al.,* 1990). Subsequently many other lectins were reported in the Amaranthaceae family, for instance, *A. caudatus, A. spinosus, A. leucocarpus* and *A. cruentus. Amaranthus caudatus* lectin was first solved at 2.2 Å resolution (Transue *et al.,* 1997). The monomeric subunit of the lectin consists of *N*- and *C*-domains linked by a short 3_{10} helix. Each of these domains exhibited a β-trefoil structure similar to that found in the B-chains of the type II RIPs ricin and abrin (Rutenber & Robertus, 1991, Tahirov *et al.,* 1995). This β-trefoil fold consists of 6 strands of antiparallel β-sheet capped by three β-hairpins to form β-

barrel. Apart from lectin, this fold is also observed in the soybean trypsin inhibitor (Murzin *et al.,* 1992). The two monomers of the lectin associate in head to tail fashion wherein the N-terminal domain faces the C-terminal domain of the other monomer, forming a dimer molecule. Thus, this dimeric organization provides two exposed surfaces for carbohydrate binding formed at the interface between the N- and C-terminal domains of the two facing monomers (Transue *et al.*, 1997).

- ***Cucurbitaceae phloem lectins***

Cucurbitaceae phloem lectins are a small group of GlcNAc-oligo-specific lectins which are generally localized to the phloem sap of the Cucurbitaceae family. They are structurally unrelated to the previously mentioned chitin binding lectins (having hevein domains) or seed lectins of any Cucurbitaceae plants (majority are type II RIPs). Many such lectins have been identified from the phloem exudates of *C. maxima* (Narahari & Swamy, 2010), *Citrullus*, *Cucumis*, *Sechium*, *Luffa* (Anantharam *et al.*, 1986) and *B. hispida* (Ota *et al.*, 2013). They are dimeric and are non-glcosylated in nature (Bostwick *et al.*, 1992).

Chitin oligosaccharides are the potent inhibitors of these lectins in terms of sugar binding activity while Glucosamine and *N*-acetylglucosamine show weak binding. This inhibitory activity of the sugar increases with increase in carbon chain length as demonstrated in the case of *L. acutangula* (Anantharam *et al.*, 1985). Glycoproteins like fetuin, ovalabumin and mucin can also inhibit these lectins, since Cucurbitacecae lectin bind to the internal di-*N*-acetylchitobiosyl sequences present in the glycoproteins. Since, these lectins are mainly isolated from the phloem exudates of the plant, perhaps having anti parasitic function. If the phloem vessels get injured, these lectins (also called phloem

protein 2; PP2) will react with another abundant phloem protein, PP1, forming a rigid gel which blocks the cut vessels to prevent the microbial infection (Read & Northcote, 1983). There is no crystal structures reported yet from this family of lectins.

- ***Jacalin***

Jacalin was the first lectin to be identified in this family, isolated from the seeds of *Artocarpus integrifolia*, have characteristics β-prism I fold (Sankaranarayanan *et al.*, 1996). Now the lectins which are structurally and evolutionary related to Jacalin are termed as "Jacalin related lectins". This β-prism I fold consists of three Greek-key β-sheets with the strands running parallel to the threefold axis of the prism; this feature is reflected in the sequence of the protein. Like amaranthis lectins, jacalin also show specificity for T-antigen disaccharide (Gal-Pl,3-GalNAc) with high specificity (Kabir & Daar, 1994). The other examples of the lectins included in this family are *Maclura pomifera* (Lee *et al.*, 1998), *Artocarpus hirsuta* (Rao *et al.*, 2004), *Helianthus tuberosus* (Bourne *et al.*, 1999) and *Calystegia sepium* (Bourne *et al.*, 2004). T*hese* β-prism I fold lectins do have similar tertiary structures but varies in their quaternary structures. For instance, Jacalin, MPA and artocarpin are tetrameric in association whereas Heltuba shows octameric; calsepa and banana lectins show dimeric associations. There was another cartegory of lectins having mannose specificity belonging to the jacalin family, isolated from jackfruits (Artocarpin) and *Madura pomifera* agglutinin (MPA) (Rosa *et al.*, 1999). Unlike other Jacalin lectins, these do not undergo proteolysis to form α and β chains. Later, it was also found that, like other lectins these lectins also exhibit insecticidal properties (Van Damme *et al.*, 1998a). For example, MPA and Jacalin could inhibit larval growth of cowpea weevil (*Callosobruchus maculatus*) (Murdock *et al.*,

1990) and southern corn rootworm (*Diabroticaun decimpunctata*)(Czapla and Lang 1990), respectively.

1.6 Biological role of lectins

The reversible sugar binding nature of lectins plays an important role in cell recognition. This concept of cell recognition is solely based when a complementary structure fit to each other at the molecular level between the interacting partners. In other words, one molecule is carrying the encoded biological information and the other molecule is capable of deciphering the code, like an enzyme-substrate interactions.

I) Role of Plant lectins

As discussed before, plant lectins are present in abundance in different tissues and storage organs, but their exact biological role or *in vivo* functions are not yet defined. Through various findings, the wide spectrums of roles were implicated for plant lectins. For example they are involved in seed maturation or germination (Howard *et al.*, 1972), cell wall extension (Kauss & Glaser, 1974), carbohydrate transport (Boyd, 1963), growth regulation (Howard *et al.*, 1972) as lectins have adenine binding sites as well (Roberts & Goldstein, 1983). Few lectins act as mitogens for lymphocytes (Goldstein & Hayes, 1978), although they have no mitogenic effect on self cells from which they have been isolated.

Since the whole plant is continually exposed to surrounding pathogens and pests, some vital tissues or storage organs with these defensive proteins could provide protection to the plant. Majority of plant lectins could maintain their structural and functional integrity under unfavourable conditions, since they are stable over a wide pH range, extreme temperatures and are resistant to animal/insects proteases.

The following are the available evidences enlisting the important functions of lectins.

(i) Forms symbiotic relationships between rhizobia of legume plants and nitrogen fixing bacteria:

This symbiosis plays a mandatory role in carrying out the nitrogen fixation in both nitrogen cycle of both terrestrial and agriculture. Here, the main species-to-species interaction is facilitated by small fatty acylated and sulfated tetrasaccharides produced by the *Rhizobium* and *Bradyrhizobium* species (Lerouge *et al.*, 1990). The function of lectins over here is to specifically agglutinate the bacteria at the root hair surface making them immobile. Thus, the soyabean nodulating rhizobia cannot nodulate garden peas or white clover. This implied that these symbionts are specific for their interacting species. For example, when pea lectin gene was expressed in white clover roots, it enabled nodulation by rhizobium strain which is supposed to be specific to pea plant (van Eijsden *et al.*, 1992). This theory was named as "lectin recognition hypothesis" (Kijne, 1996, Hirsch, 1999). However, there was no direct evidence for the presence of lectins and sugar binding receptor on the bacteria at the expected location and time when rhizobium infects. Moreover, the connection between the host lectin sugar specificity and its potential to recognize own nodulating bacteria was not found strict (Lis & Sharon, 1998).

(ii) Protection from predators and pathogens:

Plant lectins induces toxicity towards insects and higher animals, also acts as fungal growth inhibitors; playing as defence proteins (Peumans & Van Damme, 1995, Pusztai & Bardocz, 1996). Ricin and abrin are type II RIPs which are toxic in nature. These toxins were recognized as defence proteins against herbivorous animal and phytophagous invertebrates. Toxic lectins isolated from *Phaselous vulgaris, Robinia*

pseudocacia and *Sambuscus nigra* have been reported to cause severe toxicity in higher animals (Peumans & Van Damme, 1995). Similarly WGA and GNA are toxic to the rice plant pests, but Solanaceae lectins had no effect on them (Gatehouse *et al.*, 1995). WGA also inhibits *Trichoderma viride* by binding to chitin and interfering with its growth (Mirelman *et al.*, 1975).

Similarly, majority of plant lectins possessed anti-insecticidal activity also, for instance, peanut agglutinin (PNA), WGA, snowdrop lectin, garlic lectin and Solanaceae lectins kill cowpea weevil and tobacco hornworm (Hilder *et al.*, 1995). It has been demonstrated that GNA could pass through the midgut epithelium entering into the haemolymph of the rice brown plant hopper, thus exerting its toxic effects systemically (Gatehouse *et al.*, 1998). The exact anti-insecticidal mechanism is still unknown, but many reports suggest the lectin toxicity is due to binding to gut lining glycoconjugates, like carbohydrates present on the peritrophic membrane (Harper *et al.*, 1998), epithelial cells of the digestive tract (Pusztai & Bardocz, 1996) or glycosylated digestive enzymes.

The digestive tract in animals provides wide spectrum glycoconjugates presenting a stack of lectin targets. The mechanism of lectin causing toxicity in animals is well understood unlike with insects. For example, *P. vulgaris* agglutinin is highly resistant to hydrolytic enzymes present in the gut. Once PHA enters the system, it binds to glycan moiety present on the small intestine surfaces and gets endocytosed. This leads to severe distress in animals causing hyperplasia and hypertrophy in small intestine (Pusztai *et al.*, 1990). Since many lectin-gut interactions show deleterious effects on consumption by animals, many of these lectins are not incorporated into transgenic crops.

II) Role of Animal lectins

Many researchers have demonstrated a variety of functions for animal lectins. Based on similarity of carbohydrate recognition domain animal lectins have been classified into: C-type lectins (CTLs), galectins, I-type lectins, pentraxins, P-type lectins, tachylectins etc (Gabius, 1997).

a) Galectins

They have affinity for β-galactoside sugar independent of Ca^{2+} binding, found in the cell cytoplasm and nucleoplasm of various cells. Galectins are directly involved in biological process such as development, differentiation, immunity, apoptosis, metastasis of malignant cell as well as extracellular matrix recognition (Leffler, 2001, Leffler *et al.*, 2004). The galectin expression is highly regulated at different stages in the development of physiological conditions. For instance, the expression of galectin-1 is high at early stages of embryo development (Poirier & Robertson, 1993) and that of galectin-3 is high in murine and human cancer cells required for host cell adhesion (Akahani *et al.*, 1997). Several other galectins were identified and characterized from fishes, like Japanese eel *Anguilla japonica* (Tasumi *et al.*, 2002), electric eel *Electrophorus electricus* (Levi & Teichberg, 1981), windowpane flounder *Lophopsetta maculate* (Kamiya & Shimizu, 1980) and zebrafish *Danio rerio* (Ahmed *et al.*, 2004).

b) C-Type Lectins

These lectins have requirement of calcium ions for sugar binding, hence designated C-type lectins. They have wide range of functions including eliciting the

immune response, cell adhesion and apoptosis. Their further classification is shown in **Table 1.4.**

Table 1.4 Classification and functions of C-type animal lectins

Sl.No	Types	Cell types	Specificity	Functions	References
1.	*Endocytic Lectins*	Mammalian hepatic asialoglycoprotein receptor	Gal/N-acetyl galactosmine	Serum glycoprotein clearance	(Ashwell & Harford, 1982)
		Macrophages	Mannose	Antimicrobial defense, lectinophagocytosis Innate immunity	(Drickamer & Taylor, 1993, Ezekowitz *et al.*, 1990, Ofek *et al.*, 1995)
2.	Collectins	Mammalian serum and liver cells	Oligo-Mannosides	Activates complement pathway on microbe recognition and its subsequent lysis.	(Drickamer & Taylor, 1993).(Epstein *et al.*, 1996, Holmskov *et al.*, 1994)
3	Selectins L-selectins	Leukocytes		Recirculation of Leukocytes and its trafficking to inflammation sites	(Rosen & Bertozzi, 1994, Lasky, 1995, Springer, 1995, McEver *et al.*, 1995, Nelson *et al.*, 1995)

c) P-Type Lectins

This includes receptors present on lysosomes to bind mannose-6- phosphates targeted lysosomal enzymes (Kornfeld, 1992, Ludwig *et al.*, 1995, Munier-Lehmann *et*

al., 1996). Any mutation in this receptor leads to I-cell disease, an inherited lysosomal storage disease, resulting in accumulation of undigested glycoconjugates (Neufeld, 1991).

d) I-Type Lectins

These mammalian lectins belong to immunoglobulin superfamily. I-type lectins, for example, sialoadhesin present on macrophages, CD22 on B cells and CD33 are present on early myeloid cells. They are sialic acid specific lectins and have been implicated in intercellular adhesion, recognizing ligands on activated lymphocytes as an immune response (Lis & Sharon, 1998).

e) *Other Lectins*

Based on sequence homologies, calcium binding proteins were found to have lectin like activity; for example, calnexin and calreticulin are non-glycosylated membrane and soluble protein, respectively, responsible for proper folding of glycoprotein, thus acting as chaperone. The other example is MR60/ERGIC-53, a mannose-specific lectin that shuttles newly synthesized glycoproteins between Golgi apparatus and endoplasmic reticulum.

III) Role of Microbial lectins

The major function of the microbial lectins is to first adhere to the host cells and then cause infections. Sometimes, these lectins are also called *adhesins*. For example, *E. coli* K99 can recognize and bind to glycolipids containing *N*-glycolylneuraminic acid present on intestinal cells of newborn piglets and disappears when the animal grows. This also explains why *E. coli* K99 can cause lethal diarrhea in small piglets (Lis & Sharon, 1998). Once the sugar specificity is known, the bacterial infection can be prevented by incubating the adhesins with suitable sugars (Ofek *et al.*, 1996, Zopf & Roth, 1996).

Thus, oral administration of mannose and methyl R-mannoside inhibited the infection caused by type 1 fimbriated *E. coli* and *K. pneumonia strains,* respectively. Similar case was also reported in case of human clinical trials; patients with otitis externa (caused by *P. aeruginosa*) were treated with mixture of sugar solution (includes mannose, galactose and sialic acids), obtained better results as compared to local antibiotic treatment (Lis & Sharon, 1998). Hence, carbohydrates have great potential for anti-adhesion therapy against microbial infections.

Bacterial adhesion proteins can also attach to human polymorphonuclear cells or macrophages followed by their ingestion and killing of bacteria, a process termed as lectinophagocytosis (Ofek *et al.*, 1995). It refers to a process where the lectins present on the defensive cells binds to the carbohydrate moiety of the infectious agent.

1.7 Applications of lectins

As discussed before, the amount of lectin present in different organisms varies. Apart from exploiting their antimicrobial, anti parasitic, anti-tumor and anti-viral activities, it can also be employed for other applications as follows:

(1) ***Blood typing***: it is one of the oldest and the unique applications of plant lectins displaying blood group selectivity. For example, the lectin from *Vicia cracca*, *Dolichus biflorus* and *Griffonia simplicifolia* are shown to act as good anti-A, anti-A1 and anti-B, respectively. For identification of blood type O cells, fucose specific lectins from *L. tetragonolobus* and *U. europaeus* are employed. *Vicia graminea* lectin was found to be selective for blood type N from the mixture of M and N type cells. The cryptantigens

present on erythrocytes surfaces are more sensitive to lectin agglutination than by polyclonal antibodies, such lectins include Peanut agglutinin (PNA), Polybrene, and Glycine max lectin (Khan *et al.*, 2002). Many other lectins have been identified and investigated for immunohemotology, summarized in **Table 1.5.** These lectins were also employed for the studies of red blood cell topography (Judd, 1980).

Table 1.5 List of different lectins having different blood antigen specificity

Sl.No.	**Lectins**	**Carbohydrate specificity for blood group cells**
1.	*Griffonia simplicifolia* (GS-I)/ *Sophora japonica* (SJA)	Anti-B
2.	*Griffonia simplicifolia* (GS-II)/ *Salvia sclarea* (SSA)	Anti-Tk/ Anti-Tn
3.	*Helix aspersa* (HAA)/ *Helix pomatia* (HPA)/ *Vicia villosa* (VVA)/ *Phaseolus lunatus* (LBA)	Anti-A
4.	*Iberis amara* (IAA)	Anti-M (M+N)
5.	*Lotus tetragonolobus* (Lotus)/ *Ulex europaeus* (UEA-I)	Anti-O(H)
6.	*Vicia graminea* (VGA)	Anti-N

***(2) Analysis and purification of glycoconjugates* :** Lectins are used to separate glycans or glycoproteins from their mixtures. Glycoproteins consisting of mannose, galactose and N-acetylneuraminic acid are purified using lectins like, Con A, PNA and WGA, respectively, in many laboratories (Liener *et al.*, 1986). Similarly, Jacalin binds to many human plasma glycoproteins like IgA, IgD, C4 binding gp120, plasminogen, hemopexin, α lantitrypsin, 8S α3-glycoprotein and α-2-macroglobulin (Hagiwara *et al.*, 1988, Kondoh *et al.*, 1986, Aucouturier *et al.*, 1987, Hortin & Trimpe, 1990, To *et al.*, 1995).

(3) Histochemical and cytochemical probes: Lectins can be used as developmental markers during which the cell surface carbohydrates undergoes considerable changes (Gabius, 1991). At laboratory level, peanut agglutinin, soybean agglutinin and jacalin are used for cell identification and separation. For instance, PNA can differentiate between mature and immature thymocytes for bone marrow transplantations (Reisner, 1987). On the other hand, PNA is widely exploited for differentiating between normal and malignant cells, due to its high specificity for Thomson-Friedenreich antigen (T-antigen (O'Keefe & Ashman, 1982, Zabel *et al.*, 1983, Ching & Rhodes, 1989, Zebda *et al.*, 1994). Similarly, Jacalin has been used to differentiate and study benign and malignant lesions present in the breast and thyroid (Remani *et al.*, 1989, Vijayakumar *et al.*, 1992). The binding affinity of jacalin increases with advancement in the tumor progression. Many researchers have derivatized the lectins either with gold nanoparticles, fluorescent dyes or enzymes, employed as histochemical and cytochemical reagents for glycoconjugates detection present in cells, tissues and subcellular organelles (Lis & Sharon, 1998, Bies *et al.*, 2004).

(4) Mitogenic stimulation of lymphocytes: Certain lectins also act as potent mitogens, inducing lymphocytes to divide. For instance, Con A stimulates T lymphocytes and pokeweed lectin activates both T and B cells (Di Sabato *et al.*, 1987, Ashraf & Khan, 2003). This property of the lectins enables to assess the immunocompetence of patients suffering from diseases like AIDS. Jacalin is known to stimulate human CD4+ T cells selectively and hence, used in AIDS research (Pineau *et al.*, 1989, Corbeau *et al.*, 1995, Favero *et al.*, 1993). Few lectins like *Canavalia brasiliensis* (ConBr), *Pisum arvens* (PAA+) and *Artocarpus integrifolia* (KM+) are also used in vaccination against

Leishmania amazonensis infection where these lectins induce the expression of IFγ resulting in the increased expression of MHC II, CD80, and CD86 reducing the level of parasite (Teixeira *et al.*, 2006).

(5) Lectin mediated drug targeting and delivery: The main biological barriers in the drug targeting and delivery is the actual site of drug action and drug delivery, which are the main factors responsible for the development of effective and safe medicines (Lehr & Gabor, 2004, Bies *et al.*, 2004). The concept of employing lectin for delivery of various drugs was first demonstrated by tomato lectin (TL), which was used to target the small intestinal luminal surface. Later, the lectin-saccharide interactions were further explored for other biological barriers like the nasal mucosa lining, the buccal cavity and the blood brain barrier (Bies *et al.*, 2004). Lectins like Con A, PNA, Jacalin and WGA are known to mediate mucocoadhesion, cytoadhesion and cytoinvasion of drugs (Yi *et al.*, 2001). Lectin conjugated drugs enters the vesicular pathway via receptor mediated endocytosis where the drug gets accumulated within the lysosomes. Due to poor bioavailability of the drugs, its conjugation with lectin enhances the drug delivery to the epithelial cells. The lectins from mistletoe (ML I-III), stinging nettle (UDA) and tomato (TL, LEA) are used for targeting human intestinal eptithelial Caco-2 cells. WGA has demonstrated its higher affinity towards cerebral endothelium cells; it could cross the blood-brain barrier, without disturbing the brain function (Bies *et al.*, 2004, Gao *et al.*, 2006).

(6) Mapping of neuronal pathways: Another major area where application of lectins has been exploited is neuroanatomy, where lectins are used as tracer molecules for mapping neuronal connections. *P. vulgaris* leuco-agglutinin used to trace anterograde axonal tract showed the projections of the substantia nigra and the paraventricular hypothalamic

nucleus (Gerfen & Sawchenko, 1985). WGA is transported in both ways; anterogradely as well as retrogradely. Here, first the lectin is conjugated with enzymes, like horse radish peroxidase (lectin-HRP complex), which is readily taken up by the neurons and distributed throughout the axon thus helping us to locate or trace the central neuronal pathways. The lectin bound to the cell surface receptor is taken up by the cells via endocytosis via caveolae-dependent pathway (Broadwell & Balin, 1985, Gao *et al.*, 2008). If types II RIPs are used, then it functions as suicide transporter, providing a new strategy to treat neurobiological disorders.

(7) Laboratory applications: Similar to antibody actions, lectins can be used conjugated with fluorophore, chromophore, enzyme or by secondary antibody. Examples of such techniques are known as enzyme-linked lectin assay (ELLA) (Ching & Rhodes, 1989), lectin microarrays (Hirabayashi *et al.*, 2013) or lectin staining/blotting. In ELLA, only specific carbohydrates are detected and quantified which might be present on the surface of the unfixed cells (McCoy *et al.*, 1984).

Lectin microarrays are use to analyze certain glycoproteins with complex carbohydrates moiety attached. In this case, the panel of fluorescent tagged-lectins is immobilized on a single chip. The specific carbohydrate gets bind to the chip, giving information on carbohydrate composition of the sample, even if the glycosylation of samples is less (Pilobello & Mahal, 2007). Recently, the lectin microarray has been used in distinguishing infected mammalian cells with the intracellular apicomplexan parasite *Cryptosporidium parvum*. In this case, soybean lectin with N-acetyl-d-galactosamine binding specificity gave the largest signal difference due to the over-expression of glycoprotein on the surface of infected cells (Yang *et al.*, 2010). In order to study,

carbohydrate modifications occurred on proteins, a modified version of western/immune blotting approach was used called as lectin blotting method. Where different lectins were labeled and used for sugar detection present on the blot in glycoconjugate mixtures separated by gel electrophoresis and transferred to nitrocellulose membrane similar to western blot techniques (Turner, 1992, Youings *et al.*, 1996, Krzeslak *et al.*, 2003, Kotani *et al.*, 2004).

Proteomic glycome profilling

In this technique, proteomic quantification is done with the help of plant lectins using chromatographic or electrophoretic strategies in combination with mass spectrometry (LC-MS/MS, MALDI-TOF MS or MALDI-TOF/TOF MS). The workflow is shown in **Fig. 1.5** which often includes combination of lectin affinity chromatography (LAC), trypsin digestion, ion-pairing hydrophilic interaction liquid chromatography followed by MS/MS analysis characterizing N-linked glycosylation sites (Harvey, 2011, Nasi *et al.*, 2009). This proteomic approach has been used in identifying the biomarkers involved in aberrant protein glycosylation observed in cancer cells.

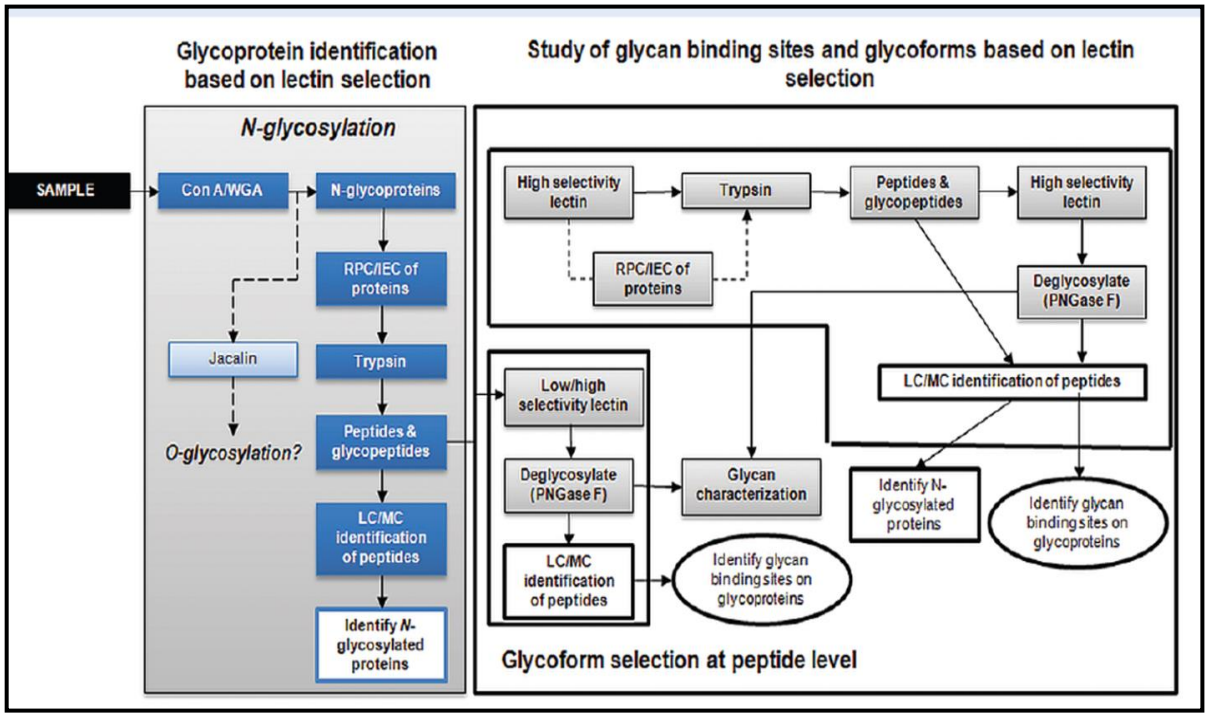


Figure 1.5 Glycome profiling using lectin affinity chromatography *(Adopted from (Regnier et al., 2007)*

(8) <u>Lectins as anti-bacterial agents</u>: There are three main reasons to find strong alternative antimicrobial agents present in the natural sources. Firstly, the synthetic drugs available in the market are of great concerns due to their high toxicity and side effects (Hafidh *et al.*, 2009). The second reason is that many of the existing antimicrobials are resistant to the pathogens. Lastly, with the advent in the biotechnological knowledge, it is possible to synthesize natural substance available by carrying out the chemical reactions imitating its mechanism. In the last 15 years, a large number of antimicrobial proteins (AMPs) have been identified in different parts of the plants (Broekaert *et al.*, 1997). These AMPs constitute a heterogenous class of proteins playing important role in defense system, like thionins, lipid transfer proteins, plant defensins, chitinases, glucanases, 2S albumins, ribosome inactivating proteins and lectin (Ye *et al.*, 2002, Zhang & Halaweish,

2003). Lectins with different carbohydrate specificities, not present on bacterial cell surfaces show growth inhibition and cytotoxic effects on micro organisms. These deleterious effects due to lectin interaction with bacterial cell wall components like teichoic and teichuronic acids, peptidoglycans and lipopolysaccharides can be effectively utilized. For instance, *Lathyrus ochrus* seeds isolectin I can bind to muramic acid and muramyl dipeptide by forming a network of hydrogen bonds between hydroxyl oxygen atoms of sugar and lectin-sacchharide binding site (Bourne *et al.*, 1994). Lectins like Con A can precipitate bacterial polysaccharides, thus used for detection of *E.coli, Shigella and Salmonela* strains (Goldstein & Staub, 1970). Many lectins have been reported to possess anti microbial activity, few are discussed here. For instance, *Curcuma amarissima lectin* could inhibit *Bacillus subtilis, Candida albicans, Escherichia coli*, and *Staphylococcus aureus* at concentration ≥ 0.446, 0.446, 0.223, and 0.892 mg/ml, respectively. No inhibitory action against *Pseudomonas auroginasa* was observed in the absence of polysaccharide ligands for *Curcuma amarissima* lectin (Kheeree *et al.*, 2010). Apart from plant lectins, animal lectins also show inhibitory activity, for example, galactose binding lectin isolated from the skin of amphibian, *Bufo arenarum*, showed anti-microbial activity against Gram negative bacteria (*E. coli* K12 4100 and wild strains of *E. coli* and *P. morganii*) and Gram positive bacteria (*Enterococcus faecalis*) (Sanchez Riera *et al.*, 2003). Examples of lectins possessing anti bacterial activity are summarized in **Table 1.6.**

Table.1.6 Examples of antibacterial lectin

Sl.No	Lectin source	Source	Effective against	Sugar specificty	References
1.	*Eugenia uniflora*	Seeds	*S aureus*, *P aeruginosa* and *Klebsiella* sp.	Complex sugars and glycoproteins	(Oliveira *et al.*, 2008)
2.	*Phthirusa pyrifolia*	leaf	*K. pneumoniae*, *S epidermidis*, *Streptococcus Faecalis and B subtilis*	fructose-1-6-biphosphate	(Costa *et al.*, 2010)
3.	*Araucaria angustifolia*	Seeds	*Clavibacter michiganensis*	GlcNAc	(Santi-Gadelha *et al.*, 2006)
4.	*Solanum tuberosum*	Seeds, stem, leaves	*E. coli* O157:H18, *Listeria monocytogenes*, *S. enteridis*, and *S. boydii*	GlcNAc oligomers	(Hasan *et al.*, 2014)
5.	*Moringa oleifera*	Seeds	*E. coli* and *S. aureus*	Complex	(Ferreira *et al.*, 2011).

Anti-biofilm activity

Biofilms are communities formed by microorganisms by getting attached to the surface and playing important role in the persistence of bacterial infections. Inside the biofilm, the pathogenic bacteria are several orders of magnitude resistant to antibiotics. Hence, this is the major cause for the reduction in the effectiveness of drug. During biofilm formation, bacteria start integrating the information from the immediate surroundings, such as about nutrients availability, quorum sensing, autoinducers, biofilm-related gene expression etc. In 1977, *P. aeruginosa* was found in the sputum of the cystic fibrosis patients, implying the role of sessile bacteria in causing infections (Hoiby *et al.*, 1977). In 1978, the term "biofilm" was formally introduced by Costerton (Costerton *et al.*, 1978).

It was found that the biofilms formed by ESKAPE (*E. faecalis, S. aureus*, *K. pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter* spp.) caused high mortality rate. The other major bacteria are *S. sobrinus* and *S. mutans* causing dental caries. Furthermore, biofilms are also found to be formed on many medical implants such as catheters, artificial hips, and contact lenses, and are resistant to antimicrobial agents. Due to their increased resistance towards biocides and antibiotics, it complicates the management of device-associated infections and thus limits its applications in therapeutic options (Blackledge *et al.*, 2013, Nett, 2014). Recently, lectins are found to be active agents against bacteria and fungi in both planktonic cells and biofilms (Paiva *et al.*, 2010, Klafke *et al.*, 2013). For both cases, inhibiting the interaction between lectin and host glycan is the necessity. This phenomenon is also known as anti adhesion therapy; can be carried out by the application of interfering drug (saccharide or glycomimetics) that would bind to the lectin stronger than the host-origin ligand. This will make the pathogen more vulnerable to treatment or can be even removed from the body through natural cleansing mechanisms, e.g. air flow in lungs, urine flow or immunity system attack (Ofek *et al.*, 2003). An elegant possibility for medicine is using lectin or sometimes lectin inhibitors as a weapon. For example, lectins LecA and LecB found in *P. aeruginosa* which are fucose-specific and galactose-specific, respectively, helps in cell adhesion and lung infections. These lectins can be blocked by using competitive inhibitors (fucose and galactose moieties, respectively). Use of these lectin inhibitors along with antibiotics is reported to facilitate biofilm dissolution or prevent biofilm formation (Diggle *et al.*, 2006, Kadam *et al.*, 2011, Johansson *et al.*, 2008). On the other hand, chitin binding lectin purified from the *S. tuberosum* prevented biofilm formation by *P. aeruginosa* (Hasan *et*

al., 2014). One study has evaluated the anti biofilm activity of various lectins like Con A, WGA, PNA, PHA, PSA, CCL (*Cajanus cajan* lectin), these lectins have inhibited the *Streptococcus* mutans biofilm formation on saliva-coated surface (Islam *et al.*, 2009). C-type lectin isolated from the snake venom, of *Bothrops jararacussu*, also disrupted 75 % and 80 % of *S. aureus* and *S. epidermidis* biofilm formation, respectively, without affecting bacterial cell viability (Klein *et al.*, 2015).

(9) Lectins as antifungal agents: Despite a large number of lectins have been identified and characterized; only few of them are manifested with potent anti fungal property. Lectins show its antifungal activity by binding to the hyphas which causes poor nutrient absorption and interferes with spore germination process (Lis & Sharon, 1981). The main constituent of fungal cell wall is chitin, and thus the chitin binding lectins mainly show antifungal activity by disturbing the synthesis/deposition of chitin in the cell wall (Selitrennikoff, 2001). As already discussed plant lectins play a major role in defense mechanism against phytopathogens. The detailed mechanism by which lectin inhibits fungal growth is not yet clear but they might be involved in modifying the fungal membrane structure or permeability. The only plant lectins that can be considered as true fungicidal proteins belongs to the chimerolectins i.e, class I chitinases. However, the main antifungal activity of these proteins is ascribed to their catalytic domain.

(10) Lectins as antiviral drugs: Anti viral lectins acts by blocking the entry of the virus into host target cells and halt virus transmission from virus-infected cells to non-infected cells. *Aspidistra elatior* lectin (AEL) purified from the rhizomes showed MIC of 4 μg/mL against the vesicular stomatitis virus, Coxsackie virus B4, and respiratory syncytial virus (Xu *et al.*, 2015). A mannose specific lectin isolated from *Gerardia savaglia* was

reported for the first time to prevent infection of human immunodeficiency virus (HIV)-1 in H9 cells. Furthermore, the same lectin could inhibit syncytium formation in the HTLV-IIIB/H9-Jurkat cell system and HIV-1/human lymphocyte system by binding to HIV-1 gp120 envelop molecule (Muller *et al.*, 1988). Later, Con A, WGA, *Lens culinaris* agglutinin, *V. faba* agglutinin and *P. sativum* agglutinin were also reported to bind gp120, and thus preventing fusion of HIV-infected cells with CD4 cells (Hansen *et al.*, 1989). Similarly, the *Musa acuminata* (banana) lectin was shown to inhibit HIV replication by directly binding to the HIV-1 envelope protein (gp120), thus blocking the entry of the virus into the host cells (Swanson *et al.*, 2010). Few lectins were reported to inhibit HIV-1 reverse transcriptase activity, for example, extra long autumn purple bean lectin (Fang *et al.*, 2010) and mushroom *Russula delica* lectin (Zhao *et al.*, 2009). Hence, lectins are future potential candidates for the treatment of AIDS.

(11) Lectins in crop-protection:

Lectins have become the most promising tool for insect/ pests management and have been genetically engineered successfully into a variety of crops like wheat, rice, tobacco, and potatoes. Moreover, the implementation of transgenic insecticidal and herbicide-tolerant plants does not cause any negative effects on the ecosystem as well (Velkov *et al.*, 2005). For instance, transgenic tobacco plants expressing *Allium sativum* lectins reduced the weight gain and development and the metamorphosis of *Spodoptera littoralis* larvae when fed on its leaves. When lectin is ingested by the insects, it interacts with the glycoproteins present on the gut lining causing permeability disruption, thus killing the insects. Galectin-1 treatment of *Plutella xylostella* larvae caused microvilli

disruption and induced abnormalities in these epithelial cells (Chen *et al.*, 2009). At larval stage, lectin can also delay the development of the insects. For instance, when *Arisaema jacquemontii* lectin was incorporated into the artificial diet, it adversely affected the development of *Bactrocera cucurbitae* larvae (Kaur *et al.*, 2006). Similar case was observed for *Arisaema helleborifolium* lectin exhibiting anti-insect activity towards the second instar larvae of *B. cucurbitae* (Kaur *et al.*, 2009). The *Helicoverpa armigera* and *Aphis* craccivora larvae are inhibited from developing into adults by avidly binding to larval brush border and peritrophic membrane by *Dioscorea batatas* lectin (Ohizumi *et al.*, 2009) and *Arum maculatum* tuber lectin (Majumder *et al.*, 2005), respectively.

1.8 Other potential applications of lectins

a) Lectins with Pro-healing activity

Recently, lectins with immunomodulating effect have known to show some peculiar pro-healing activity. The exact mechanism has not been elucidated yet, but interesting observations were seen. For instance, the lectin isolated from *Bauhinia variegata* seeds (BVL) was employed in the topical surface of the surgical-induced wound in a murine model, lectins behaved as potential pro healing molecules. This might be due to mitogenic stimulation of nearby cells, activating them to release cytokines and recruit neutrophils (Neto *et al.*, 2011). In other studies, the same lectin was responsible for stimulating differentiation of fibroblasts into myofibroblast in order to remodel the underlying connective tissue (Li & Wang, 2011). Similar healing observations were also made by Cramoll 1,4 lectin in healthy and immunocompromised mice (de Melo *et al.*, 2011).

b) Plant lectins are novel Toll-like receptor agonists

Very recently it is known that certain plant lectins exhibit TLR stimulation, this implied that the innate immune system can detect and respond to specific lectins. These observations were shown by SBA, Con A and PHA lectin which stimulated the extracellular TLRs (2/6, 4 and 5). More precisely, SBA only stimulated TLR4, Con A, TLR2/6 whereas PHA-L stimulated all TLR2/6, 4 and 5. This might be due to difference in the sugar specificity (Unitt & Hornigold, 2011).

c) Lectins as antitumor drugs

Lectin can have wide applications in biomedical sciences including cancer research. From the past few decades, it is well known that lectins have an anti-neoplastic property, also representing novel nontraditional source of anticancer compounds. It also helps in understanding the various aspects of cancer and its mode of spreading, i.e metastasis. Since cancer is still a major worldwide health issue, with routine changes in lifestyle including the genetic and environmental risk factors adding to the high risk of developing cancer. Hence, a never ending demand is there for new cancer drugs. Many of the ethnomedicinal plants are rich source of bioactive compounds which are known to possess anticancer properties (Amri, 2014). In the current decade, many reports have shown that these lectins are very dynamically contributing as cancer cell biomarker, helps in cell adhesion and localization plays important role in signal transduction, acts as mitogens, cytotoxic and apoptotic agents. Among the legume lectins, the widely characterized one, Con A was reported to induce mitochondrial membrane disruption and cytochrome c release leading to apoptosis and autophagy through a mitochondria-

dependent signaling pathway in cancer cells (Li *et al.*, 2010). Some of the other lectins include *S. flavescens* lectin (SFL) inducing apoptosis via death-receptor pathway(Liu *et al.*, 2008a). Glycine max lectin showed anti proliferative activity against breast cancer MCF7 cells and hepatoma HepG2 cell (Lin *et al.*, 2008). The **Table 1.7** summarizes the list of other lectin with anti neoplastic activities.

Table 1.7 List of lectins possessing anti neoplastic activity

Sl.No.	Lectin Source	Cell lines	References
1.	*Viscum album L. coloratum*	B16-BL6 melanoma cells/ Human A253 cancer cells	(Park *et al.*, 2001, Choi *et al.*, 2004)
2.	*Inocybe umbrinella*	HeLa cells	(Zhao *et al.*, 2009)
3.	*Abrus precatorius*	Dalton's lymphoma cells	(Bhutia, Mallick, Maiti *et al.*, 2008)
4.	*Sophora flavescens*	HeLa cells	(Liu *et al.*, 2008b)
5.	*Polygonatum odoratum*	Murine fibro sarcoma L929 cells	(Liu, Zhang *et al.*, 2009)
6.	*Pseudomonas aeruginosa*	Breast cancer cells (MDA-MB-468, and MDA-MB-231HM cells)	(Liu, Hou *et al.*, 2009)
7.	*Phaseolus vulgaris*	Breast cancer MCF-7 cells	(Lam & Ng, 2010)
8.	*Pisum sativum*	Ehrlich ascites carcinoma cells in vivo mice	(Kabir *et al.*, 2013)
9.	*Triticum aestivum*	Pancreatic cells	(Schwarz *et al.*, 1999)
10.	*Artocarpus integrifolia*	Colon cancer	(Yu *et al.*, 2001)

Although the induction of apoptotic pathways by lectins looks different, in all the cases the caspases activation is definitely involved. The lectin induced apoptosis can either caspase-8 dependent (extrinsic pathway) or -9 dependent (intrinsic pathway) but

both leads to final activation of caspase -3. In few cases, both caspase-8 and -9 get activated (Kobayashi *et al.*, 2010, Liu, Zhang *et al.*, 2009). In case of caspase-8 dependent, apoptosis is mediated by lectin interactions with death receptors like Fas receptor (Lam & Ng, 2010). Once the fate of the cell has been decided to be apoptotic, the anti apoptotic factors belonging to the Bcl family gets down-regulated (Bhutia, Mallick, Maiti *et al.*, 2008, Bhutia, Mallick, Stevens *et al.*, 2008, Lam & Ng, 2010). The next step in the cascade is the disruption of the mitochondrial membrane releasing cytochrome c and calcium ions into the cell cytoplasm. Lectins induced anti tumor effect were also observed *in vivo* conditions as well. For instance, *Pleurotus citrinopileatus* lectin (Li *et al.*, 2008) and *R. lepida* lectin (Zhang *et al.*, 2010) when administered intraperitoneally showed potent antitumor activity in white Kunming mice bearing sarcoma 180. Few lectins like Con A and Con Br showed remarkable anti tumor activity by inducing intrinsic apoptotic pathway and producing high levels of ROS (Reactive Oxygen Species) (Faheina-Martins *et al.*, 2011). To increase its efficacy in cancer therapy, the suitable delivery system is required to enhance its bioavailability and to reduce its toxicity. For instance, a toxic lectin from *Cratylia mollis* was encapsulated with liposomes showing improved antitumor activity in Swiss mice having sarcoma 180, with no toxicity caused to liver and kidney (Andrade *et al.*, 2004). Later, mistletoe lectin was encapsulated with biodegradable polymer of alginate/chitosan in order to protect the lectin getting degraded in the acidic pH present in the stomach (Lyu *et al.*, 2004). The other main reason for tumor growth and metastasis is because of angiogenesis triggered by chemical signals secreted by tumor cells (Folkman, 1971). Angiogenesis process is regulated by both activator and inhibitor molecules. For cancer therapeutics, angiogenesis

process also becomes the good target, where angiogenic inhibitors should be up-regulated and activators should be down-regulated (Dameron *et al.*, 1994).

Autophagy: an alternative strategy for lectin-mediated cancer therapy

Autophagy, also called PCD II (programmed cell death II), provides an alternative possibility for lectin mediated mechanism for inhibiting neoplasm. In the autophagy process, a cell destroys its own organelles by fusing with lysosomes. Many studies have shown that any defect in autophagy leads to tumorous growth through various genes. For example, Beclin 1, a part of a type-III phosphatidylinositol 3-kinase complex, important for autophagic vesicle formation to suppress tumor. Inactivation of this gene leads to human breast and ovarian cancer (Chen & Karantza-Wadsworth, 2009). Similarly, Tumor suppressor genes, p53 and PTEN, stimulates autophagy and are also common target for cancer therapy. Sometimes induction of apoptosis in cancer cells is defective, then autophagy-induced cell death is the alternate solution. With reference to lectin binding, for example, Con A is known to induce autophagic pathway in heptatoma ML-1 cells (Lei & Chang, 2009). Treatment of Con A lectin to heptatome ML-1 cells resulted in the formation of LC3-II (autophagic marker), vesicles and BNIP3 induction. These observations supported the fact that lectin are also responsible for induction of autophagy pathway (Chang *et al.*, 2007, Zhang & Ney, 2009). Only few lectins have been known so far inducing autophagic mechanisms. Thus, more scientific contributions are required in determining successful mechanisms of neoplastic cell death.

1.9 Scope and Motivation for the Work

In the last few decades, there has been rapid advancement in the plant lectin biochemistry. Due to lectins' ability to decode the information encrypted in the form of carbohydrates, it has become very important cell recognition molecules to be studied. Worldwide efforts to discover and characterize new lectins with unique sugar specificity are carried out by researchers. Advancements in the biophysical techniques, molecular biology and possibility of synthesizing oligosaccharides are responsible for this progress in lectin characterizations. Despite many lectins with novel activity have been discovered still many new lectins with unique sugar specificity and biological applications are yet to be discovered.

In the last decade, our lab has been working on many lectins and made several interesting discoveries. These include detailed biophysical characterization and structure determination of complex sugar specific lectin from *Cicer arietinum, Moringa oleifera* (Katre *et al.*, 2005, Katre *et al.*, 2008a, b) and *Trichosanthes dioica* (Dharkar *et al.*, 2006).

The present thesis describes the structure-function relationship of two hemagglutinins purified from different plant families, Cucurbitaceae and Solanaceae, both lectins found to be chito-specific in nature. GlcNAc-specific lectins play an important role in cancer therapy and acts as good anti-microbial agents. In this regard the objectives of the present study were to purify lectins and characterize them with respect to sugar specificity and secondary structure under extreme denaturing conditions. In addition, we carried out fluorescence quenching in order to probe tryptophan residues and its micro environment. The other biophysical studies were done using CD, Fourier

Transform Infra red (FT-IR) and Dynamic Light Scattering (DLS) under denaturing conditions to determine lectin structural and functional stability.

The first hemagglutinin studied is *Bh*L, lectin purified from *Benincasa hispida*, a homodimer of 34 kDa with chito-specificity. The protein retains its structural and functional activity in a wide range of pH, stable at higher temperature and in the presence of chemical denaturants (Chapter 3). Similarly, another agglutinin studied was purified from the seeds of *Datura innoxia* (*Di*L9), a monomeric 9 kDa glycoprotein with 4 % carbohydrate content. The lectin showed carbohydrate specificity towards GlcNAc oligomers, and found very stable at extreme denaturing conditions similar like *Bh*L (Chapter 4). Further, these lectins were investigated for their biomedical application potential like antimicrobial and anti cancer activities.

Chapter 2

Material & Methods

This chapter presents the details of the approaches undertaken to purify the lectins from plant sources *Benincasa hispida* and *Datura innoxia* and the different methods employed to study the lectin activity, structure, application potential and functional stability of the purified lectins. The materials and different experimental methods utilized in the entire study are described below.

2.1 Materials

2.1.1 Biological materials

Benincasa hispida fruit was purchased from the local market near Pashan, Pune, India. *Datura innoxia* seeds were collected locally from the premises of CSIR-National Chemical Laboratory (NCL), Pune, India. The rabbit erythrocytes were procured from National Toxicology Centre (NTC), Pune, India. For microbial assays, the bacterial and fungal strains were obtained from National Collection of Industrial Microorganism (NCIM), CSIR-NCL, Pune, India.

Cell lines and culture conditions: The cell lines were obtained from the European Collection of Cell Cultures (ECCC, Salisbury, UK)

- Primary Human umbilical vein endothelial cells (HUVECs),
- Mouse fibroblast cell line (L929),
- Lung adenocarcinoma (A549),
- Cervix adenocarcinoma (HeLa),
- Acute monocytic leukemia cell line (THP-1),
- Pancreatic adenocarcinoma (PANC-1),
- Human pancreatic ductal adenocarcinoma cell line (CFPAC-1),

- Human pancreatic epithelial carcinoma cell line (MIA PaCa-2).

These cell lines were cultured using different media supplemented with 10 % fetal bovine serum (FBS; Gibco) and the cells were incubated at 37 °C and 5 % CO_2 in a humidified atmosphere. THP-1 was maintained in RPMI 1640 media; L929, A-549, PANC-1, CFPAC-1 and MIA PaCa-2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM). HeLa and macrophages were cultured in Eagle's Minimum Essential Medium (EMEM). HUVECs were maintained in M200 Media supplemented with 50 X LVES (Gibco, Invitrogen).

2.1.2 Chemicals and reagents

Q-sepharose, Sephacyrl S-200, chitin matrix (from crab shells), bovine serum albumin (BSA), urea, guanidine hydrochloride (GDn-HCl), guanidine thiocyanate (GDn-SCN), Ethylenediaminetetraacetic acid (EDTA), chemical modifiers like diethylpyrocarbonate (DEPC), N-Bromosuccinimide (NBS), 2,4,6-trinitrobenzenesulphonic acid (TNBS), Woodward's reagent K (WRK); phenylglyoxal (PGO), N-acetylimidazole (NAI), phenylmethylsulfonyl fluoride (PMSF), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB); gel filtration markers, sugars and sugar derivatives were purchased from Sigma Chemicals Co, USA. Protein markers used in SDS-PAGE were procured from Bio-Rad. Chemicals used for quenching experiments such as acrylamide, cesium chloride (CsCl), potassium iodide (KI), 8-anilino-1-naphthalne sulfonic acid (ANS) were purchased from Sigma, USA. For binding studies various sugars/lignads and their derivatives were also procured from Sigma, USA. All other reagents of the highest

purity were obtained from local suppliers. Solutions were prepared in deionised (DI) water for spectroscopic studies.

2.2 Hemagglutination assay

2.2.1 Rabbit erythrocyte preparation

Rabbit RBC's were washed 5 times with 1 X PBS (20 mM potassium phosphate buffer at pH 7.2) containing 150 mM NaCl by centrifuging at 3000 rpm. A 3 % (v/v) suspension of the erythrocytes in the above buffer was treated with 100 µl of pronase enzyme (10 mg ml^{-1}) at 37 ºC for 1 h, washed 5 times with the same buffer and used for further studies. The pronase treatment of blood cells was tried wherever the hemagglutinating activity was found low.

2.2.2 Hemagglutinating activity

2-fold serial dilution method was employed to carry out hemagglutination activity (HA) (Lis *et al.*, 1970) using 3 % rabbit erythrocytes l X PBS (*v/v*). The protein sample (50 µl) was serially diluted in PBS, mixed with 50 µl of erythrocytes suspension and agglutination activity was observed after 1 h incubation. The hemagglutination unit (HU) is expressed as the reciprocal of the highest dilution (titre) exhibiting visible agglutination. The specific activity of the agglutinin is expressed as the number of HU mg^{-1} of the protein.

2.2.3 Hemagluttination inhibition assay

For hemagglutination inhibition assays, a prior incubation of the protein sample with different sugar solutions was performed. 25 µl of sugar solution is serially diluted

and were pre-incubated with 25 µl of lectin sample for 30 mins at room temperature (RT). Rabbit RBC's (50 µl) was added then mixed and resultant solution was analyzed after 1 h incubation.

2.3 Protein purification

The lectins from *B. hispida* fruit and *D. innoxia* seeds were isolated and purified. The following describes the details of the purification process of each lectin studied.

2.3.1 Extraction of lectins

A) Benincasa hispida lectin (BhL)

The extraction of the lectin from *B. hispida* fruit (500 g) was carried out by homogenizing the pulp in deionised (DI) water at 28 °C. The slurry obtained was filtered through double layer of muslin cloth and centrifuged at 10,000 rpm for 20 min at 4 °C.

Activation of chitin matrix

The flakes of chitin (Crab shells, Sigma) was ground into fine powder and kept in DI water for swelling. For 5 g of chitin matrix, treatment with 1N NaOH (100 ml), 1N HCl (100 ml) followed by DI water washing was given till neutral pH was reached. This activated chitin matrix was mixed with the clarified supernatant for binding at 10 °C with continuous stirring for 12 h.

Chitin affinity chromatography

Affinity chromatography is a method which separates out the protein depending on the interactions with ligands bound to the column. Here, chitin matrix was used for the isolation of lectin from the crude extract. It is a naturally occurring polymer of GlcNAc

which serves the purpose both as matrix and ligand for affinity chromatography. After the extraction step, the protein bound to chitin matrix was packed in a column and washed with DI water to remove unbound protein. The protein of interest was eluted with 50 mM glacial acetic acid and immediately dialyzed against the DI water. Hemagglutination assay was performed at each stage of purification to confirm the presence of lectin in the collected fractions.

B) Datura innoxia lectin (DiL9)

Dried seeds of *D. innoxia* (20 g) were finely powdered and soaked in 20 mM sodium phosphate buffer, pH 7.4 for 2 h. The homogenate was filtered through double layered muslin cloth and centrifuged at 10,000 rpm for 20 min at 4 ºC.

Ammonium sulphate precipitation:

The supernatant with lectin activity was harvested and precipitated with 60 % ammonium sulphate at 4 ºC, and kept overnight undisturbed. Later, the precipitated solution was centrifuged at 10,000 rpm for 20 mins. The precipitate thus obtained was dissolved in 50 mM sodium phosphate buffer pH 7.4 and dialyzed against the same buffer for 24 h with three consecutive changes of buffer. The supernatant with sufficient lectin activity obtained after centrifuging the dialysate was used for further purification.

Ion-exchange chromatography:

The ion-exchange chromatography depends upon protein charge due to ionizable side chains of amino acids. Each ionizable side chain group has its distinct pK_a, at that pH, the population of the ionizable group is dissociated. Thus, different proteins with different amino acids composition will have difference in overall charges at a particular

pH, and hence fractionation can be carried out. So here, the dialyzed supernatant was then subjected to Q-sepharose column chromatography, equilibrated with 50 mM column equilibrated with sodium phosphate buffer pH 7.4. The column was washed with NaCl gradient (0-0.5 M) and the fractions were collected until the absorbance at 280 nm was less than 0.01. Final wash was given with 1 M NaCl to remove all other bound and unbound matter to the column and re-equilibrated with the same buffer as mentioned before. Each fraction with good absorbance was checked for hemagglutinating activity and were pooled, dialysed against TBS (50 mM Tris-Cl, pH 8.0 containing 150 mM NaCl), concentrated and stored at -20 ºC for further use.

Gel filtration chromatography (Sephacryl S-200):

In gel filtration chromatography, the matrix used for column packing contains pores of different sizes permitting small protein molecules to pass through it. The higher molecular weight protein or complexes do not enter the matrix pores and remains in the void spaces of the column. Therefore, larger protein molecules can migrate faster as compared to smaller ones, and hence this chromatographic technique helps in determining the size, weight or homogeneity of the proteins.

Sephacryl S-200 matrix, bead size 25-75 μm (Sigma,USA) was packed in column (150 ml, 1 m length, 5 mm radius) and equilibrated with 50 mM Tris-HCl containing 150 mM NaCl, pH 8.0 (TBS). In each purification step (for *Bh*L and *Di*L9), the final homogenous solution was obtained after loading the concentrated sample (from chitin affinity/ Q-sepharose column) on gel filtration column (Sephacryl S-200) equilibrated previously with TBS. Fractions with detectable hemagglutinating activity were pooled, dialysed against DI water and stored at -20 ºC for future use.

2.3.2 Protein estimation

Protein concentrations were determined according to the method of Lowry (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as standard.

2.4 Molecular weight determination

2.4.1 SDS-PAGE

Reagents: Appendix-I

Procedure

SDS-PAGE was carried out using 12-15 % gel according to the method described by Laemmli (Laemmli, 1970). 20 µl of the sample was mixed with 5 µl of the sample loading buffer (5X) and kept in a boiling water bath for 5 min. The heated samples were loaded onto the gel and were allowed to run at constant 90 V. On completion of the run, the gel was stained with Coomassie brilliant blue R-250 for 30 minutes and then destained to visualize the bands.

2.4.2 Size exclusion / Gel filtration chromatography

Sephacryl S-200 column was standardized and all biomarkers were run to calculate Ve/Vo. Blue dextran (Sigma, USA) at a concentration of 5 mg ml^{-1} dissolved in TBS was loaded on the column to calculate the void volume (Vo) of the column. The absorbance was taken at 280 nm using UV-VIS spectrophotometer (Shimadzu, Japan) and calculated by measuring the eluent volume (Ve) collected from the point of application of the blue dextran to the center of the peak obtained in the plot of Absorbance vs eluent volume. Similarly, other standard biomarkers (BSA, α-amylase,

Carbonic anhydrase; Trypsin inhibitor; Cytochrome c, Aprotinin) were loaded and Ve was calculated. The graph was plotted between Ve/Vo vs $\log_{10}$ (Molecular weight) to determine the mass of the purified lectins.

2.4.3 MALDI-TOF/TOF and MS/MS

The utility of MALDI-TOF/TOF for protein and peptide analyses lies in its ability to provide highly accurate molecular weight information of molecules. This accurate information can be extremely useful for protein identification as well as for its characterization. The ideal sample preparation in MALDI would require a homogenous layer of small matrix crystals containing a solution of the lectin samples. The choice of the matrix depends on the protein molecular weight range. Mainly for peptides and proteins, Sinapinic acid (SA) provides better coverage for the higher-mass proteins (>10 kDa). It is prepared by dissolving 10 mg of SA in 70:30 water/acetonitrile with 0.1 % TFA final concentration.

MALDI-MS/MS

In-gel trypsin digestion protocol was carried out using mass spectrometry (Shevchenko *et al.*, 2007), in order to identify the protein. The trypsin digested peptides were mixed with a solution of α-cyano-4-hydroxycinnamic acid (10 mg ml^{-1}, CHCA) prepared in 50 % acetonitrile and 0.3 % trifluoroacetic acid. The samples were analyzed in a mass spectrometer MALDI-TOF/TOF in reflection mode. On an average 100-150 MS/MS spectra were measured for the digested protein and were analyzed using Protein pilot software. Uniprot software was used to create the plant lectin database.

2.5 Biochemical techniques for protein characterization

2.5.1 Determination of Carbohydrate content

Phenol-Sulphuric acid method was used to determine the neutral carbohydrate content with slight modification using mannose as standard, as previously reported (DuBois *et al.*, 1956).

2.5.2 Chemical modification studies

Chemical modification is one of the most widely used methods to study ligand/sugar binding sites and identification of the residues involved in substrate binding. For this, various chemical modifiers are used which are specific for each amino acid as described below.

a) To determine the number of trptophans (Trps) residues present in the lectin molecules, NBS titration was performed (Spande & Witkop, 1967). Aliquots of 2 µl of NBS (2 mM) were added to the native protein solution till the decrease in absorbance at 280 nm remained stable. By measuring the reduction in OD_{280}, the number of modified tryptophan residues was determined, by taking the molar absorption coefficient as 5,500 M^{-1} cm^{-1}. This gives number of Trps present on the surface of the protein. In order to estimate the total number of tryptophans, the protein was initially denatured in the presence of 6 M Gdn-HCl for 16 hrs and the NBS titration was carried out as described above.

b) Modification of histidine residues with DEPC was carried out according to the method of Ovadi (Ovadi, 1967) by dissolving in absolute ethanol. The lectin was treated with 1-10 mM DEPC for 30 min and the residual hemagglutination activity was determined at each concentration.

c) PMSF was used to modify serine as reported previously (Gold & Fahrney, 1964). The lectin solution was incubated with 5-10 mM PMSF in 50 mM Tris-HCl buffer, pH 8 at room temperature for an hour. The residual activity was determined by hemagglutination assay for each aliquot.

d) Modification of lysine with TNBS was carried out as reported by Habeeb (Habeeb, 1966). The reagent stock of 0.1M was prepared in 0.1 M Tris buffer, pH 8.0. Protein solution was dialysed with the same buffer and allowed to react for 1 hr with different concentrations of TNBS. The residual activity was then determined.

e) Modification of the carboxylate groups with WRK was carried out using the reported procedure (Sinha & Brewer, 1985). For this the lectin was incubated in 50 mM citrate-phosphate buffer pH 6.0, with different concentrations of WRK in the range 5-20 mM for 30 mins. Aliquots were removed and in each case the residual activity was determined.

f) Arginine residues were modified by incubating the protein with PGO (methanol dissolved) in the range of 1-5 mM, for 30 min at room temperature and the residual hemagglutination activity was determined for each aliquot according to the protocol described elsewhere (Takahashi, 1968).

g) Free cysteines can be modified by using DTNB (0.1M stock). DTNB in the concentration range 2-50 mM was incubated with lectin solution for 30 mins. The residual hemagglutination activity was determined for each aliquot (Habeeb, 1972).

h) NAI was used to modify tyrosine residues (Riordan *et al.*, 1965). NAI in the concentration range 2-10 mM was used for incubation and the hemagglutination assay was performed with the modified protein.

The native lectin without any reagent was used as a positive control for hemagglutination activity in each case of chemical modification.

2.5.3 Effect of pH on lectin activity

Different buffers were used in the range of pH 1-12 to determine the lectin stability and activity, carried out at room temperature for 12 h. Buffers used were: 20 mM glycine-HCl (pH 1-3), 20 mM citrate-phosphate (pH 4-6), 20 mM sodium- phosphate (pH 7), 20 mM tris-HCl (pH 8-9) and 20 mM glycine-NaOH (pH 10-12). The lectin sample in DI water at room temperature served as positive control and buffer was taken as negative control. Each assay was performed in triplicates.

2.5.4 Thermal denaturation profile of lectin

Thermal kinetics measures the effects of protein structure, folding and stability, which is crucial for protein function. Heating a sample above its critical temperature, increases the chemical potential energy enough to break the weak bonds which maintains the tertiary structure of the protein. The disruption of these chemical bonds leads to denaturation of the protein and thus inactivating it. The effect of temperature on lectin activity was studied by incubating the sample at different fixed temperatures between 25 ºC and 100 ºC for 30 min and residual activity determined after cooling the sample and carrying out the hemagglutination assay. To deduce thermostability of the lectin, kinetics of inactivation was carried out by first incubating the sample at higher temperatures in PBS at fixed intervals of time (20-120 min) and then the aliquots were taken followed by cooling it to room temperature and assaying the activity, as mentioned earlier. Using

Arrhenius equation (Leite *et al.*, 2005), the Gibbs free energy change (ΔG) of activation was estimated as a simple way to understand lectin heat denaturation process.

$$\Delta G = RT \ln(kT/k_1h) \qquad Eq.1$$

where R is the gas constant (1.987 cal mol^{-1} K^{-1}); T is the absolute temperature; k is the Boltzmann constant (1.37 x 10^{-6} erg K^{-1}); k_1 is the velocity constant and h is Planck's constant (6.25 x 10^{-27} erg s^{-1}).

The velocity constant (k_1) can be calculated from the slope of the curve obtained from plotting values in the following equation,

$$k_1t = - \ln (A/A_o) \qquad Eq.2$$

where t is the time of heat treatment (s), A is the residual hemagglutinating activity and A_o is the initial hemagglutinating activity before heat treatment.

2.5.5 Effect of EDTA and metal ions

To determine the metal dependency of the lectin, the samples were dialysed against 10 mM EDTA and then dialysed against DI water. The hemagglutination activity was evaluated before and after the addition of different divalent ions (1-50 mM). All the assays were done in triplicates.

2.5.6 Effect of chemical denaturants

Chemical denaturants disrupt the protein structure by breaking hydrogen bonds, salt bridges, disulfide bonds and non-polar hydrophobic interactions involved in secondary and tertiary structures, but are not strong enough to disrupt peptide bonds.

Therefore, a wide range of chemical reagents can cause disruption of protein structure. Most proteins, mainly enzymes lose their catalytic activity when denatured, since the position of amino acid residues involved in binding to substrates in transition states are disrupted. In some cases, the protein denaturation is reversible on removal of the denaturing agent. This notion was important, as it is considered that the information required for proteins folding is encoded in its primary structure. Information from denaturing studies can be utilized to assess the structural and functional stability of proteins.

Chemical denaturants such as urea, guanidine hydrochloride (GDn-HCl) and guanidine thiocyanate (GDn-SCN) as well as reducing agents like β- mercaptoethanol (βME) and dithiothreitol (DTT) were used here to study lectins stability towards retaining activity and structure. For this, the protein solution was incubated with different concentrations (0-8 M) of the chaotropic agents for 24 h. After incubation, 50 µl aliquot was withdrawn and residual activity determined after 1 h as described above.

2.5.7 Effect of organic solvents

The stability of both the lectins in various organic solvents was also studied. Proteins would have evolved in aqueous conditions and water molecules could be part of their three-dimentional structure and often involved in function and dynamics. In turn these solvent molecules play an important role in determining the specificity, co-operativity and thermodynamics of ligand binding (Mattos & Ringe, 2001). The behavior of the protein/enzyme will be different with respect to the solvent properties like

hydrophobicity, capacity to form hydrogen bonds and water miscibility which will have significant influence on the structural and activity aspects of the protein/enzyme.

It is thought that as a result of kinetic trapping, i.e the strong hydrogen bonding between the protein atoms, in the presence of hydrophobic solvents a protein can retain their native structure where water molecules are excluded and present on the surfaces only (Ru *et al.,* 1999). Whereas, polar solvents can easily remove the water molecules from the protein surface by competing for hydrogen bonds between protein atoms, and thus denaturing the protein. For example, alcohols have hydrophilic part which can disrupt the tertiary structure of proteins (Babu & Douglas, 2000).

In this study, both the lectins were incubated in water miscible solvents like ethanol, methanol, acetonitrile (ACN), dimethylsulphoxide (DMSO) and propanol (25 % and 50 % v/v) at pH 7.4 for 24 h. This study was carried out to observe the effect of organic solvents on the sugar binding capacity of lectins. The mixture of lectin and the organic solvent was incubated at room temperature in tightly closed vials to prevent evaporation. 50 µl aliquots were withdrawn and the hemagglutination assay was performed.

2.5.8 Proteolytic digestion studies

Proteolytic enzymes such as trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), pepsin (EC 3.4.23.1), papain (3.4.22.2) and protease (Nagarse; 3.4.21.62) were used for proteolytic digestion of lectins at 37 °C in 20 mM sodium phosphate buffer at pH 7.4. This study was carried out to evaluate resistance of lectins towards these enzymes showing structural rigidity. The mixture of lectin and each of the proteolytic enzymes

was incubated at 10:1 molar ratio for 24, 48 and 72 h. 50 µl aliquots were used for hemagglutination assay.

2.6 Biophysical techniques for characterization of protein and tryptophan microenvironment

2.6.1 Steady-state fluorescence studies

The fluorescence studies of lectin samples were carried on Perkin Elmer Life Sciences LS50 spectrofluorimeter equipped with a Julabo F20 water bath. The intrinsic fluorescence of the protein solutions were analyzed by exciting at 295 nm and the resulting emission spectra were recorded between 310-400 nm by setting scanning speed of 100 nm min^{-1} and slit width 7 nm for both monochromators. Each recorded spectrum was an average of 3 accumulations and in each case the baseline corrections were done by subtracting the emission produced by either buffers or denaturants before carrying further analysis.

To study the effect of certain denaturing conditions on the intrinsic fluorescence property of the lectin, wide range of pH, temperatures and different chemical denaturants were used to carry out the experiments.

a) To study the effect of pH on fluorescence of lectins, the samples were incubated in 25 mM buffers of pH ranging from 1-12 for 24 h after which the fluorescence scans were recorded.

b) For studying thermal unfolding profiles of lectins, the samples were incubated at temperatures ranging from 25 °C to 95 °C for 5 mins and fluorescence spectra were recorded.

c) The denaturing effect of chaotropic agents like urea and GDn-HCl was studied by incubating the protein samples at different concentrations (0-8 M) at room temperature, pH 7.4 for 4, 8, 16 and 24 h.

2.6.2 Tryptophan decomposition analysis

The fluorescence spectrum of tryptophan in protein shows emission maxima in the range of 308 to 358 nm. Maximum number of proteins contains more than one component of tryptophan. PFAST program (http://pfast.phys.uri.edu/pfast/) based on SIMS and PHREQ algorithm (Burstein *et al.*, 2001) was used to carry out decomposition analysis of tryptophan fluorescence. Observed spectrum could be divided into 5 spectral classes namely A, S, *I*, *II* and *III* (Burstein *et al.*, 1973, Reshetnyak *et al.*, 2001). Each of this spectral form is listed in **Table 2.1.**

Table 2.1 Classification of Trptophan spectral classes

Trp class	**Position of maxima (nm)**	**Characteristic features**	**Example protein**
A	308	Extreme non-polar environment, No H-bonding	Azurin from *Pseudomonas* and *Bordetella*
S	318-330 nm	Non-polar environment, with few polar groups available. The excited indole group forms H-bond with the surrounding free polar atoms	L-asparaginase (Shifrin *et al.*, 1971)
I	331.0±4.8 nm	Polar environment. H-bonds are formed in 2:1 stoichiometry with surrounding polar molecules	Chymotrypsinogen, B-lactoglobulin and trypsin

II	340 ±5 nm	Maximum emission. Direct contact of indole group with structured-water molecules	Myosin
III	350 nm	Maximum emission similar to free Trp in solution. Trps residues present on protein surfaces.	-

2.7 Solute quenching studies

Quenchers are molecules which decrease the fluorescence intensity of a fluorophore containing substance. Reactions like, excited state, complex formation or energy transfer can result in quenching process. Chemicals like acrylamide (neutral), iodide and cesium (charged) are well known quenchers. Fluorescence quenching studies provide useful insight into the study of changes in tryptophan environment of a protein. Acrylamide is an effective collisional quencher being small in size can diffuse into the interior of the proteins and thus, can decrease the tryptophan fluorescence (Eftink & Ghiron, 1976). The ionic quenchers iodide and cesium, due to their large size cannot penetrate into the protein interior and thus, quenches tryptophan residues near the protein surfaces in the vicinity of positively and negatively charged residues, respectively (Lakowicz, 1983). The mechanism of iodide quenching is a result of intersystem crossing to an excited triplet state, which is promoted by the spin-orbit coupling of the excited fluorophore and halogen (Kasha, 1952).

The lectin samples, both in native and denatured form, was titrated with 5 M stock solutions of quenchers on a Perkin-Elmer LS 50B spectrofluorometer at 28 °C. Fluorescence quenching studies were carried out by adding 3-5 µl aliquots of quencher after each scan from stock till final concentration of quencher reached 0.5 M in 2 ml of

lectin solution. Iodide stock solution contained sodium thiosulphate (0.2 M) to suppress tri-iodide formation (I^{-3}). Baseline correction was done to eliminate background noise. Stern-Volmer (Eq.1) and modified Stern-Volmer equations (Eq.2) were used to estimate K_{sv} and fractional accessibility of a quencher (f_a), respectively (Lehrer & Leavis, 1978). The following equations were used to analyse the data.

$$F_0 / F_c = 1 + K_{sv}[Q] \qquad \textit{Eq. 3}$$

$$F_0 / \Delta F = f_a^{-1} + (K_q f_a)^{-1} [Q]^{-1} \qquad \textit{Eq. 4}$$

Where $\mathbf{F_0}$ and $\mathbf{F_c}$ are fluorescence intensities, in the absence and presence of quencher **[Q]**, respectively; $\mathbf{K_{sv}}$ is the Stern-Volmer quenching constant and $\mathbf{K_q}$ is the corresponding quenching constant.

2.8 Saccharide/ligand binding studies

Specific sugar/ligand binding to lectins might result in fluorescence quenching or its enhancement (Gaikwad & Khan, 2006). These changes in the fluorescence intensity are exploited in order to obtain the association constants for various sugars and ligands. To a definite concentration of protein (2 ml) dialyzed in 20 mM phosphate buffer, pH 7.4, 3-100 µl aliquots of sugar solutions were added till saturation was achieved and the change in fluorescence intensity was measured after exciting the sample at 295 nm. Similarly, ligands such as adenine and its derivatives were identified to quench the protein fluorescence intensity. Change in fluorescence intensity (ΔF) was recorded with increasing concentrations of adenine. Binding constant (K_a) was calculated from the following equation (Chipman *et al.*, 1967).

$$Log[C]_f = -log[K_a] + log[(F_0 - F_c) / (F_c - F_\infty)] \qquad \textit{Eq. 5}$$

Here, $\mathbf{F_0}$ is the fluorescence intensity of a free unbound protein whereas $\mathbf{F_c}$ is that with bound sugar of concentration **[C]**, $\mathbf{F_\infty}$ is the fluorescence intensity upon saturation of all sugar/ligand binding sites. In the plot of log $[(F_0-F_c) / (F_c-F_\infty)]$ versus log[C], the abscissa intercept gives the dissociation constant (K_d), reciprocal of which gives the association constant (K_a). The free energy parameters for binding were calculated using thermodynamic equation:

$$\Delta G = - RT \ln(K_a) \qquad \textit{Eq. 6}$$

2.9 Parameter A and Phase Diagram Analysis

Parameter A is defined as the ratio between the fluorescence intensity at 320 nm to that at 365 nm (Turoverov *et al.*, 1976). It is calculated to understand the changes in conformation of a protein under denaturation conditions (He *et al.*, 2005, Su *et al.*, 2007). The phase diagram plotted between the fluorescence intensity at 320 nm vs that at 365 nm at different Gdn-HCl concentrations identifies the presence of folding intermediates.

2.10 FTIR structural studies

Fourier Transform infrared (FTIR) spectra were recorded using Bruker Optics ALPHA-E spectrophotometer in the range of 1000-2000 cm^{-1}. The protein concentration of 1mg/ml was used for the IR scan. The characteristic secondary structure changes at different denaturing conditions were also studied by incubating the sample in extreme pH condition, in different concentrations of GDn-HCl and at higher temperatures.

2.11 Circular dichroism studies

Circular dichroism (CD) spectra were recorded on a JASCO J-815-150S (Jasco, Tokyo, Japan) spectropolarimeter connected to a Peltier Type CD/FL cell circulating water bath at 28 °C. In studies at higher temperatures the quartz cuvettes were sealed to prevent any evaporation losses. The lectin sample of 0.15-0.20 mg ml^{-1} concentration was used for far-UV CD spectra, measured in the wavelength range of 190-250 nm (scanning speed = 100 nm min^{-1} and response time 1s) and an average of 3 accumulations were recorded. The near-UV CD spectrum was recorded using 1 mg ml^{-1} protein concentration in the range of 250-300 nm. Blank spectrum in each case was subtracted from the recorded protein sample data and analysed in terms of mean residual ellipticity (MRE) in deg cm^2 $dmol^{-1}$ which is defined as:

$$MRE = M\,\theta_\lambda / 10\,d\,c\,r \qquad \text{Eq. 7}$$

Here, **M** is the molecular weight of protein, θ_λ is observed ellipticity in millidegree, **d** is cell path length in cm, **c** is protein concentration in mg ml^{-1} and **r** is the average number of amino acid residues. Using CDPro software (http://lamar.colostate.edu/~sreeram/CDPro/main.html) available online, the secondary structure elements were calculated considering the lowest NRMSD values.

The effect of temperature on the stability of lectin secondary structure was studied, firstly by increasing the temperature at the rate of 5 °C min^{-1} ranging from 25 °C -95 °C. In order to study the thermal reversibility of the lectin structure, the denatured samples were cooled back to room temperature and new scans were recorded. The temperatures of the protein samples were increased at the rate of 1°C $min^{-1}$and the ellipticity at different wavelengths were recorded. The effect of pH on the secondary structure was monitored by incubating the lectin sample in 25 mM buffers of different

pH (1-12) as mentioned above for 4- 24 h before recording the spectra. The effect of denaturant on the protein structure was studied by incubating the lectin in 0-6 M Gdn-HCl for 4 h and the far UV CD spectra were recorded. Similarly, the effect of organic solvents (25-50 % v/v) and amino acid chemical modifiers were studied by incubating at pH 7.4 for 4 h at room temperature with respective solvents.

2.12 Hydrophobic dye Binding

8-anilino-1-napthalene sulfonic acid (ANS) is a small hydrophobic dye that specifically binds to hydrophobic patches present in proteins and hence ~~it~~ is mainly used for characterizing and detecting the presence of partially unfolded states in proteins (Ali *et al.*, 1999, Gasymov & Glasgow, 2007). ANS emission spectra were recorded for lectin samples by exciting at 375 nm measuring the emission in the range of wavelengths 400-550 nm using a slit width of 7 nm. The dye binding to protein was analyzed by recording the change in ANS fluorescence intensity at extreme pH values, at higher temperatures and in the presence of different concentrations of denaturants. Aliquots from 15 mM ANS stock was mixed with 2 ml protein of same concentration. Spectrum of ANS in blank was subtracted from the spectrum of the protein-ANS combination for further studies.

2.13 Rayleigh light scattering studies

The propensity to form protein aggregation upon thermal denaturation was studied using Rayleigh light-scattering experiments in time drive module of Perkin Elmer LS50 spectrofluorimeter connected to a Julabo F20 water circulating bath. The

wavelength of 400 nm was used for both excitation and emission monochromators. The excitation slit width was kept at 10 nm and emission slit width at 2.5 nm. Scattering was recorded for 30 s.

2.14 DLS measurements

Dynamic light scattering (DLS), also known as quasi-elastic light scattering and photon correlation spectroscopy (Schurr, 1977), a technique widely used for analyzing the size, shape and other structural characteristics of protein molecules in solution. It also allows us to study the effects of various denaturing conditions on the structure and activity of protein in question. The change in protein size/shape due to pH, temperature or chemical denaturants can be attributed to protein unfolding or aggregation. The wide application of DLS studies include inter- and intramolecular interactions of proteins and other biopolymers, gelation, micellization, and molecular conformation (Harding *et al.*, 1992). For example, rod shaped protein molecule will move slower as compared to spherical molecules, since the friction or drag force on the former will be more. Also, the scattered intensity by larger protein molecules will be more than by the smaller ones. However, increase in hydrodynamic radius/ diameter of the protein size; it would imply the onset of denaturation/ aggregation. Whereas, no change would indicate maintenance of the native structure of protein (Sluzky *et al.*, 1992). Proteins, especially those of therapeutic importance are highly sensitive to a wide range of parameters like concentration, high temperature, pH of the buffer used and chemical environment used during storage. Without acquiring information on the stability of therapeutic protein, its application for human health will be difficult to realize. One major disability factor is

aggregation, a result of protein instability and self-association, thus reducing the efficacy of protein drugs.

DLS measurements were done to study the protein size/shape and presence of aggregates, using Particle Sizing Systems, Inc. (Santa Barbara, CA, USA). The samples (1 mg ml^{-1}) were centrifuged at 10,000 rpm 10 min and were filtered (0.22 μm) to remove any solid particulate matter present. Generally, the geometrical information like protein shape or size can be estimated by using the translation diffusion coefficient. DLS studies were carried out to determine protein particle size/shape in the presence of different denaturing conditions to study its unfolding pattern. Extreme denaturant concentrations were not used due to solvent irregularity.

Using estimated diffusion coefficient provided by the DLS software, the hydrodynamic radius (R_h) can be calculated by Stoke's-Einstein relationship, as given below:

$$\boldsymbol{D = kT / 6\Pi\eta R_h} \qquad \textbf{Eq. 8}$$

where $\boldsymbol{D}$ is the diffusion coefficient, $\boldsymbol{k}$ is the Boltzmann's constant, $\boldsymbol{\eta}$ is the solvent viscosity, $\boldsymbol{T}$ is the absolute temperature, and $\boldsymbol{R_h}$ is the hydrodynamic radius.

DLS experiment can also be used to distinguish between a homogenous monodisperse and an aggregated sample; an aggregated sample will show increase in their hydrodynamic radii. In addition, it provides information regarding polydispersity which is an important parameter for predicting protein crystal formation. It has been reported that a monodisperse protein sample (polydispersity measured is less than 15 %) can crystallize easily as compared to a sample that contains non-specific aggregates (Zulauf & D'Arcy, 1992, Ferre-D'Amare & Burley, 1994).

2.15 Antimicrobial and Anti-biofilm studies

To fight bacterial and fungal infections, the molecules mostly used are antibiotics. Their introduction in the day to day life of humans had greatly benefited the health-related quality of human life. However, over the past few decades, due to resistance developed by pathogenic organisms against the antibiotics and due to their toxic side-effects, health benefits of antibiotics are under threat. This has put pressure on researchers to discover/investigate newer drug molecules having lesser resistance and preferably of biological origin which can play a significant role in the prevention and treatment of human diseases.

In countries like India, many indigenous plants are used as traditional medicines to treat several disorders; also they are used as part of the primary healthcare systems. From 1981 till now, many natural products have been isolated and characterized, found applications especially in the areas of infectious disease and cancer (Newman, 2005). With the increase in drug resistance and decline in the rate of discovery of new molecules as drug candidates, the pressure is on to search for natural compounds possessing novel mechanisms of action (Shahidi, 2004, Runyoro *et al.*, 2006). The antimicrobial effects of chemical compounds against many pathogenic bacteria and fungi have been studied by a very large number of researchers around the world and the hunt for novel molecules is always going on. In this context efforts are going on to broaden the spectrum of available antimicrobial agents from natural plants: secondary metabolites like tannins, alkaloids, flavonoids, terpenoids, glycosides etc or to use defensin proteins like lectins, trypsin inhibitors, and proteases etc. which are previously known to have *in vitro* antimicrobial activity (Dahanukar *et al.*, 2000, Cowan, 1999).

2.15.1. Testing Microorganisms and Growth Media

Depending on the medical and pharmacological importance certain pathogenic microbes and opportunistic pathogens were selected for testing effects of lectins on them. Bacterial strains like, *Staphylococcus aureus* (NCIM-2079), *Bacillus subtilis* (NCIM-2010), *Escherichia coli* (NCIM-2065), *Enterococcus faecalis* (NCIM-2403), *Pseudomonas aeruginosa* (NCIM-2036), *Bacillus cereus* (NCIM-2155), *Bacillus megaterium* (NCIM-2032), *Bacillus sphaericus* (NCIM-2478), *Salmonella typhimurium* (NCIM-2501), *Klebsiella pnemoniae* (NCIM-2706), *Proteus morganii* (NCIM-2040), *Serratia marcescens (NCIM-2078), and Corynebacterium sp.(NCIM-2640);*and fungal strains like *Aspergillus niger (NCIM-501), Aspergillus flavus* (NCIM-519), *Fusarium moniliforme (NCIM*-1099), *Fusarium oxysporum* (NCIM-1281), *Trichoderma reesei* (NCIM-992), *Saccharomyces cerevisiae* (NCIM-3044), *Penicillium notatum* (NCIM-741), *Cephalosporium sp*. (NCIM-676), *Candida albicans* (NCIM-3100) were chosen for the assay. The nutrient agar and potato dextrose agar (PDA) slants were used for maintaining the stock cultures of bacteria and fungi, respectively, with storage at 4 °C.

2.15.2. Antibacterial assay

Antimicrobial activity of lectins was examined using disc diffusion method (Cole, 1994) on sterile nutrient agar (NA) plates. The selected bacteria were seeded separately onto the surface of NA plates. MRS agar plates were used only for *E. faecalis*. The lectin sample was filter sterilized and aseptically loaded on the sterile disc placed on the nutrient agar plates and the organisms were allowed to grow at 37 ºC for 24 h. The antimicrobial activity of the lectin is estimated by measuring the diameter in millimeters

(mm) of the transparent ring around the disc which is the zone of inhibition. Ampicillin was used as a positive control. These lectins were also tested for their anti-mycobacterial activity in terms of their *in vitro* effects against dormant and active phases of *M. tuberculosis* H37Ra using XTT Reduction Menadione Assay (XRMA) protocol (Singh *et al.*, 2011). All experiments were performed in triplicates.

2.15.3 Antifungal Assay

Antifungal bioassays were performed on already mentioned fungal strains using agar well diffusion method (Cole, 1994), in order to determine the susceptibility of these pathogenic fungi towards lectins. Amphotericin B (as positive control) and lectin were dissolved in deionized water and loaded onto the well (8 mm in diameter) of potato dextrose agar plates, incubated at 28 °C. The diameter of the clear ring formed around the well was considered as a measure of the inhibitory power of the lectin against the particular test pathogen.

2.15.4 Anti biofilm assay

Communities of similar or different microbe's adherent to solid surfaces like medical, industrial instruments and natural surfaces. These biofilms are covered by extracellular matrix (polysaccharides) which is known to provide structural protection to the microbe community, a main reason for antibiotic resistance. So here, to determine the anti-biofilm activity we have used the microtiter dish assay (O'Toole, 2011), an easy tool to study early stages of biofilm formations. This assay does not include flowing conditions, however we can identify the factors (enzymes, adhesions or genes) involved

in it. This assay makes it convenient to screen biofilm formation by variants of micro-organisms under different growth conditions. In the protocol used here to determine the anti-biofilm activity, we have focused our study to inhibit biofilm formation by the model organisms mentioned above by lectins purified (*Bh*L and *Di*L9).

Procedure:

1. Biofilm forming microbes:

The selected organisms (mentioned above) were grown in a nutrient media (LB) for 24 hr. The culture was diluted in 1:100 ratios using fresh media and 100 µl was dispensed into microtitre plates in triplicates. The plates were incubated for 24-72 h depending on the assays at 37 °C.

2. Biofilm staining:

The liquid was dumped out by turning back the plate, after required incubation period. Using sterile water, the plates were washed thrice to remove any unattached cells and media components present without disturbing the biofilm formed.

3. Quantification of biofilm formed:

Crystal violet solution (0.1 % in water) was added (125 µl) to each microtitre well and incubated at room temperature for 15 mins. After incubation wash the plates thrice with water and keep for drying at 50 °C for 15-20 mins. For qualitative assays, the photographs were taken. To quantify the amount of biofilm formed, 125 µl of glacial acetic acid (30 % in water) was added to each well and incubated for 15 mins to solubilize the crystal violet. The absorbance was taken at 550 nm in a plate reader. The effect of drugs on biofilm inhibition was determined

by calculating the percentage of absorbance reduction determined according to the formula given as

Percentage of Abs reduction = Abs of (Control- Test)/ Abs of control *100 Eq. 9

2.16 Cell line studies

2.16.1 Cytotoxicity assay

The cell cytotoxicity studies were carried out *in vitro* to determine the effects of lectins on various cell lines using reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye (Mosmann, 1983). For this, 96-well flat bottom micro titre plates were seeded with cells with density of $1x10^5$ cells ml^{-1} and incubated at 37 °C in an atmosphere of 5 % CO_2 and 95 % relative humidity within a CO_2 incubator. Once the cells were adhered, lectin treatment in dose dependent manner was given (*Bh*L and *Di*L9, 150-5 µg ml^{-1}) where Carboplatin (1 mg ml^{-1}) was used as positive control. The cells with no drug treatment were the negative control. At the end of the incubation, the cells were washed thrice with phosphate buffered saline (PBS) subsequently, followed by the addition of 10 µl of MTT solution (5 mg ml^{-1}, Sigma Chemicals, USA) in each well. The plate was incubated for 4 h at 37 °C which resulted in the formation of the formazan product. Later the formazan crystals were solubilized by the addition of acidified isopropanol (200 µl) and absorbance was measured on a SPECTRAmax PLUS 384 plate reader (Molecular Devices Inc, USA), at 570 nm. Here, the untreated cells were considered as 100 % viable and percentage of cell viability (or Growth inhibitory concentration, GI_{50}) was calculated according to the formula given as below:

Percentage of cell growth inhibition = $(A_C - A_T) /(A_C – A_B) * 100$ Eq. 10

where A_C is the absorbance of the control cells, A_T is the absorbance of the lectin/drug treated cells and A_B is the absorbance of the blank (MTT dye).

2.16.2 Microscopic observation of cell morphology

Two different staining methods, *Acridine orange* and *Annexin-V FITC* staining, were employed to confirm the cell morphological features.

Acridine orange (AO) staining:

It is an organic vital fluorescent stain that binds to nucleic acids and is useful for studying cell cycle or cell death pattern induced by the drugs. In this study, exponentially growing cancer cells were taken and treated with lectins (*Bh*L and *Di*L9) for 24 h. The microtitre plate was centrifuged, washed with PBS and the cells were fixed using 3.7 % paraformaldehyde. Later, the fixed cells were stained with AO (8.5 μg ml^{-1}) incubated for 20 min under dark conditions. The cells were visualized and images were collected using EVOS® FL Cell Imaging System by setting the filter sets at 470 nm excitation and 525 nm for emission.

Annexin V-FITC Apoptosis Assay:

In brief, the cancer cells were treated with lectins for different time intervals (2, 24, 48 and 72 h). After incubation, the microtitre plate was centrifuged and resuspended sequentially in binding buffer (0.01 HEPES, pH 7.4, containing 140 mM NaCl and 25 mM $CaCl_2$) containing Annexon V-Fluorescein isothiocyanate **(**FITC, 3 μg ml^{-1}), 4',6-Diamidino-2-pheylindole (DAPI, 1 μM ml^{-1}) and Propium iodide (PI, 10 μg ml^{-1}) (Rieger *et al.*, 2011). This protocol also helped us in estimating the proportion of apoptotic and necrotic cells separately i.e, the number of cells positive for Annexin V-

FITC and PI were determined using a laser-scanning confocal microscope (LSCM), magnification 20 X (Olympus FV1000) and 3D multichannel-image processing was done using Thermo Scientific™ High Content Screening (HCS) Studio™ 2.0 cell analysis software. The number of cells undergoing apoptosis was estimated.

2.16.3 Cell Cycle Analysis

In brief, the cancer cells were treated with lectin and incubated for 6, 12, 18 and 24 h. At each selected time point, sub G_0/G_1 distribution of cells were determined by DAPI staining and measured using LSCM at 386 nm and the data was analyzed as mentioned before (Roukos *et al.*, 2015).

2.16.4 Determination of Mitochondrial membrane potential ($\Delta\psi_m$)

Many researchers have reported that the mitochondria play a crucial role in the onset of apoptosis in many cells by losing its membrane potential (Wilson, 1998, Zoratti & Szabo, 1995), whereby releasing cytochrome c and calcium ions. These molecules act as mediators in the apoptotic pathway. To confirm this, lectin-stimulated cancer cells were incubated for 4, 8 and 12 h. The cells were centrifuged and resuspended in MitoTracker Red (0.1 μmol l^{-1}, Invitrogen) containing DMEM media incubated for 15 min at 37 °C. Later, the cells were washed, fixed and stained with DAPI (1 μM ml^{-1}) analyzed using a LSCM/ HCS. Any disruption in the Δψm of lectin treated cells was quantified by the sequestering of fluorochrome MitoTracker Red dye by the viable cells. A decrease in red fluorescence intensity was taken as an indication of mitochondrial membrane dysfunction.

2.16.5 Estimation of cytoplasmic calcium release concentration $[Ca^{2+}]_i$

As mentioned above, on disruption of mitochondrial membrane the calcium ions are also released. Fluo-4 acetoxymethyl ester (Fluo-4/AM, 4 μmol l^{-1}, Invitrogen), is a fluorescent Ca^{2+} binding indicator, was used to quantify the concentration of the cytoplasmic [Ca^{2+}] using LSCM. In brief, the cancer cells were treated with lectins for different time intervals. After incubation, the plate was centrifuged and incubated with Fluo-4/AM for 30 min at 37ºC. Later, the cells were washed with cold PBS, fixed and stained with DAPI. Any change in the mitochondrial calcium level was quantified by measuring the Fluo-4/AM fluorescence intensity at a detection spectrum of 488 nm.

2.16.6 Detection of caspase activity

All animal cells contain caspases, which are **C**ysteine-dependent **ASP**artyl-specific prote**ASE** present in inactive form inside the cells. Certain stimulus triggers the activation when the cell fate is decided to be suicidal. Thereby, these caspases act on a variety of cell signal transduction proteins, nucleic acid modifying proteins, cytoskeletal and nuclear proteins and endonucleases to target a cell for its final destruction by disintegrating its own contents. These proteases are subclassified by their mechanism of action involved in apoptosis as either initiator (caspase-8 and -9) or executioner caspases (caspase-3, -6, and -7) (McIlwain *et al.*, 2013).

In this study also we have tried to check the activation of caspases of lectin stimulated cancer cells response. Fluorimetric assay kit, like Apo Alert Caspase Luminescent Assay Kit (Promega, USA) and EnzChek® Caspase-3 Assay Kit (Molecular probes, USA) was used for caspase-8, -9 and -3 detection, respectively. Briefly, the lectin treated cells were incubated for indicated time periods. After the

incubation, the cells were harvested and lysed with lysis buffer provided in the kit at 4 °C, centrifuged and supernatant was collected. The extracted supernatant (50 µl) was mixed with equal volume of 2 X reaction buffer and specific substrate conjugate (Z-DEVD–AMC) for caspase-3, Acetyl-Ile-Glu-Thr-Asp p-nitroaniline (Ac-IETD-pNA) for caspase-8 and acetyl-Leu-Glu-His-Asp-p-nitroaniline (Ac-LEHD-p-NA) for caspase-9 were added. The plate was incubated for 1 h at 37 °C, during this time the caspases cleave and release p-nitroaniline (p-NA) from the substrates giving fluorescence. Lysis buffer (50 µl) was taken as control (no-enzyme) to determine the background fluorescence of the substrate. The fluorescence intensities were measured to calculate the fold increase in the activity of caspases using a plate reader (VarioskanFlash, using SkanIt Software 2.4.5 RE, Thermo Scientific.) by setting the filters 496/520-nm for caspase-3, 400/500-nm for caspase-8 and 380/460-nm for caspase-9, respectively.

2.16.7 Anti-angiogenesis activity of lectins

Angiogenesis can be defined as the birth or formation of new blood capillaries from the preexisting blood vessels (Folkman, 2004), a fundamental process involved in wound healing, embryonic developmental and during reproduction (Zadeh & Guha, 2003). During neovascularization process, the endothelial cells get activated by the binding of VEGF factor and undergo migration towards angiogenic factors and proliferate to increase in cell number. Finally, these cells get reorganized into 3 D tubular structures. It is now known to be responsible for the spread of a tumor, hence becoming the promising target for cancer therapy.

To evaluate the anti-angiogenic activity of lectins (*Bh*L and *Di*L9), the three-dimensional tubular vessel formation by HUVECs was used for the *in vitro* assay

(Angiogenesis Starter Kit, Life technologies). The HUVECs were grown on the Marigel coated plates suspended in appropriate media in the presence of pro-angiogenic compound VEGF. The cells were subsequently given lectin treatment and incubated for 24 h at 37 °C in a 5 % CO_2 humidified atmosphere. A known anti-angiogenic agent, called *Suramin* (5 µg ml^{-1}) was used as positive control. After incubation with lectin, rhodamine conjugated phalloidin staining was used to detect the endothelial tubes formed and the nuclei was stained with DAPI, to count the number of nuclei per tube. Automated imaging was done on Cellomics' ArrayScan® HCS Reader.

Chapter 3

Study of the conformational transitions and functional characterization of *Benincasa hispida* Lectin

Benincasa hispida belongs to Cucurbitaceae family (Commonly known as Ash gourd), the plant requires very warm weather to grow and the fruit can be stored for many months. Its texture varies from place to place, like those grown in Indian subcontinent the fruit appears white with rough texture whereas in South East Asian varieties it has a smooth waxy texture. It is also called 'winter melon' because of its availability during winter in areas of deciduous vegetation. In Indian cuisine the vegetable is traditionally used to prepare a wide variety of dishes. In north India, ***Petha*** (a kind of sweet) is prepared from Ash gourd and in south it is used in many curries or prepared with buttermilk.

In India, the traditional medical treatment such as Ayurveda has mentioned many medicinal properties of *B. hispida* such as using as a laxative, diuretic, tonic, anti-diabetic, aphrodisiac, cardio tonic, urinary calculi, kills intestinal worms, blood disease, insanity, epilepsy, and also in cases of jaundice, dyspepsia, fever, and menstrual disorders (Kirtikar *et al.*, 1993). The crude extract/juice of the raw fruit is known to be beneficial for treating mercury poisoning and snakebites; methanolic extract is reported to possess antiulcer (Grover *et al.*, 2001), anti-inflammatory, antihistaminic, and antidepressant activities (Anil kumar & Ramu, 2002)

Figure 3.1 *Benincasa hispida* fruit, flower and plant.

In addition to the above characteristics, several bioactive proteins have been identified in this plant, for example, osmotin like protein, a member of pathogenesis related proteins (Shih *et al.*, 2001) and a serine proteinase inhibitor (Atiwetin *et al.*, 2006), α- and β-benincasins possessing antifungal activity (Ng *et al.*, 2003) and hispin, a ribosome inactivating protein (Ng & Parkash, 2002). The Phloem lectin-like protein with anti-insecticidal property has been cloned and purified from *B. hispida* (Ota *et al.*, 2013). Hence, such proteins' being present in a medicinally important plant such as ashgourd makes it worthwhile to characterize their structure-function relation. Previously, the characterization of a few lectins from Cucurbitaceae family has been reported. In 1978, Sabnis & Hart had reported high hemagglutination activity of lectins from the phloem exudate of 3 cucurbit species, namely *Cucurbita maxima, Cucumis sativus* and *Cucumis melo,* found to be specific for chitooligosaccharides (Sabnis & Hart, 1978). Recent reports include physico-chemical characterization of lectins purified from phloem of *T. anguina* (Narahari *et al.*, 2011), *L. acutangula* (Anantharam *et al.*, 1985) and *C. maxima* (Narahari & Swamy, 2010), but no 3-D X-ray structure is yet available.

This chapter describes the study of a newly identified lectin, a homodimer protein with subunit molecular weight 17 kDa, from *B. hispida* fruit extract. As mentioned before, a phloem lectin of 35 kDa was cloned from the seeds of *B. hispida* but it was monomeric in nature. This might have been an isolectin present in the same plant but isolated from different parts of the plant.

3.1 Isolation and Purification of Benincasa hispida Lectin (BhL)

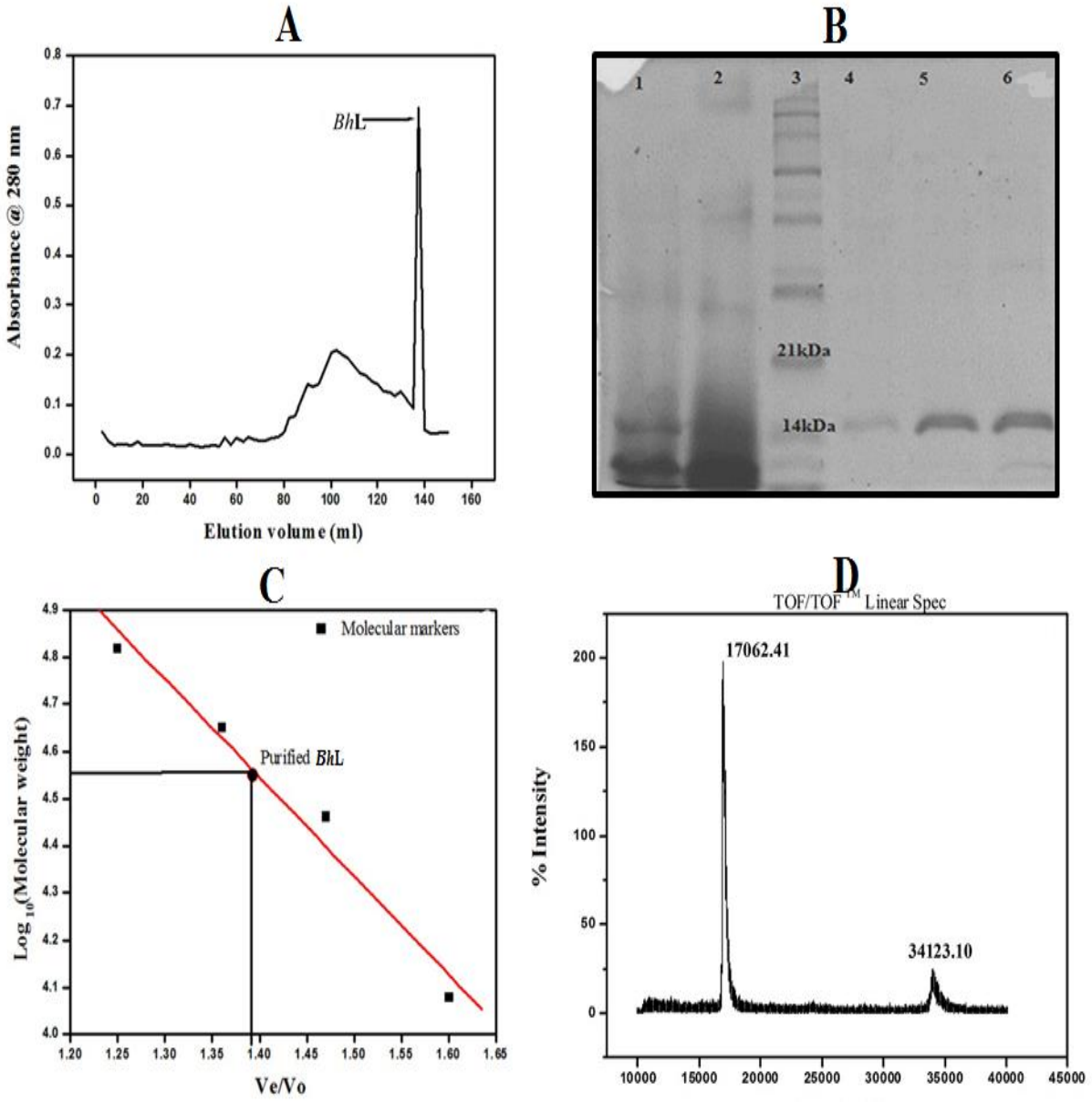
Total yield of pure *Bh*L from 500 g of fruit extract was 2.6 mg ml^{-1}; the lectin was purified to homogeneity using chitin affinity chromatography as a first step followed by gel filtration on Sephacryl S-200 column **(Fig. 3.2.A)**. The increase in purification fold after each step is calculated and summarized in **Table 3.1**. The purified fractions of *Bh*L were resolved on 12 % SDS-PAGE, where the lectin showed a single band corresponding to molecular mass near to 14 kDa **(Fig. 3.2.B)**. The molecular mass of native protein was determined to be approximately 35,481 Da **(Fig. 3.2.C)** from Sephacryl S-200 chromatography. When the lectin was subjected to MALDI-TOF/TOF, the scan showed 2 peaks corresponding to MW 17 kDa and 34 kDa confirming that the lectin exists as a homodimer **(Fig. 3.2.D)**.

Table 3.1 Summary of purification of *Benincasa hispida* lectin (*Bh*L)

Purification step	Total protein (mg)[a]	Total activity (titre x ml)	Specific activity (HU mg^{-1})[b]	Purification (fold)[c]	Yield (%)
Aqueous Crude extract (From 500 g)	728	62400	86	1.0	100
Affinity chromatography (Chitin)	10.5	8400	800	9.3	13.5
Gel filtration (Sephacryl S-200)	**2.6**	2800	1000	12.0	4.5

[a] *Crude extract of protein from 500 g of Ashgourd fruit.*

[b] *Specific activity: ratio of hemagglutination unit divided by total protein concentration(mg ml^{-1}).*



[c] *Purification fold: the ratio of minimum concentration of crude protein able to show hemagglutination and that of the protein fraction purified at each step.*

Figure 3.2 Purification and molecular mass estimation of *Bh*L. A) Purification profile of lectin on gel filtration column (S-200). **B)** Purity check on 12 % SDS-PAGE. Lane 1: chitin affinity column fraction; 2: loaded on Sephacryl S-200 column; 3: Protein molecular marker; 4-6: Pure *Bh*L. **C)** Plot of Molecular mass vs Ve/Vo for the estimation of native *Bh*L**. D)** MALDI-TOF/TOF spectra.

Biochemical and structural studies were carried out using spectroscopic methods to investigate the structure-function relation of the lectin. However, attempts to crystallize *Bh*L using the purified sample were not successful.

3.2 Hemagglutination and sugar inhibition studies

The carbohydrate binding specificity of *Bh*L was determined by carrying out the sugar inhibition assay. The potential of monosaccharides, disaccharides and different sugar derivatives to inhibit lectin activity were investigated and tabulated **(Table 3.2)**. It was found that the simple sugars of monosaccharides, disaccharides and acetylated sugars were ineffective in inhibiting hemagglutination activity of *Bh*L even at very high concentrations (1 M), whereas only *N, N',N''*- Triacetylchitotriose (10 mM) was found to be potent inhibitor but not GlcNAc or chitobiose among the sugars used. All glycoproteins showed complete hemagglutination inhibition of *Bh*L. These observations suggest that the sugar binding site of *Bh*L must be large enough to accommodate larger sugar molecules.

In glycoproteins, the presence of GlcNAc oligomers or those covalently attached with other sugars are oriented in such a way as to make additional interactions with the *Bh*L, thus inhibiting the activity. Our study suggested that with increase in the chain length and complexity of the sugar the inhibitory power for lectin also increased. In this study, no other sacchharide showed similar inhibitory effect, implying that *Bh*L has specificity for *N, N',N''*- Triacetylchitotriose or sugar with higher chain length. The chito sugar binding behavior was found similar to other cucurbitaceous lectins from *T. anguina*

(Narahari *et al.*, 2011), *C. maxima* (Narahari & Swamy, 2010) and *L. acutangula* (Anantharam *et al.*, 1986).

Table 3.2 Carbohydrate inhibition studies of *Benincasa hispida* lectin (*Bh*L)

Sl.No	Sugar	Minimum concentrations needed to inhibit the hemagglutination (µg ml^{-1})
1.	Glucosamine	NA
2.	*N*-acetyl glucosamine	NA
3.	*N, N',N*"- Triacetylchitotriose	48
4.	Fetuin	625
5.	Ovalbumin	1250
6.	Bovine submaxillary mucin	78
7.	Casein	780
8.	Thyroglobulin	40
9.	Rabbit serum	ND

NA: No agglutination *ND: Not determined*

3.3 Effect of metal ions

Dialyzing against EDTA did not affect lectin activity indicating that metal ions had no role in its hemagglutination activity. Interestingly, 50 % of the residual hemagglutination activity was observed only in the presence of Hg^{2+}, Zn^{2+} and Cd^{2+}. 12.5 % of the lectin activity was retained in the presence of Fe^{2+} **(Fig. 3.3)**. The reduction in activity could be due to precipitation of the lectin in the presence of heavy metals rather than them modifying the sugar binding site.

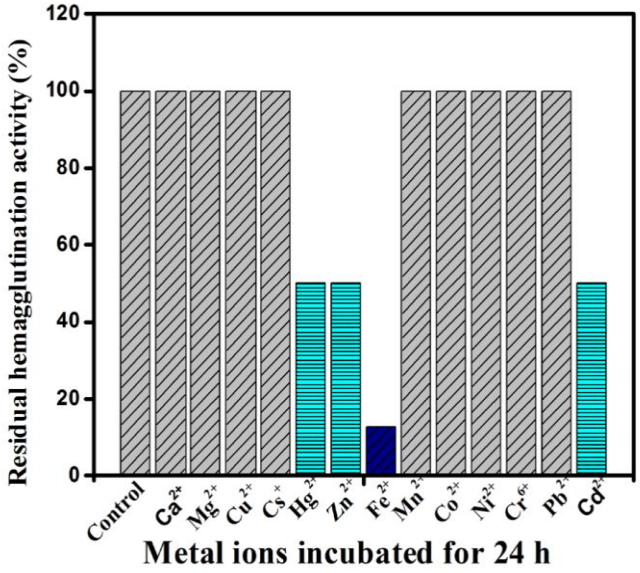


Figure 3.3 Effect of heavy metal ions on *Bh*L (0.1 mg ml^{-1}) hemagglutinating activity.

3.4 Structural analysis

3.4.1 Steady state fluorescence

The scan of intrinsic fluorescence of native *Bh*L was measured with emission maxima (λ_{max}) at 349 nm suggesting the polar environment of tryptophan residues. Total of six Trp residues were estimated through NBS titration of native and denatured protein, and it was found that two Trps are present on the protein surface and four buried. The scan of denatured *Bh*L showed red shift of 7 nm implying exposure of buried Trps in unfolded lectin **(Fig. 3.4.A)**. The tryptophan residues of *Bh*L could be classified into spectral class II (100 %) determined by PFAST decomposition analysis of fluorescence spectra. Class II represents fluorophores in the protein hydrogen bonded with structured water molecules (Reshetnyak & Burstein, 2001).

3.4.2 Circular dichroism (CD) studies

Far-UV CD spectrum of *Bh*L was recorded to estimate the composition of secondary structural elements at 28 ºC. The spectra showed the minima for negative ellipticity at 218 nm indicating high β-sheet content of the protein **(Fig. 3.4.B)**. The analysis of CD data using CONTNLL program of CDPro suite predicted α-helix: 5.5 %, β-sheet: 40.5 %, turns: 21.6 % and random coil: 32.5 % (NRMSD = 0.042). Thus, *Bh*L possesses high β-sheet content and several turns as well as unordered elements. This composition is similar to that found in the structures of SGPL and PPL (Narahari *et al.*, 2011, Narahari & Swamy, 2009). The near-UV CD spectrum showed presence of ordered structure **(Fig. 3.4.C).**

3.4.3 Fourier Transform infrared analysis

FTIR spectrum of *Bh*L (1 mg ml^{-1}) was found to correlate with CD analysis. The spectra showed prominent transmittance minima at 1640 cm^{-1} corresponding to β-sheet (amide I band) and 1698 cm^{-1} for β-turns (amide I band). Interestingly, minima were also observed at 1545 cm^{-1} corresponding to α-helix (amide II) and at 1278 cm^{-1} belonging to unordered structure (amide III) (Adochitei & Drochioiu, 2011) **(Fig.3.4.D)**.

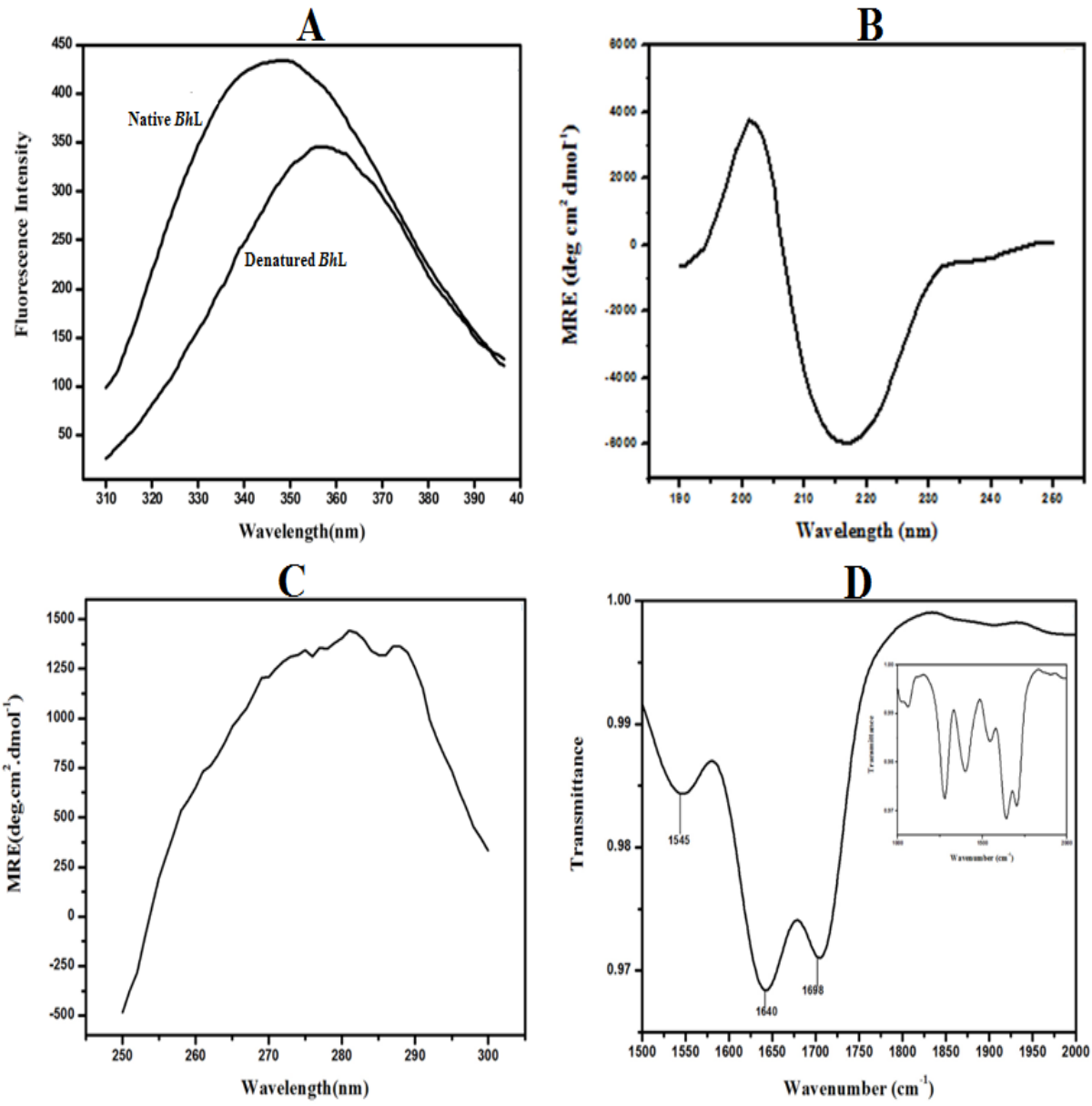


Figure 3.4 Biophysical characterization of *Bh*L. A) Intrinsic fluorescence spectra of native (λ_{max} at 349 nm) and denatured (λ_{max} at 356 nm) *Bh*L (30 µg ml^{-1} at pH 7, 28 °C). **B)** Far-UV CD spectrum (150 µg ml^{-1} at pH 7.0). **C)** Near-UV CD spectra of *Bh*L (1mg ml^{-1}). **D)** FTIR spectrum of *Bh*L in amide I band region (1.5 mg ml^{-1} at pH 7.0). Inset shows spectrum over the range 1500-2000 cm^{-1}.

3.5 Influence of organic solvents on the stability of lectin structure

It is now well known that many proteins undergo folding process through series of a few partially folded intermediate species (Kim & Baldwin, 1990, Fink, 1995, Privalov, 1996). On characterizing such intermediates one can obtain valuable insights into mechanisms involved in protein folding processes (Creighton, 1990). Due to high co-operativity involved in protein folding, it is difficult to obtain structural information directly about these intermediate species (Kim & Baldwin, 1982). Many techniques have been developed to trap these intermediates, but poses difficulties in interpretation of the data because of the existence of multiple unfolded conformations and effect of final refolding conditions (Hughson *et al.,* 1990, Sosnick *et al.*, 1994).

Consequently, it was found that characterizing partially folded state with those at equilibrium would be probable. The unfolding process can be induced in a controlled manner to study intermediate folded states, providing valuable insights into protein folding behavior, structural interactions, and stability. Recently, a potential approach to modulate protein structures has been suggested by many researchers called as ‘‘solvent engineering’’(Klibanov, 1989). Here in this approach, solvents like short chain alcohols (methanol, ethanol, and propanol) and other miscible solvents such as DMSO and ACN have been used as denaturants. These solvents are known to not only stabilize the native like secondary structures (Nelson & Kallenbach, 1989) but can transform other structural elements to non-native structures (Fan *et al.*, 1993). For example, DMSO acts as a strong structure perturbing agent for proteins; where sulfoxide group compete for H-bond and two methyl groups strongly interact with the hydrophobic residues of proteins (Lehmann & Stansfield, 1989).

The present study provides characterization of *Bh*L towards assessing structural stability and tolerance to organic solvents. Interestingly, it was found that *Bh*L showed good tolerance towards high concentrations of organic solvents. Incubating the lectin in 50 % methanol for 24 h had no affect on hemagglutination activity, partial stability was observed in the presence of acetonitrile, while the protein was denatured in ethanol, propanol and DMSO (all 50 % v/v). Far-UV CD spectra also showed no significant change in secondary structure of the lectin in presence of methanol, propanol and acetonitrile whereas complete loss of structure was seen in the presence of ethanol and DMSO **(Fig. 3.5)**. The alteration of the structure in presence of propanol was not favorable for the activity of the protein.

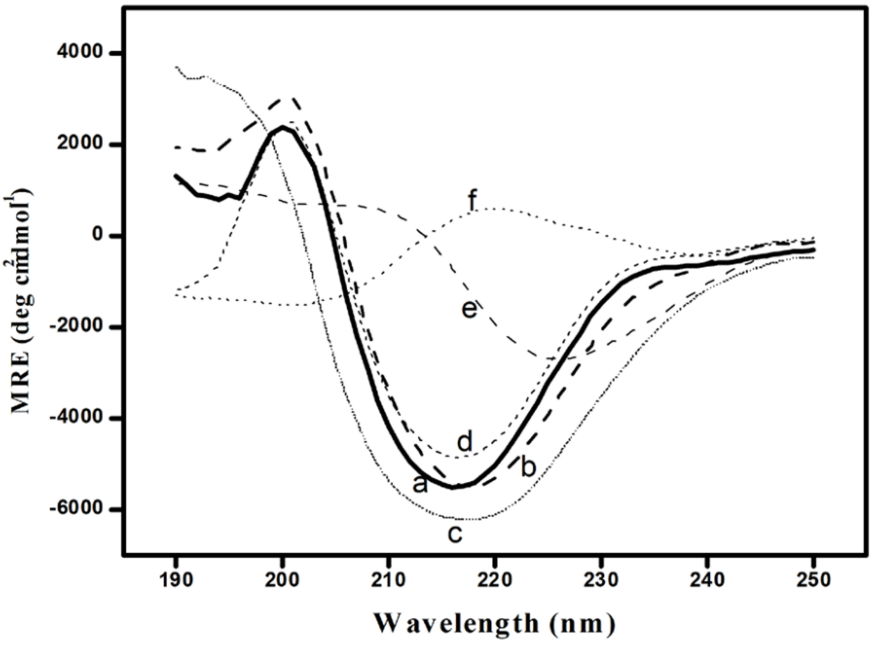


Figure 3.5 Effect of organic solvents on *Bh*L activity. Far-UV CD spectra of *Bh*L treated with 50 % of organic solvents after 4 h; **a**-control, **b**- methanol, **c**- propanol, **d**-acetonitrile, **e**-ethanol and **f**-DMSO.

3.6 Steady state solute quenching studies

The solute quenching studies were carried out to understand the topological and microenvironment of tryptophan residues as the lectin was found to lose hemagglutinating activity on modification by NBS. The obtained fluorescence quenching data was analyzed by using Stern-Volmer and modified Stern-Volmer equation. The fluorescence quenching profile by different quenchers, represented by Stern–Volmer and modified Stern-Volmer plots, are shown in **Fig. 3.6(A-D).** Quenching of intrinsic fluorescence with acrylamide gave linear Stern-Volmer plot for native *Bh*L and showed upward curvature with denatured lectin, indicating presence of both static and collisional conformers of tryptophan. For *Bh*L, the fluorescence quenching efficiency was highest with acrylamide. The K_{sv} value for acrylamide was 8.04 M^{-1} while that for iodide is 2.74 M^{-1} for native lectin. Cesium showed no quenching, implying a positive environment near tryptophan residues **(Table 3.3)**.

The modified Stern-Volmer plot depicted significant increase in the fractional accessibility of tryptophans for KI after denaturation indicating change in the conformation of the protein. A slight increase in K_{sv} value by acrylamide quenching was observed on lectin denaturation. Interestingly, no full accessibility was observed even after denaturation of protein (incubated in 6 M Gdn-HCl) by acrylamide (82 %) indicating presence of some residual structure. The higher K_{sv} and fractional accessibility obtained by acrylamide could be due to its smaller size and capacity to penetrate into the interior of the protein.

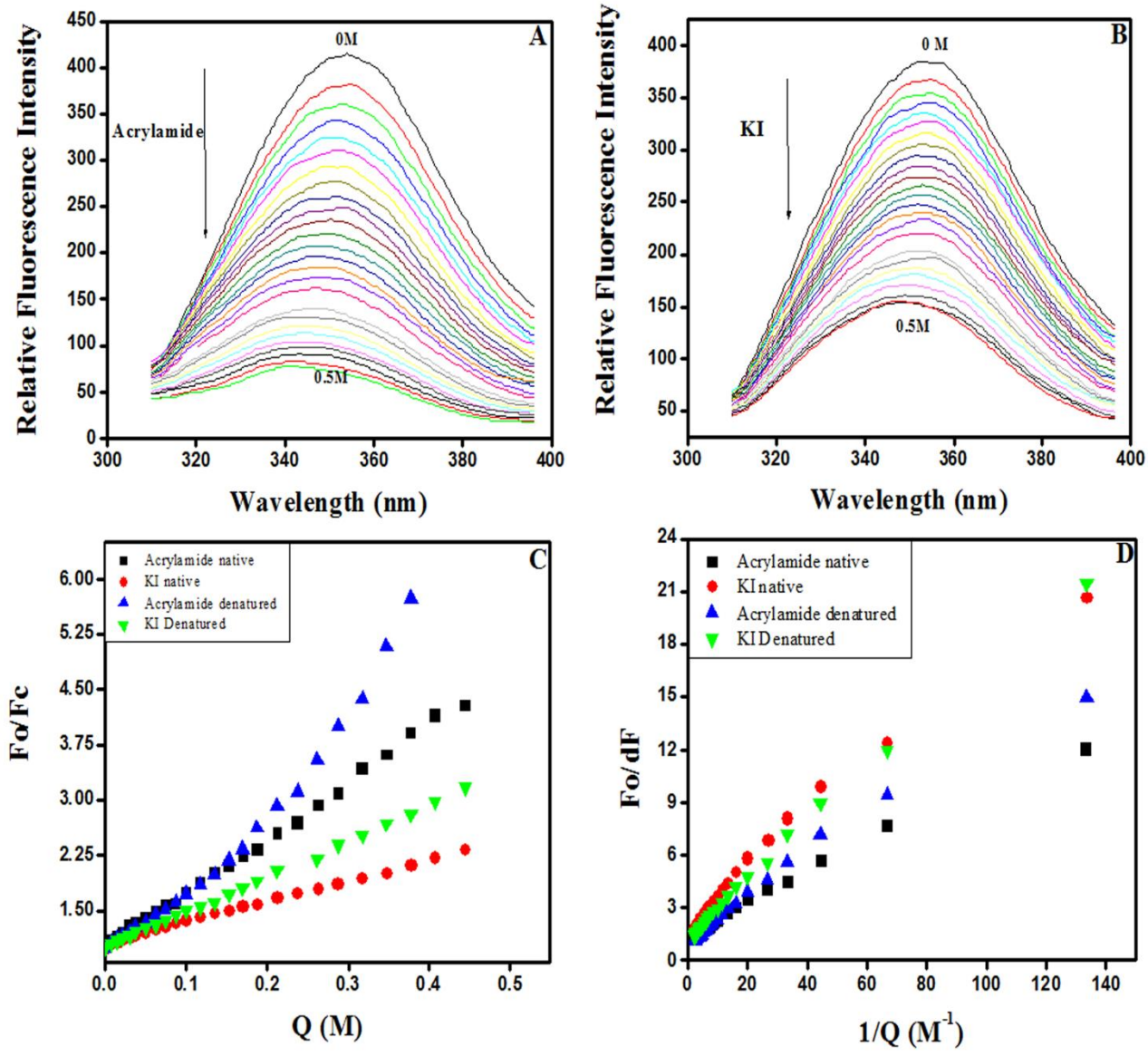


Figure 3.6 Fluorescence quenching studies. Quenching of the intrinsic fluorescence of *Bh*L with **A)** Acrylamide, **B)** Iodide, **C)** Stern-Volmer plot of native and denatured *Bh*L quenched with acrylamide and KI and **D)** modified Stern-Volmer plot.

Table 3.3. Summary of parameters obtained from Stern–Volmer and modified Stern–Volmer analysis of the intrinsic fluorescence quenching of *Bh*L with different quenchers

Quencher and Samples	**K_{sv} (M^{-1})**	***f_a***
Acrylamide		
Native	8.04	0.76
Denatured with 6M GDn-HCl	8.42	0.82
KI		
Native	2.74	0.48
Denatured with 6M GDn-HCl	3.79	0.63

3.7. Fluorimetric analysis of Sugar/ligand binding

3.7.1 Sugar Binding

Fluorescence titration of the purified lectin with sugars might vary tryptophan microenvironment by fluorescence intensity quenching/enhancement process (Mahanta *et al.*, 1992). These changes in the fluorescence intensities were exploited to determine the association constants of various sugars and ligands bound. The *B. hispida* lectin did not bind to any monosaccharides or disaccharides as there was no change in fluorescence intensity on additions of these sugars. From the hemagglutination inhibition studies (***Section 3.2***), it is clear that *Bh*L is a chito-oligosaccharide-specific lectin, but the titration of the lectin with glucosamine and GlcNAc did not show any significant changes in the fluorescence intensity. However, titration with higher chain length sugars like *N,N'*-Diacetylchitobiose and *N,N',N''*-Triacetylchitoriose, resulted in 23 % and 37 % enhancement in the fluorescence intensity, respectively, without any change in emission

maxima. The slope of the plot of log $[(F_0 - F_c)/(F_c - F_\infty)]$, versus log $[C]_f$ was near unity, implying presence of a single binding site on the protein molecule **(Fig. 3.7)**. Association constants estimated for the binding of various sugars at 25 °C are listed in **Table 3.4**. It was found that association constant (K_a) calculated for chitotriose (K_a = 1.0 x 10^4 M^{-1}) was 83 fold more than that of chitobiose (K_a = 1.2 x 10^2 M^{-1}) indicating presence of extended sugar binding site on *Bh*L, as already discussed. The negative ΔG values indicated spontaneous nature of binding **(Table 3.4)**.

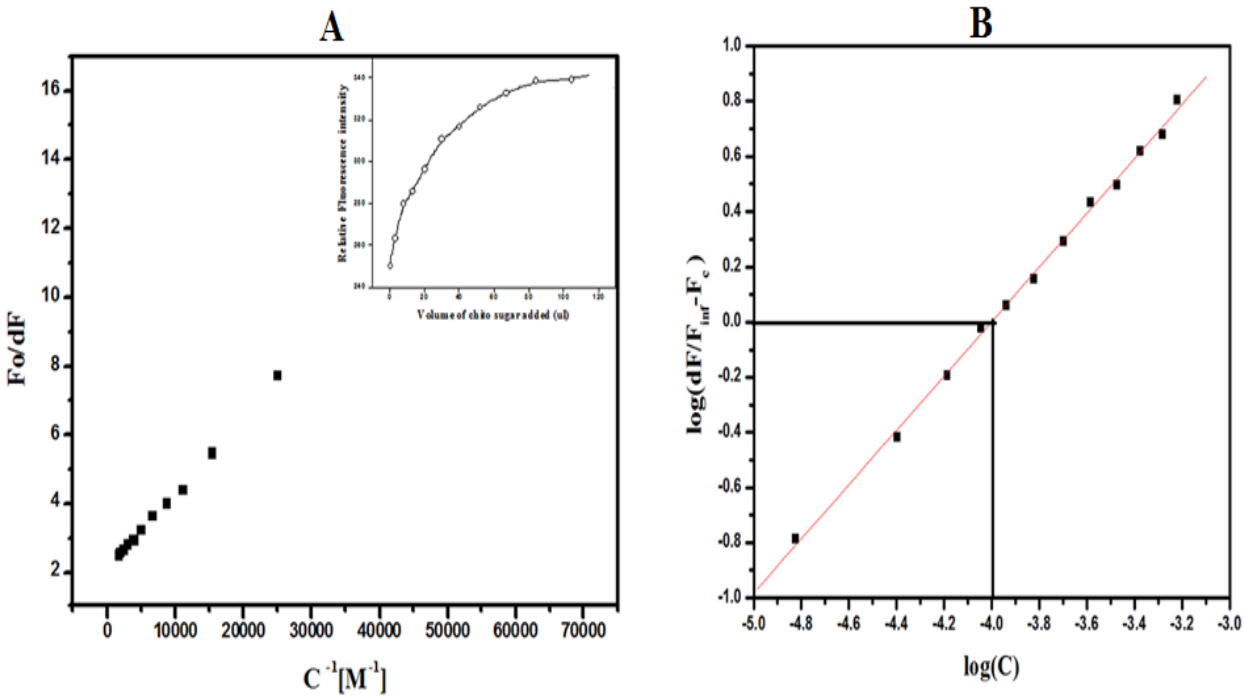


Figure 3.7 Plots used for the calculation of binding constants for the sugars binding to *Bh*L (0.03mg ml^{-1}). A) F_0/dF vs C^{-1}, **B)** log $[dF/(F_\infty - F_c)]$ vs log(C).

Table 3.4 Association constants, K_a, calculated for various chito-sugars and ligand at 25 °C with *Bh*L and the corresponding Gibb's free energy values

S.No	Sugar/Ligand	K_a (M^{-1})	-ΔG kJ mol^{-1}
1	Gluocosamine	NB	-
2	*N*-Acetyl Gluocosamine	NB	-
3	*N,N'*-Diacetylchitobiose	1.2×10^2	11.8
4	*N,N',N''*-Triacetylchitoriose	1.0×10^4	17.1
5	Adenine	1.0×10^4	17.1

NB: No binding *ND: Not determined*

3.7.2 Adenine Binding

Binding of adenine resulted in quenching of the fluorescence intensity along with a red shift in λ_{max} indicating conformational changes in protein. The estimated binding constant calculated for adenine binding to *Bh*L was 1×10^4 M^{-1} **(Table 3.4).** In case of legume lectins, they are known to bind adenine effectively suggesting some physiological importance for this property in these plants. For example, lima bean lectin from *Phaseolus lunatus* showed maximum affinity towards adenine ($K_a = 8.3 \times 10^4$ M^{-1}) (Roberts & Goldstein, 1983) whereas winged bean lectin has K_a close to 1.5×10^4 M^{-1} (Puri & Surolia, 1994). Adenine and adenine derived plant growth regulators are hydrophobic molecules and bind to lectin at sites different from saccharide binding site (Etzler *et al.*, 1981, Gegg *et al.*, 1992). As far as adenine binding is concerned, this is the first report of a chito-oligosaccharide-specific lectin binding to adenine.

3.8 Conformational transitions studies

3.8.1 pH induced changes on the structure and activity of BhL

a) Effect of pH on hemagglutination activity

*Bh*L was found to be highly stable in the broad pH range (1-12) retaining full hemagglutination activity on incubating with different pH buffers for 24 h**.** This observation implied the high stability and compactness of the lectin at extremes of pH. There are a few reports on plant lectins possessing stability in a wide range of pH, for instance, lectins from *Alocasia cucullata* (Kaur *et al.*, 2005) and *Allium sativum* (Clement & Venkatesh, 2010) are reported pH stable.

b) Intrinsic fluorescence

The effects of pH induced changes on the structure of *Bh*L were studied using spectroscopic techniques. As discussed before, native *Bh*L at pH 7.4 showed the maximum at wavelength 349 nm in the intrinsic fluorescence studies indicating solvent exposed tryptophan with Class II conformer. The fluorescence intensity of the lectin decreased at highly acidic or highly alkaline pH as compared to neutral pH. As there was no change in the emission maxima (λ_{max}), except some minor changes in the Trp microenvironment, no major structural transition was observed in the protein molecule at any of the pH. Thus, no loss in lectin activity was observed. The reduction in fluorescence intensity was due to protonated and deprotonated forms of the amino acids present on the surface, which eventually affects the emission of the Trp residues **(Fig. 3.8)**.

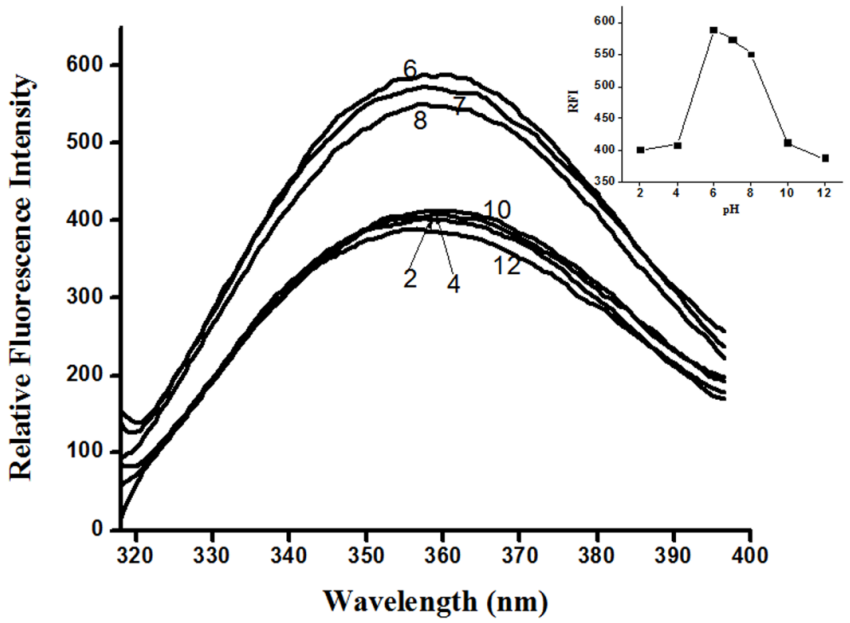


Figure 3.8 pH induced changes of *Bh*L. Fluorescence intensity spectra of *Bh*L incubated in different pH buffers for 24 h (Inset: The relative fluorescence intensity (RFI) curves at various pH).

c) CD analysis

Structural changes induced by the extremes of pH on *Bh*L were analyzed by recording the CD spectra of protein incubated in different pH buffers (1-12) for 16 h. The far-UV CD spectra also revealed no change in the secondary structure throughout the pH range. The protein could retain the major part of the structure, but few variations were seen at MRE 200 nm implying slight decrease in ordered structure **(Fig. 3.9)**. Thus, from above studies we can conclude that *Bh*L is stable in all pH conditions with no loss in structure and function.

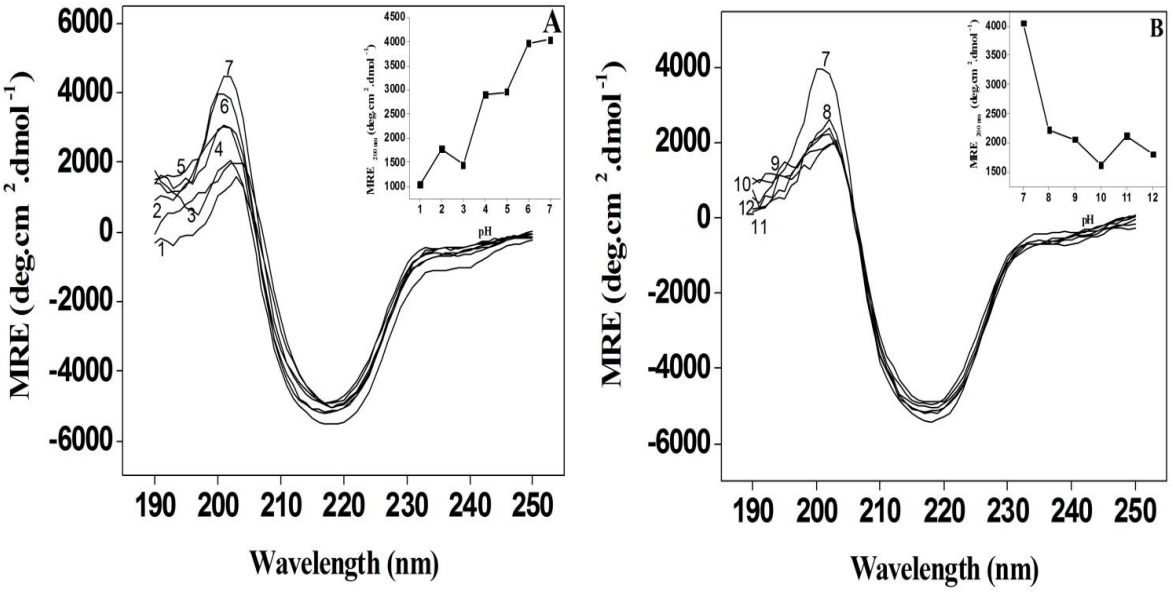


Figure 3.9 pH induced structural changes in *Bh*L as seen in the far-UV CD spectra of *Bh*L (150 µg ml^{-1}) incubated for 24 h at respective pH. The numbers indicate pH value.

3.8.2 Thermostability of BhL

a) Effect of temperature on lectin activity

The hemagglutination activity of *Bh*L was monitored at different temperatures (25-95 °C), and found to be stable till 60 ºC for 1 h retaining 100 % of its activity. On increasing the temperature, a slow decay curve was seen implying denaturation of protein. Sigmoidal fit (R^2 = 0.95) analysis of the thermal denaturation curve indicated T_m of the protein to be 82.6 °C **(Fig. 3.10.A).**

b) Intrinsic fluorescence

Fluorescence intensity gradually decreased with increasing temperature. At 90 °C the fluorescence intensity of the lectin remaining was 40 % of the intensity at room temperature. This could be due to the deactivation of the singlet excited state **(Fig.**

3.10.B). No light scattering intensity was observed till 90 °C, however, a sudden increase was seen at 95 °C when measured in time drive module. This observation implied that only at very high temperatures the lectin had undergone aggregation. On decreasing the temperature back to 25 °C, more protein aggregated indicating irreversible thermal aggregation **(Fig. 3.10.B)**.

c) CD analysis

Structural transitions occurring in *Bh*L on incubation at different temperatures at fixed pH 7.4 were studied. It was observed that till 80 ºC there was no significant change in secondary structure except an increase in the content of turns by 7 % **(Fig. 3.10.C)**. This evidence supports the existence of a rigid and compact structure of the lectin; thus, some residual activity was observed at higher temperatures also. The sigmoidal fit (R^2 = 0.99) analysis of change in ellipticity at 218 nm gave an estimated T_m value of 82 ºC, closer to value estimated using hemagglutination assay. The decrease in MRE values at 200 nm implies an increase in disorder as the temperature rises **(Fig.3.10.D)**. The thermal unfolding and resultant aggregation observed at pH 7.4 was found to be irreversible, since the lectin started aggregating further on re-cooling. This thermostability of *Bh*L was found to be higher compared to other Cucurbitaceae lectins such as lectin from *C.maxima,* which is stable till 70 °C (Narahari & Swamy, 2010) or that from *T. anguina* phloem exudates stable till 50 °C (Narahari *et al.*, 2011).

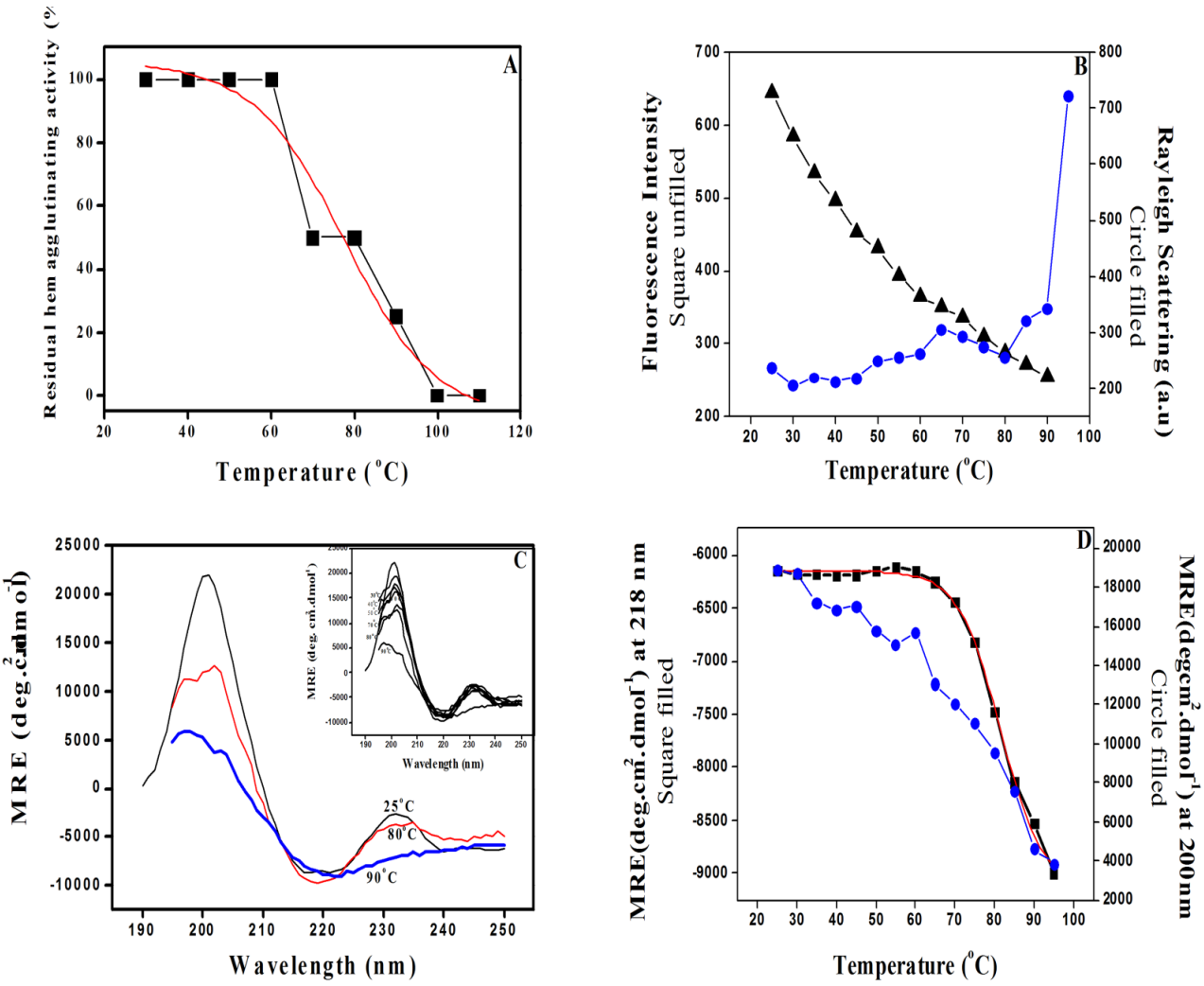


Figure 3.10 Thermal denaturation of *Bh*L. A) Effect of temperature on the hemagglutinating activity and Sigmoidal fit. **B)** Plot of fluorescence intensity and Rayleigh scattering vs temperature. **C)** Far-UV CD spectra of *Bh*L (150 µg ml^{-1}) incubated at respective temperatures for 5 mins. The numbers on the spectra indicate temperatures. **D)** Change in ellipticity at 200 and 218 nm during thermal denaturation is shown. Plot consists of MRE_{200} and MRE_{218} vs temperature, and Sigmoidal fit analysis.

3.8.3 Chemical denaturation of BhL

a) Effect of denaturants on lectin activity

*Bh*L was incubated with increasing concentrations of Urea and GDn-HCl (0-6 M) for 24 h. It was found that the lectin retained 25 % hemagglutination activity in the presence of 6 M GDn-HCl **(Fig. 3.11.A)**, whereas in the presence of urea no loss in activity was observed. This implies that the lectin has rigid and compact structure maintaining its activity. The denaturant in the lectin gets diluted during serial dilution for hemagglutination assay; hence denaturation could be reversible to some extent.

b) Intrinsic fluorescence studies:

A 6 nm red shift was observed in the λ_{max} of the intrinsic fluorescence of *Bh*L in the presence of 5 M GDn-HCl, indicating complete exposure of tryptophan residues due to unfolding of the lectin **(Fig. 3.11.A)**. The shape and position of tryptophan spectrum was characterized using Parameter A (I_{320}/I_{365}) analysis, monitoring the sensitivity of lectin to different GDn-HCl concentrations. The tryptophan conformer class also changed from II to III, further indicating complete exposure of tryptophan residues due to unfolding of the lectin molecule **(Fig. 3.11.B)**. The existence of intermediate species during unfolding process was inferred from a phase diagram plot (I_{320} vs I_{365}). The point of intersection of the two linear portions corresponds to the existence of an intermediate at 3.5 M concentration of GDn-HCl **(Fig. 3.11.C)**. Interestingly, both a pronounced secondary structure and hemagglutinating activity were simultaneously observed at this concentration (3.5 M) of GDn-HCl.

c) CD analysis

From the far-UV CD spectra, it is seen that the structure gradually opens up with increasing concentration of GDn-HCl. A complete loss of *Bh*L secondary structure at 4 M GDn-HCl was observed **(Fig. 3.11.C)**. At this denaturant concentration, the lectin retained complete hemagglutination activity as that of the native lectin already discussed. This could be due to the presence of residual structure retained in the protein which helps to correctly refold the protein on increasing denaturant dilution. Based on the alteration seen in the structure of protein in presence of GDn-HCl, as observed in the fluorescence and far UV-CD spectra, it could be suggested that this denaturation is reversible in nature.

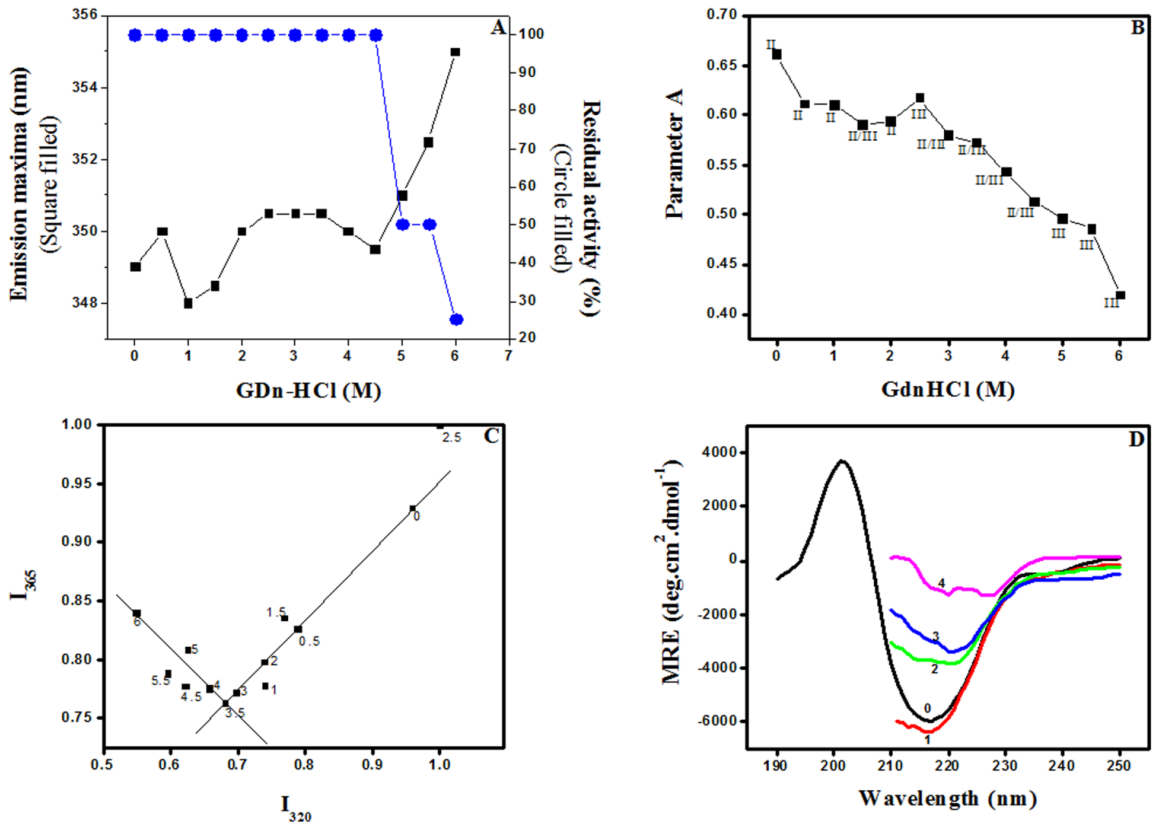


Figure 3.11 Effect of GDn-HCl on *Bh*L. A) λ_{max} shift and hemagglutination activity vs GDn-HCl concentration. Lectin was incubated with required GDn-HCl concentration, 20 mM

Phosphate buffer, pH 7.4 for 16 h at room temperature. **B)** Parameter A analysis showing tryptophan conformer classes. **C)** Phase diagram. The numbers indicate the GDn-HCl concentrations. **D)** Far UV-CD spectra of *Bh*L in presence of GDn-HCl. The protein sample was incubated for 4 h in respective denaturant buffers. The numbers on the spectra indicate GDn-HCl concentration.

3.9 Hydrophobic dye binding

ANS binding to the lectin was not observed at any of the higher temperature range, at extreme pH conditions or during chemical denaturation (data not shown) indicating no exposure of the hydrophobic patches on the surface or absence of that in the proteins. May be due to this, the lectin is highly soluble with less propensity for aggregation.

3.10 Mass spectrometry analysis

MALDI TOF-MS/MS and LCMS were carried out to find the sequence of the lectin. The data obtained are the average peaks of peptides after running the trypsin digested products. For database analysis (run on PLGS software), the lectin database was used (Downloaded from Uniprot). Unfortunately very few sequence similarities were found but seems to be unrelated **(Table.3.5)**. For example, *Bh*L fragments showed similarity *Brassica napus* and some stress responsive protein which are highly unlikely for lectin characteristics. It was not possible to deduce the primary sequence of the lectin due to lack of sufficient homology for sequences in the databases. Hence, different methodologies need to be tried to determine the primary sequence.

Table. 3.5 Analysis of LCMS data using PLGS software

Sl.No.	Species	Accessions	Coverage
1.	BnaA06g01980D protein OS Brassica napus GN BnaA06g01980D PE 4 SV 1	A0A078GC10	9
2.	Uncharacterized protein OS Beta vulgaris subsp vulgaris GN BVRB 6g130310 PE 4 SV 1	A0A078J6J8	15
3.	Ricin type beta trefoil lectin domain protein OS Streptomyces turgidiscabies Car8 GN STRTUCAR8 02463	L7FB30	34
4..	Stress responsive protein OS Zea mays GN Zm 78408 PE 2 SV 1	B6TF92	65

3.11 Conclusion

In the present study, a Cucurbitaceae plant, *B. hispida* was selected for isolation of one of the lectins present in it and its characterization. Purification of *B. hispida* lectin (*Bh*L) was carried out using chitin affinity and gel filtration chromatography techniques. The lectin identified forms a homodimer with subunit molecular weight of 17 kDa, as shown by SDS-PAGE and MALDI-TOF/TOF analysis. Hemagglutination inhibition studies interpreted that *Bh*L is specific to GlcNAc oligomers only. pH stability studies showed that the lectin is active in all pH. The temperature stability studies have shown that *Bh*L is a thermostable protein retaining 12.5 % of its activity at 90 °C. Also, the hemagglutination activity of *Bh*L is not inhibited by EDTA nor increased by the addition of any divalent metal ions suggesting that *Bh*L does not need any of these ions for its agglutination activity. Chemical denaturation studies of *Bh*L by using urea and GDn-HCl has indicated that the lectin is stable in the presence of urea but loses 50 % of its

hemagglutination activity at 5 M GDn-HCl. Fluorescence spectral analysis revealed that the Trp conformer belonged to Class II with emission maxima at 349 nm. The far UV-CD spectra indicated the predominant β-sheet structure of *Bh*L and less of α-helical structure, a feature of Cucurbitaceae lectins, similar to PPL (Narahari & Swamy, 2010). *Bh*L was also stable in the presence of organic solvents indicating compactness of the structure. Absence of hydrophobic patches and low propensity for aggregation makes *Bh*L worthy for its application in many biomedical strategies. The lectin seems to possess a rigid structure and does not get easily unfolded at applied denaturing conditions. Binding affinity of *Bh*L towards chito oligosaccharides does establish the potential applicability of the lectin. Adenine binding might be correlated to physiological function of *Bh*L in plant. Crystallographic studies were attempted but not successful in crystallizing the protein.

Chapter 4

Biochemical and biophysical characterization of a cytokinin binding agglutinin purified from the seeds of *Datura innoxia*

Datura innoxia belongs to Solanaceae family, also called toloache, an annual shrub plant with white flowers, and typically grows till 1.5 to 2 m in height. *D. innoxia* has hairy stem and leaves; and the fruits are droopy fully covered with short thorns. The seeds are white at early stage and on maturing becomes orange to brown in color (Ratsch, 1998). Most people find foul odor from the entire part of the plants whereas few finds fragrance of the flowers pleasant when it blooms at night. Its origin is in the American Southwest and Mexico, but commercially grown in Central America, North Africa, India, and England as a source of scopolamine used as drug in the pharmaceutical industry (Dafni & Yaniv, 1994).

Datura innoxia, is known to be the most ethnopharmacologically important plant among the thorn apple species; the therapeutic uses of *Datura* has been well described in the texts of Ayurveda in more than 3 samhitas, 9 samgraha granthas, 7 nighantus and other published texts. It has been reported that the root, root bark, leaves, seed, oil are used as different ingredients in 521 formulations, which are made to treat more than 50 disease conditions. For example, the main indications include *vatavyadhi* (diseases of joints), *aamavata* (rheumatoid arthritis), *vrana* (wound), *agnimandya* (loss of appetite), *jvara* (fever), *grahani* (irritable bowel syndrome) and many more (Doshi *et al.*, 2015).

In addition to these benefits, many researchers have demonstrated the anitimicrobial activity against many pathogenic organisms of the methanolic and ethanolic extracts prepared from *D. innoxia* (Eftekhar *et al*., 2005, Gachande & Khillare, 2013). Using plants as a source of medicine has been inherited by generations as an

important part of the health care system. There are more than 45,000 identified plant species in India, with 3000 reported to be of medicinal importance.

Figure 4.1. *Datura innoxia* flower and fruit with seeds.

Very few reports are available, regarding the bioactive proteins present in *Datura* species such as lectins and their medical importance. For instance, a lectin purified from *D. stramonium*, a closely related species, was reported to be mitogenic in action (Crowley & Goldstein, 1982). Lectins with high molecular weight, around 150 and 300 kDa, have been reported earlier from the same with mitogenic activity (Levitskaya *et al.*, 1985). Other high molecular weight *D. innoxia* lectins (82, 54, 49 and 41 kDa) with sugar specificity towards GlcNAc oligomers are also reported (Petrescu *et al.*, 1993).

This chapter describes the isolation, purification and characterization of the smallest agglutinin from the seeds of *D. innoxia o*f MW 9 kDa (*Di*L9). This lectin is being reported for the first time and we have characterized the protein using biochemical

and biophysical techniques as part of an effort to expand the knowledge of lectins for novel applications.

4.1 Isolation and purification of Datura innoxia lectin (DiL9)

The *Di*L9 agglutinin was isolated and purified from the crude extract prepared from the seeds of *D. innoxia* in 20 mM sodium phosphate buffer pH 7.4. The extract was filtered using muslin cloth and centrifuged at 10,000 rpm for 20 min at 4 °C. Later, 60 % ammonium sulphate precipitation was carried out and dialyzed against the above said buffer. The dialysate was loaded on Q-Sepharose column equilibrated with the same buffer and the lectin of interest eluted with the unbound fractions. The fractions were pooled, concentrated and dialysed against 50 mM Tris-HCl buffer, pH 8.0. This preparation got resolved into 2 peaks, P1 and P2, both showing lectin activity on gel filtration column **(Fig. 4.2)**.

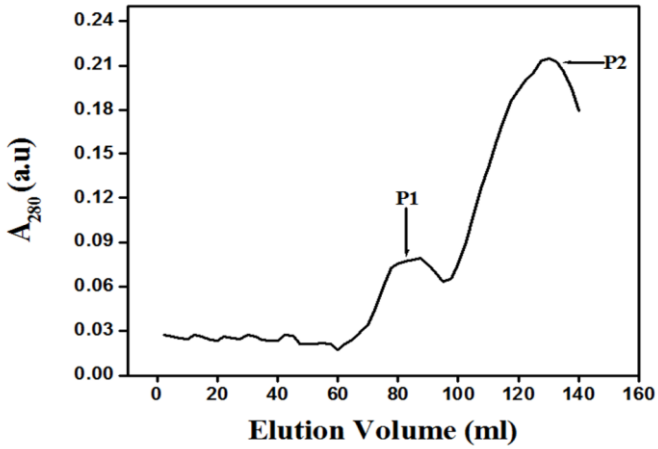


Figure 4.2 Purification profile of *Di*L9 using Sephacryl S-200 gel filtration chromatography column, equilibrated with TBS at flow rate of 1 ml min^{-1}. The column resolved the loaded preparation into two different peaks (P1 & P2). Here, the peak P2 corresponded to *Di*L9.

In **Figure 4.2** the peak P1was identified as the already reported 88 kDa lectin from *D. innoxia* seeds (Petrescu *et al.*, 1993), whereas the peak P2 was the smaller molecular weight lectin *Di*L9 of our interest. Since this lectin has not been reported yet, we have undertaken to characterize it in this study. The purification procedure, specific activity, total activity and protein enrichment fold of *Di*L9 lectin at each step is summarized in **Table 4.1**. It is interesting to note that, at each step of purification there is a considerable decrease in activity per unit weight of protein and purification fold; this may be because *Datura* seeds are rich in different types of lectins and at each chromatographic step removal of other lectins from the preparation is taking place.

Table 4.1 Summary of purification of *Datura innoxia* lectin (*Di*L9)

Fractions	Volume (ml)	Total protein(mg)	Total activity	Specific activity[1]	Fold purification[2]	% yield
Crude extract (Prepared from 20 g dried seeds)	230	529	36800	69.56	1.0	100
Q- Sepharose	70	62	11200	180.6	2.6	30.4
Sephacryl S-200	22	**26.4**	880	34	0.48	2.4

[1] *Specific activity is defined as the hemagglutination unit (HU) divided by the protein concentration (mg ml^{-1}) of the assay solution.*

[2] *Purification fold was calculated as the ratio between the minimal concentration of the crude extract able to cause visible agglutination of the rabbit erythrocytes and that of the protein fraction obtained at each purification step.*

4.2 Molecular weight determination

The molecular weight of the lectin was determined using both SDS-PAGE and MALDI-TOF/TOF technique. The purity and homogeneity of the preparation was checked at each step of chromatographic techniques using SDS-PAGE under reducing conditions. The 60 % ammonium sulphate precipitated protein showed presence of multiple bands ranging from 6 to 97 kDa. After Q-sepharose chromatography, the unbound fractions with hemagglutinating activity collected showed only two bands of approximately 9 and 88 kDa. As already mentioned, the 88 kDa lectin was previously reported, so we focused on the small agglutinin which was finally purified using Sephacryl S-200 **(Fig. 4.3.A).** The *Di*L9 moved as a single band in a native polyacrylamide gel electrophoresis run. MALDI-TOF/TOF estimated the exact molecular weight to be 9656.6 Da, a monomeric lectin **(Fig. 4.3.B)**. To distinguish this from other lectins of the same source it is designated as *Di*L9 for *Datura innoxia* lectin of molecular weight 9 kDa.

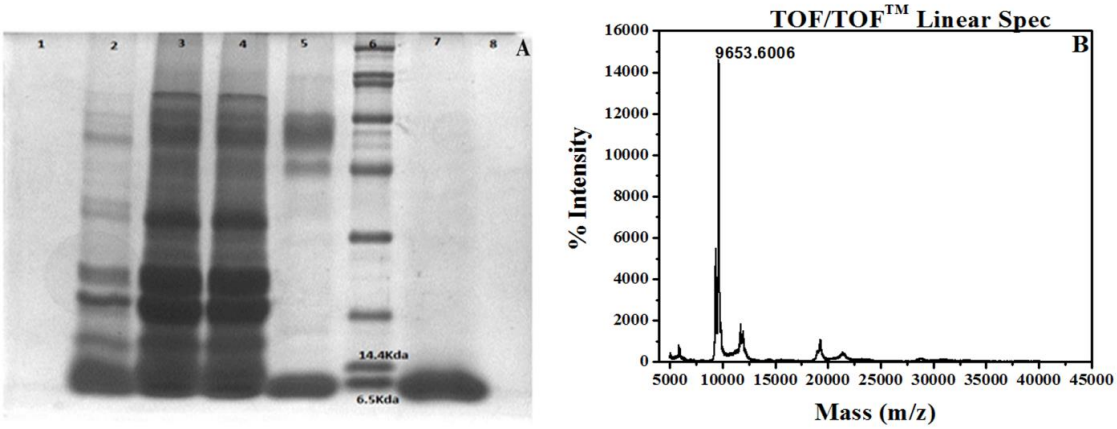


Figure 4.3 Estimation of molecular weight of *Di*L9. (**A)** Purity check on 12 % SDS-PAGE. Lane 2: crude extract, lane 3- 4: 60 % ammonium sulphate precipitate, lane 5: preparation loaded

on Sephacryl S-200 column, 6: Protein molecular weight marker, 7: Pure *Di*L9. (**B)** MALDI-TOF/TOF spectra.

4.3 Estimation of carbohydrate content

The total neutral carbohydrate content was estimated using phenol-sulphuric acid assay to be around 4 %. Hence, this lectin is considered a glycoprotein, although has lower sugar content compared to the other lectins reported from the same source. For example, 25 % sugar content was reported for 88 kDa agglutinin from *D. innoxia* seed (DIA) (Petrescu *et al.,* 1993). Lectins reported from Solanaceae family, potato lectin (Allen & Neuberger, 1973) and Jimson weed lectin (Crowley & Goldstein, 1982) had 50 % and 40 % carbohydrate content, respectively.

4.4 Sugar inhibitors of DiL9

The carbohydrate binding specificity of the *D. innoxia* lectin was studied by carrying out the hemagglutination inhibition assay. Different sugars (mono-/ di-saccharides/ derivatives) and glycoproteins were used for the investigating inhibition of lectin activity. Low activity was observed when assayed with rabbit erythrocytes even at high lectin concentration whereas a minimal concentration of *Di*L9 was required when pronase treated erythrocytes were used. This might be due to unavailability or weak interactions with the glycans present on the erythrocytes membrane by *Di*L9. The enzyme, pronase is a nonspecific protease cleaving membrane proteins randomly present on erythrocytes and exposing other glycoproteins.

It was found that only GlcNAc oligomers, i.e., *N,N',N''*-Triacetylchitotriose (2.5 mM) showed inhibitory action and not glucosamine, GlcNAc and *N,N'*-

Diacetylchitobiose **(Table 4.2)**. These observations confirmed that the sugar binding site requires at least three β (1-4)-linked *N*-acetyl glucosamine units. Similarly, other Solanaceae lectins from *D. stramonium* (Desai *et al.*, 1981), *S. tuberosum* (Kilpatrick, 1980) and *U. dioica* (Peumans *et al.*, 1984) were also reported to possess only GIcNAc oligomer sugar specificity. The glycoproteins like fetuin, ovalbumin, mucin and thyroglobulin tested against *Di*L9, also showed hemagglutination inhibition at low concentrations, similar to *Bh*L discussed in Chapter 3.

Table 4.2 Hemagglutination inhibition activity of *Di*L9 by saccharides and glycoproteins

S.No	Sugar / Glycoprotein	Minimum concentrations required to inhibit *Di*L9 activity (μg ml^{-1})
1.	Glucosamine	NI
2.	*N*-acetyl glucosamine	NI
3	*N,N'*-Diacetylchitobiose	NI
4.	*N, N',N*"- Triacetylchitotriose	750
5.	Fetuin	62.5
6.	Ovalbumin	78
7.	Bovine submaxillary mucin	156
8.	Thyroglobulin	9

NI: No inhibition

4.5 Effect of metal ions on lectin activity

On incubation with EDTA and other metal ions no change in lectin agglutination activity was observed, suggesting that *Di*L9 could be a lectin not dependant on metal ions for hemagglutination activity.

4.6 Proteolytic digestion and DiL9 activity

The effect of digestion by various proteolytic enzymes having different specificity on the hemagglutination activity of *D. innoxia* lectin was investigated. Surprisingly, no change in the hemagglutination activity of the lectin was observed when incubated with trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), pepsin (EC 3.4.23.1) and papain (EC 3.4.21.62) at 37 ºC for 24 h (data not shown). However, 50 % loss in lectin activity was detected on incubation with protease (Nagarse; 3.4.21.62). This aspect of *Di*L9 was found similar to other *Datura* lectins having proteolytic resistance (Levitskaya & Yunusov, 1996). The resistance towards proteolysis by *Di*L9 and other *Datura* lectins was accredited to the fact that they were glycoproteins and stabilized by numerous disulphide bonds capable of maintaining the native structure under stress.

4.7 Structural analysis using fluorescence and CD

The steady state intrinsic fluorescence of native *Di*L9 gave λ_{max} (emission wavelength) of 354 nm implying presence of exposed tryptophan residues on the protein surface. Using NBS titration, a total of 4 Trp residues were estimated, of which 3 were present on the surface and one was buried. Denatured *Di*L9 (incubated in 6 M GDn-HCl) showed a red shift of 3 nm in the λ_{max} **(Fig. 4.4.A)** suggesting unfolded lectin exposing

the buried Trp residues also to the polar solvent along with the shifting of λ_{max} towards longer wavelengths. Also, the PFAST decomposition analysis revealed Class III conformers of Trp residues.

To estimate the secondary structural elements, the far-UV CD spectrum of *Di*L9 was recorded **(Fig. 4.4.B)**. Using CONTINLL program of CDPro analysis, the secondary structural components were calculated as α-helix: 31.5 %, β-sheet: 17.9 %, turns: 20.7 % and random coil: 29.8 %. Previously, two high molecular weight lectins (150 kDa and 300 kDa) from *D. innoxia* were characterized using CD spectral studies and shown to have polyproline II type conformations (Levitskaya & Yunusov, 1995). As already known, the negative MRE of Cotton effect at 200 nm also implied a random-coil conformation (Fasman *et al.*, 1970). These conformations were absent in *Di*L9 and thus, differing from other *D. innoxia* lectins with respect to their structural features.

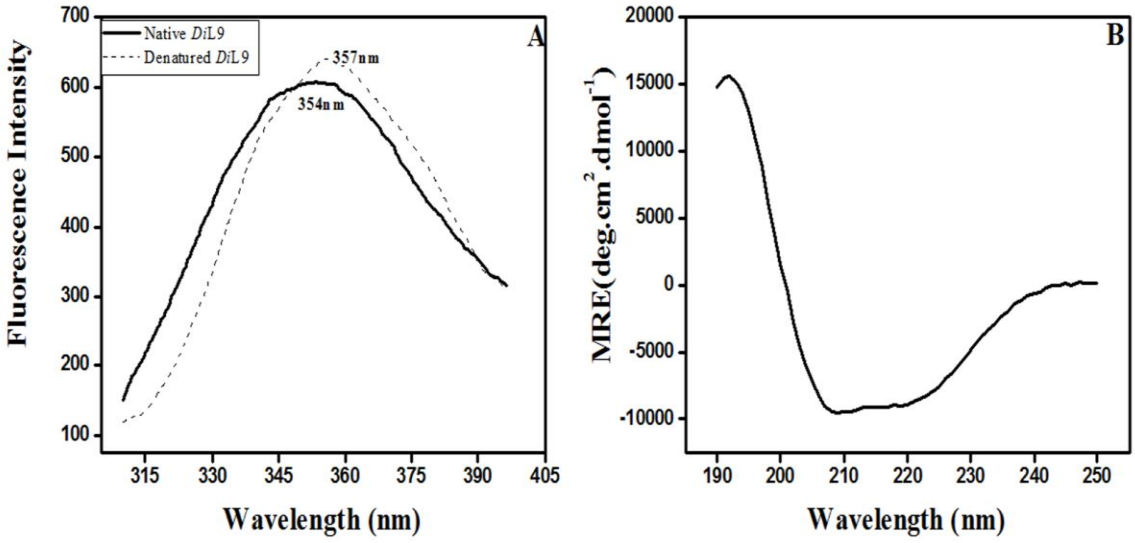


Figure 4.4 Biophysical characterization of *Di*L9. (A) Intrinsic fluorescence spectra of native (λ_{max} at 354 nm) and denatured (λ_{max} at 357 nm) *Di*L9 (0.05 mg ml^{-1} at pH 7.4, 28 °C). **(B)** Far-UV CD spectra of *Di*L9 (0.2 mg ml^{-1}).

4.8 Chemical modification studies

Within the folded structure of a protein, the micro-environment of protein side chains are quite flexible exhibiting reactivity different from those expected from computational studies of model compounds. Here, in this study, purified *Di*L9 was subjected to various chemical modifications in order to determine the amino acid residues that are involved in sugar binding site. In the presence of 2 mM NBS, a complete loss in lectin activity was observed, suggesting the direct role of tryptophan residues in sugar binding. The residual hemagglutination activity of about 50 % and 25 % was observed on NAI and WRK treatment indicating possible role of tyrosine and aspartate/glutamate residues, respectively, near the sugar binding pocket of the protein **(Table 4.3)**. The other chemical modifiers had no effect on *Di*L9 hemagglutination activity. The far-UV CD spectra also showed disruption of secondary structure in the presence of these chemical modifiers (2 mM, 4 h incubation). This loss in structure might be the reason for loss of lectin activity. Incubating the lectin with NBS resulted in complete loss of structure, and hence no activity was observed **(Fig. 4.5).** From these observations it can be concluded that Trp, Tyr and Asp/Glu are involved in carbohydrate binding region of *Di*L9 lectin.

Table 4.3. Effect of chemical modification on *Di*L9 activity

Sl.No	Chemical Modifier	Concentration (mM)	Residual hemagglutinating activity (%)[#]
1.	Native *Di*L9	0	100
2.	WRK	5	25
3.	NAI	10	50
4.	NBS	2	0

 # Total activity (100 %) of DiL9 corresponds to titre of 2^6

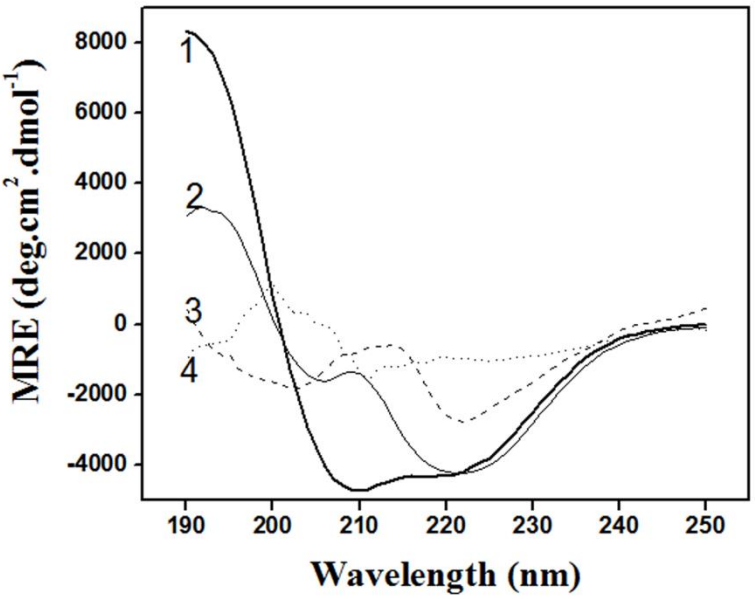


Figure 4.5 Far-UV CD spectra of chemical modification effects on *Di*L9; 1- Native *Di*L9, **2**-NAI, **3**-WRK and **4**- NBS treated *Di*L9. The lectin (0.2 mg ml^{-1}) was incubated with 2 mM of each chemical modifier for 4 h.

4.9 Stability of DiL9 in various organic solvents

Organic solvents have different physical properties like miscibility, hydrophobicity and hydrogen bonding capacity which can have drastic influence on the structural and functional aspects of proteins/enzymes. Here, the effects of various organic solvents on *Di*L9 lectin activity was investigated and found out that it showed good tolerance towards high concentrations of organic solvents. In the presence of methanol and ACN (all 50 % v/v), the lectin retained its complete activity and remained constant up to 24 h of incubation. The structural studies using far-UV CD spectra of *Di*L9 in presence of organic solvents also showed increase in negative ellipticity implying enhancement in the compactness of the lectin structure. It was observed that with increase in the carbon chain length of the solvent like ethanol or DMSO, there is a decrease in the hemagglutination activity **(Fig. 4.6.A)**. This might be due to the capacity of the solvents to compete with protein atoms for hydrogen bonding disrupting intramolecular

interactions and destabilizing the structure (Knubovets *et al.*, 1999). These observations were also reflected in the far-UV CD spectra of *Di*L9 incubated with 50 % organic solvents for 4 h, where *Di*L9 in the presence of methanol and ACN retained structure, on the other hand it lost complete structure on incubation with ethanol and DMSO **(Fig. 4.6.B)**.

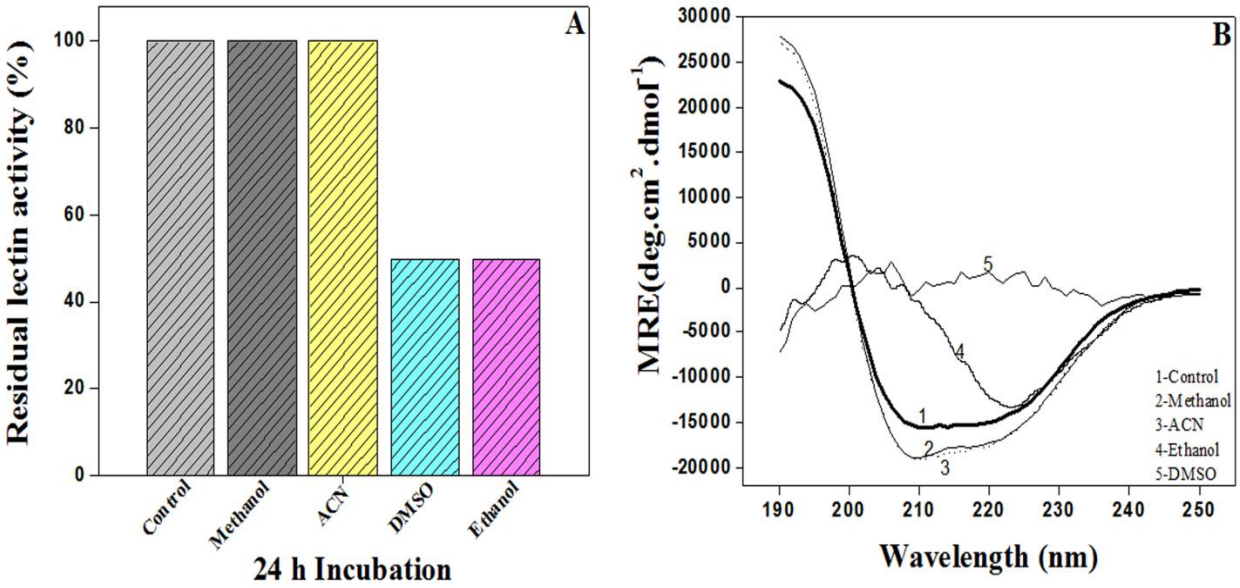


Figure 4.6 Effect of organic solvents on **A)** hemagglutination activity of *Di*L9 on 24 h incubation and **B)** far-UV CD spectra of *Di*L9. The lectin (0.2 mg ml^{-1}) was incubated with 50 % organic solvent for 4 h at 28 ºC.

4.10 Solute quenching studies of DiL9: Native & Denatured forms

Solute quenching experiments on native and denatured *Di*L9 was carried out to gather topological information by titrating the lectin against quenchers. The intrinsic florescence of *Di*L9 was quenched by both acrylamide and KI but not by CsCl, quenching was without any change in the λ_{max} of 354 nm. Only acrylamide showed

complete (100 %) tryptophan quenching of the *Di*L9 fluorescence. This suggested that the micro-environment near Trp residues was electro positive in nature. As discussed before, the quenching data was analyzed by the Stern-Volmer as well as modified Stern-Volmer equation to quantify the extent of quenching. Acrylamide quenching of native and denatured *Di*L9 showed slight upward curvature in Stern-Volmer plot **(Fig. 4.7.A),** indicating presence of both static and collisional conformers of tryptophan. The modified Stern-Volmer plot **(Fig. 4.7.B)** showed complete accessibility (100 %) of Trp residues to acrylamide while 69 % by KI. The neutral quencher was most efficient for native *Di*L9, with Stern-Volmer constant (K_{sv}) of 12.84 M^{-1} while that for iodide was 2.73 M^{-1}. The quenching response was poor towards the denatured lectin by both the quenchers, presumably due to already exposed fluorophore. **Table 4.4** summarizes the solute quenching parameters for the lectin under native and denatured conditions.

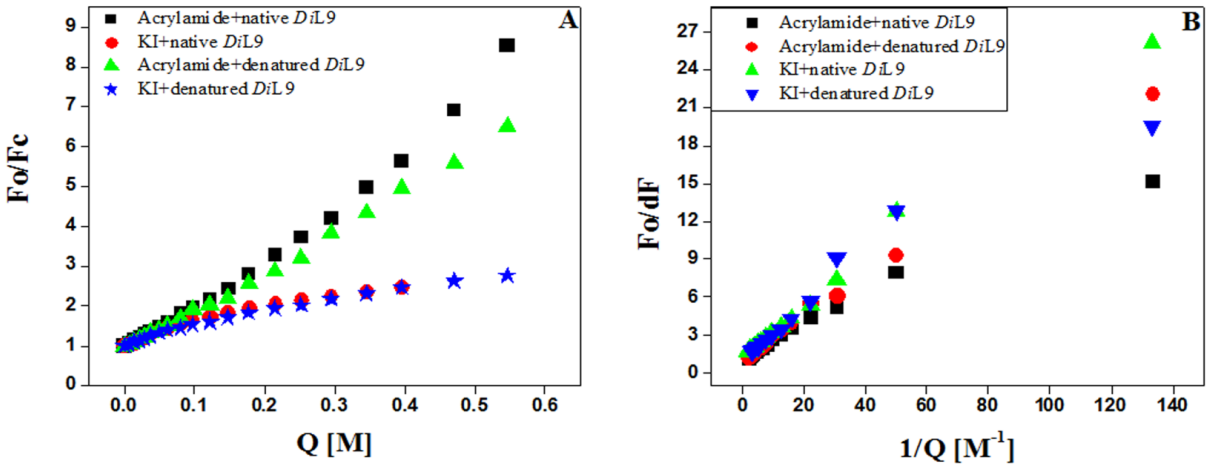


Figure 4.7 Fluorescence quenching studies. Solute quenching of the intrinsic fluorescence of *Di*L9 with acrylamide and iodide, **A)** Stern-Volmer plot of native and denatured *Di*L9 quenched with acrylamide and KI and **B)** modified Stern-Volmer plot.

Table 4.4 Summary of parameters obtained from the intrinsic fluorescence quenching of *Di*L9 with different quenchers

Quencher and Samples	**K_{sv} (M^{-1})**	***f_a***
Acrylamide		
Native	12.84	1.08
Denatured with 6 M GDn-HCl	10.9	1.01
KI		
Native	2.734	0.694
Denatured with 6 M GDn-HCl	3.316	0.64
CsCl		
Native	0.83	0.108
Denatured with 6 M GDn-HCl	1.04	0.157

4.11 Binding of Sugars & Cytokinin-derivatives to DiL9: A fluorimetric analysis

4.11.1 Sugar binding studies

Sugar binding studies were carried out by using fluorescence titration of *Di*L9 with various sugars which resulted in fluorescence enhancement without any shift in the emission maximum. Similar to *Bh*L binding studies, from the plot of F_0/dF vs 1/C; F_∞ was calculated and used to plot double logarithmic graphs for each sugars. The association constants (K_a) were estimated from the double logarithmic plots. The slope of the plot of $\log[(F_0-F_c)/(F_c-F_\infty)]$ vs $\log[C]$ was found to be near unity for all the sugars used, suggesting the presence of single binding site per protein molecule. **Table 4.5** summarizes the estimated association constants and free energy obtained for each sugar

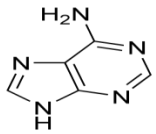
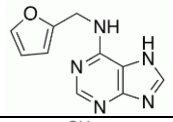
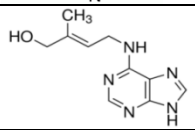
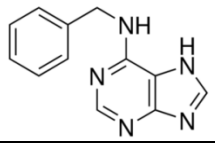
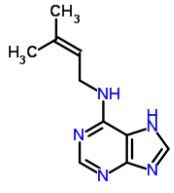
studied at 25 °C. This sugar binding property of *Di*L9 was found similar to *Bh*L, since *Di*L9 also showed no binding of monosaccharides or disaccharides, but showed affinity for higher sugar derivatives like *N,N',N''*-Triacetylchitobiose/triose. *Di*L9 showed 12 fold more affinity for chitotriose compared to chitobiose, which implies that with increase in carbon chain length, there is an increase in affinity values indicating extended sugar binding site present in *Di*L9. Different sugar derivatives were also included in the binding studies against *Di*L9. The lectin showed significant affinity for methyl 2-acetamido-2-deoxy α-D-glucopyranoside and 2-acetamido-2-deoxy-3-O-(β-α-galacto-pyranosyl)-D-glucopyranose with K_a values calculated as 5.0 x 10^2 and 1.1 x 10^3 M^{-1}, respectively, where only glucosamine does not bind to *Di*L9. These observations also confirm that the lectin has larger site for saccharide binding more specific for GlcNAc moiety. This property of the lectin can be utilized for the medical diagnostic purposes. Whereby large glycoproteins with extended sugar chains are expressed on different kinds of cancer cells and this lectin can be useful for its rapid detection purpose.

4.11.2 Binding of adenine and other derivatives

Previous reports have shown that many legume lectins, apart from sugar binding sites, also possess some hydrophobic sites for binding adenine or adenine-derived plant hormones called cytokinins (Roberts & Goldstein, 1983). The reason for the presence of these hydrophobic sites in lectins is not clear, but binding of these adenine/cytokinin-binding legume lectins might be involved in storage of plant hormones or in plant growth regulation. When carrying out binding studies, we have found that non-legume lectin *Di*L9 also binds adenine and other physiologically active compounds, like cytokinins:

kinetin, zeatin, N^6-isopentenyladenine (IPA) and N^6-benzylaminopurine (BAP) with strong affinity. The binding efficiency of these ligands was studied by monitoring the drop in the intrinsic Trp fluorescence of the *Di*L9 when excited at 295 nm. The adenine binding resulted in the fluorescence quenching, the association constant (K_a) estimated at 25 ºC, similar as in sugar binding studies, is listed in the **Table 4.5.** Interestingly, we found that *Di*L9 binds with higher affinity to the natural cytokines: Zeatin (K_a = 3. x10^3 M^{-1}) and N^6-isopentenyladenine (K_a = 6.45 x10^2 M^{-1}). Additionally, *Di*L9 showed significantly good interactions with BAP (K_a = 2.5 x10^3 M^{-1}) and kinetin (K_a = 5.55 x10^2 M^{-1}). Lima bean lectin from *P. lunatus* (Roberts & Goldstein, 1983) (K_a = 8.3 x10^4 M^{-1}) and *D. biflorus* lectin (Gegg *et al.,* 1992)(K_a = 7.31 x 10^5 M^{-1}) have been reported to have high affinity for adenine. Very few lectin structures with bound adenine such as *D. biflorus* lectin (Hamelryck *et al.,* 1999) and galactose-specific legume lectin from *Dolichos lablab* (Shetty *et al.,* 2013) only are available. Previously, Pathak et al have shown that lectins can also act as cytokinin antagonist either by directly interacting with BAP or inhibiting the delay in senescence (Pathak *et al.,* 2006). As calculated, the negative Gibbs free energy of the binding (ΔG) suggested the spontaneous nature of the binding in each case. From these observations, we can conclude that the lectin plays an important physiological role in plants growth.

Table 4.5 Binding constants, K_a, obtained for various chito-oligosaccharides at room temperature with *Di*L9 and the corresponding Gibb's free energy values

S.No	Sugar/Ligand	K_a (M^{-1})	-ΔG kJ mol^{-1}
1.	Gluocosamine	NB	-
2.	*N*-Acetyl Gluocosamine	NB	-
3.	Chitosan	ND	-
4.	*N,N'*-Diacetylchitobiose	1.2 $x10^2$	-11.86
5.	*N,N',N''*-Triacetylchitoriose	1.4 $x10^3$	-17.94
6.	Methyl 2-acetamido-2-deoxy α-D-glucopyranoside	5.0 $x10^2$	-15.39
7.	2-acetamido-2-deoxy-3-O-(β-α-galacto-pyranosyl)-D-glucopyranose	1.1 $x10^3$	-17.35
	Phytohormones		
8.	Adenine 	2.50 x 10^2	-13.67
9.	Kinetin 	5.55 $x10^2$	-15.65
10.	Zeatin 	3.33 $x10^3$	-20.09
11.	Benzylamino purine (BAP) 	2.5 $x10^3$	-19.38
12.	N^6-isopentenyladenine (iPA) 	6.45 x 10^2	-16.02

NB: No binding *ND: Not determined*

4.12 Conformational transitions studies

4.12.1 Monitoring conformation of DiL9 at different pH

a) Effect of pH on lectin activity

The *Di*L9 activity was found to be stable in whole pH range (1-12), retaining 100 % activity (2^6 titre corresponds to 100 %) even after incubating for 24 h. Similar behavior was observed for *Bh*L as discussed in the last chapter and for other reported lectins such as lectins from *Allium sativum* (Clement & Venkatesh, 2010) *and Hericium erinaceum* (Li *et al.*, 2010).

b) Fluorescence studies

Intrinsic fluorescence:

The ionic strength of the medium can drastically affect the protein stability and activity. There was a decrease in the fluorescence intensities of *Di*L9 at acidic pH range due to protonation of amino acids near tryptophan residues whereas at alkaline condition, enhancement in the intrinsic fluorescence spectrum was observed with a red shift of 3 nm, indicating partial unfolding of the lectin with increased Trp polarity **(Fig. 4.8).**

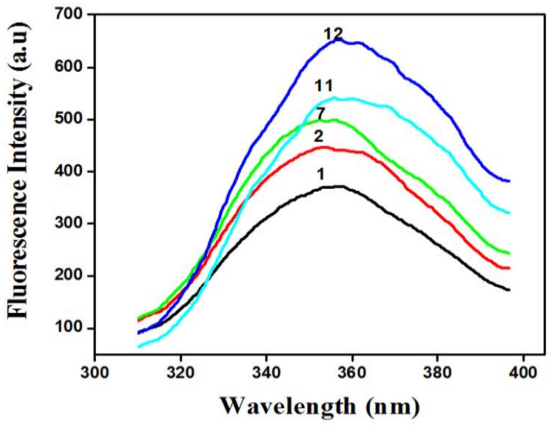


Figure 4.8 Intrinsic fluorescence spectra of *Di*L9 incubated at different pH buffers for 24 h (indicated with numbers).

Extrinsic fluorescence:

Studies on ANS binding to *Di*L9 were carried out to determine the presence of molten globule like structure at acidic pH conditions. ANS binds to native lectin itself (at pH 7.4) showing presence of already exposed hydrophobic patches on the surface. It was observed that at lower pH range (1-2), drastic increase in ANS intensity (3 fold of binding to native lectin) was seen implying enhanced exposure of hydrophobic groups with a concomitant blue shift in the emission maxima from 520 nm to 460 nm. This confirmed the presence of acid molten globule like structure of *Di*L9. On the other hand, at alkaline pH, there was reduction in the fluorescence intensity **(Fig. 4. 9)**.

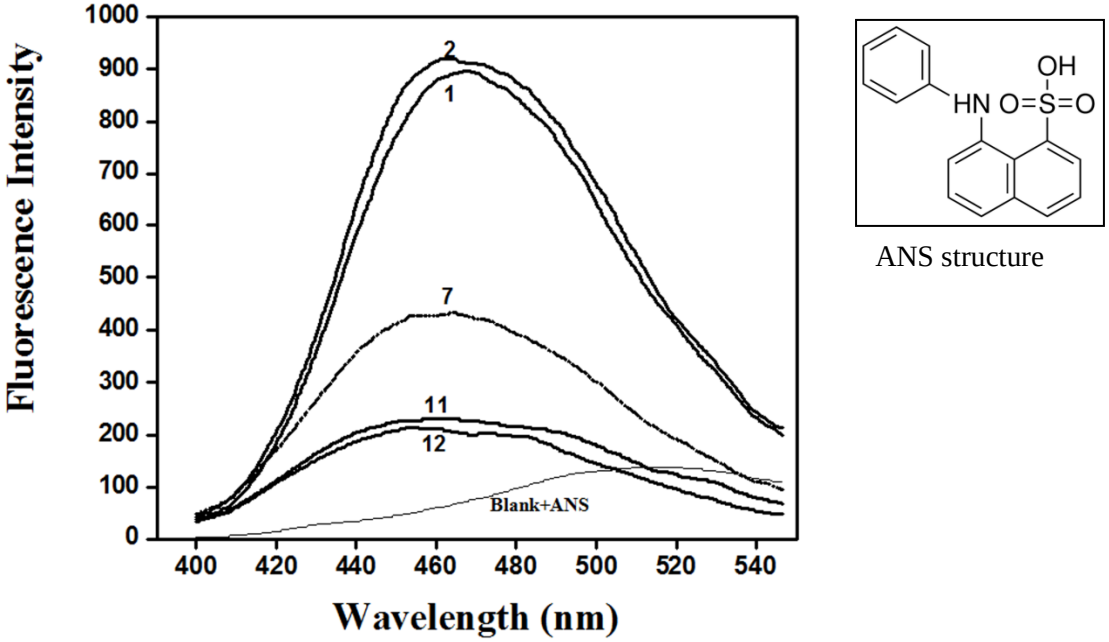


Figure 4.9 Effect of pH on ANS fluorescence emission spectra of *Di*L9. The numbers on the spectra corresponds to the pH of the protein solution.

c) CD studies

The far-UV CD spectra of *Di*L9 showed intact structure in the pH range of 1-12 for 4 h **(Fig. 4.10.A,B)**. Native *Di*L9 spectrum (at pH 7.4) showed negative minima at 208 nm and 220 nm indicating presence of prominent α-helix and β-sheets content. No major structural alterations were observed between pH 1-12, indicating the stability of the protein at broader pH range. Perturbations in the unordered structural elements of the protein were observed corresponding to changes seen in MRE at 190 nm. The near-UV CD spectrum of the native *Di*L9 showed very ordered structure with a deep trough at 275 nm. This order was intact even at acidic condition whereas, significant decrease in the MRE at alkaline condition (pH 12.0) was observed implying changes in the environment of aromatic amino acid residues. This also revealed existence of the lectin in molten globule conformation **(Fig. 4.10.C).**

Molten Globule like Structure of DiL9

Retention of the stable secondary and tertiary structure with exposed hydrophobic amino acids in *Di*L9 at pH 2.0 showed possible existence of an acid induced molten globule like structure. Its unusual folding back to native form at acidic pH might be the reason for its retention of full activity at acidic conditions. Such peculiar properties of the proteins were reported previously for immunoglobulin MAK33 (Buchner *et al.*, 1991), glucose oxidase (Haq *et al.*, 2003) and Nprot (Rohamare *et al.*, 2015).

Fink et al, had already described 3 types of acid denatured states of proteins, where at lower pH (3-4) type I proteins showed initial unfolding with immediate

refolding to the A state, i.e. a molten globule-like conformation. Type II proteins does not undergo unfolding even upon acid titration but directly get transformed into the molten globule state and type III proteins also show no significant unfolding at pH as low as 1.0, but behaves similar to type I in the presence of urea (Fink *et al.*, 1994). Here, based on our observations we can conclude that *Di*L9 could be of type I (A state) protein, which showed similar characteristics as of staphylococcal nuclease belonging to the same category (Fink *et al.*, 1993).

d) FT-IR analysis

CD spectrum did not show significant changes with respect to the secondary structure of the lectin at extreme pH conditions, but slight variations were observed at MRE 200 nm. These conformational changes induced by pH variation can be precisely monitored using FTIR, since it is very sensitive to structural changes. The **Fig.4.10.D** shows the deviations in the FTIR spectra of *Di*L9 secondary structure from the native form in the presence of extremes of pH: 2 (acidic) and 12 (basic). The bending at 1643 (cm^{-1}) corresponds to C=N and C=C bonding. At extreme pH condition, there has been decrease in the transmittance implying increase in the absorption of IR by the different bonds (C=O of amide bond) in the lectin. A shift in the peak position was also observed which corresponded to higher disordered structure, i.e, protein unfolding leads to more hydrogen bonding with the exposed solvent, changing the vibrational frequency (C=O, NH bonds) which eventually results in change in the bond strength or reduced mass and thus, resulting in the peak shift towards smaller wave number.

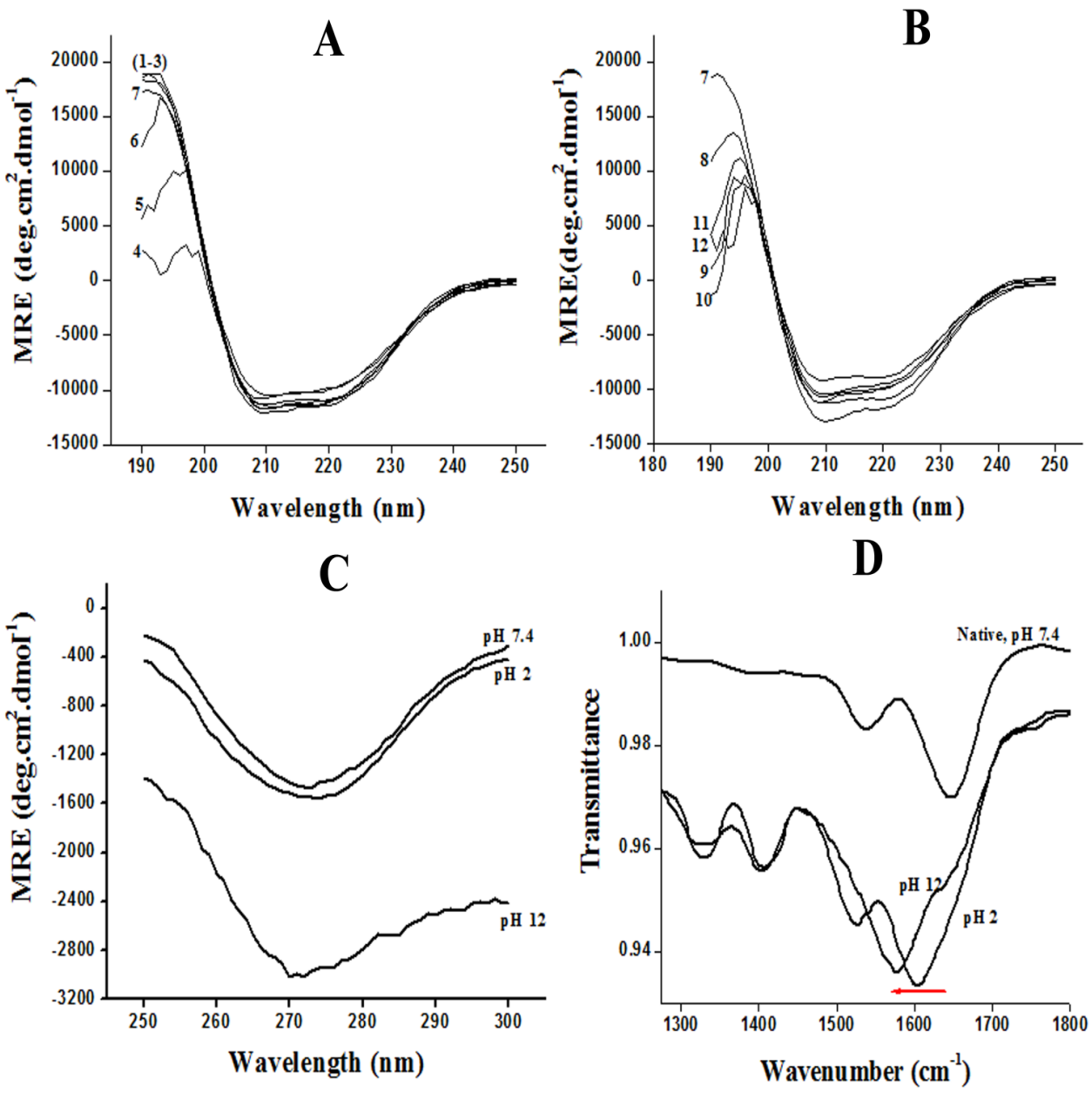


Figure 4.10 Effect of pH on *Di*L9. Far-UV CD spectra of *Di*L9 incubated at different pH range for 4 h. **A)** Acidic pH range from 1-7 **B)** Basic pH range from 7-12. **C)** Near-UV CD spectra of *Di*L9 (0.20 mg ml^{-1}) native, incubated at pH 2 and pH 12 condition. **D)** FT-IR spectrum of *Di*L9 (1 mg ml^{-1}) incubated in native condition, pH 2 and pH 12.

4.12.2 Thermo stability of DiL9

a) Effect of temperature on lectin activity

*Di*L9 was found to be significantly thermostable where it retained 100 % of its hemagglutination activity at 60 ºC for 2 h of incubation. The increase in temperature showed sigmoidal decay curve in lectin activity with 50 %, 25 % and 12.5 % activity at 70, 80 and 90 ºC, respectively **(Fig. 4.11.A)** and melting temperature was estimated at 70.8 °C. It was observed that the thermostability of *Di*L9 in terms of hemagglutinating activity varies with respect to both temperature and incubation time i.e., lectin retained 50 % of its maximum activity at 70 ºC even after 2 h of incubation, but rapidly inactivated at 100 ºC. This has suggested that the lectin undergoes complete unfolding of the structure under extreme temperatures resulting in the loss of activity **(Fig. 4.11.B)**. There are other plant lectins reported with similar thermostability property such as lectins isolated from edible mushroom *Agaricus arvensis* (Zhao *et al.*, 2011), *Pinellia ternata* (Zuo *et al.*, 2012) and *Trichosanthes dioica* (Dharkar *et al.*, 2006).

The Gibb's free energy of lectin thermostability at different incubation temperatures and time intervals were also calculated (Using Eq 1). For example, the ΔG value at 60 ºC was found to be zero, due to no change in hemagglutination activity implying no thermal enthalpy (ΔH) change. It was observed that with increase in temperature the ΔG values were found to be positive implying that energy was absorbed in a non-spontaneous process. Considering this phenomenon, the maximum residual hemagglutinating activity observed at 90 ºC for 60 min corresponded to ΔG of 43 kcal mol^{-1}; where the entropies (S) of the native and denatured states are equal **(Table 4.6).**

From the literature we now know that many lectins are toxic and show anti nutritional effects on mammals, including humans, the free energy of activation of lectin denaturation process remains an important physicochemical parameter when considering lectin's applications in various fields like medicines, agriculture and other related fields of human and animal nutrition (Vasconcelos & Oliveira, 2004).

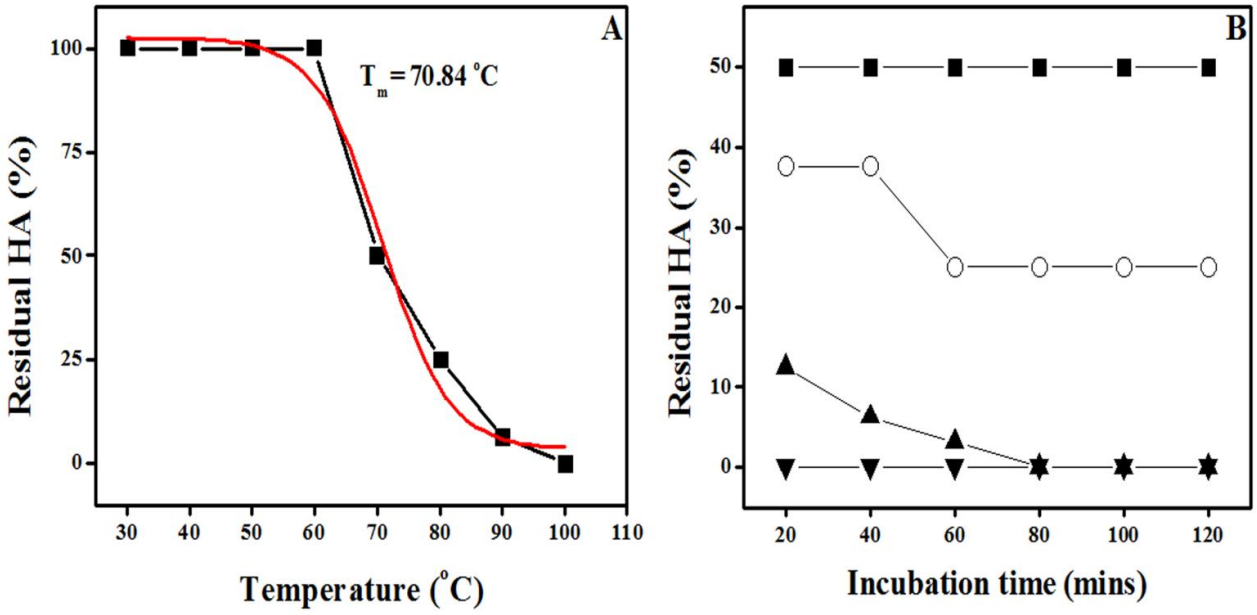


Figure 4.11 Thermal denaturation of *Di*L9. (A) Effect of temperature on agglutinating activity of purified *Di*L9 towards rabbit erythrocyte suspension in PBS. **(B)** Thermostability of *Di*L9 at: (-■-) 70 ºC; (-○-) 80 ºC; (-▲-) 90 ºC; and (-▼-) 100 ºC. Each point on the line is the mean (+1 SD) derived from three replicates. Total activity (100 %) corresponds to a titre of 2^6.

Table 4.6 Estimate of Gibbs free energy for *Di*L9 at different incubation temperatures

Temperature (^oC)	T (K)	A_o	A	Time (secs)	k_1	RT	ln(kT/k1h)	ΔG (kcal mol^{-1})
60	333	100	100	3600	0	0	0	0
70	343	100	50	3600	1.92541E-4	681.541	61.22945	41.73
80	353	100	25	3600	3.85082E-4	701.411	60.56504	42.48
90	363	100	6.25	3600	9.62704E-4	721.281	59.49436	42.91

ΔG is Gibbs free energy, R is the gas constant (1.987 cal $mol^{-1} K^{-1}$); T is the absolute temperature (K); k is the Boltzmann constant (1.37 x 10^{-6} erg K^{-1}); k_1 is the velocity constant and h is Planck's constant (6.25 x 10^{-27} erg s^{-1}), t is the time of heat treatment (s), A is the residual hemagglutinating activity and A_o is the initial hemagglutinating activity before heat treatment. (100 % corresponds to titre of 2^6)

b) Steady state fluorescence

Fluorescence studies showed that with increasing temperature there was a gradual decrease in the fluorescence intensity of the native protein, which might be due to the deactivation of the singlet excited state by non-radiative process. The observed λ_{max} values were in the range of 354–355 nm till 85 ºC while above 85 ºC, fluorescence spectrum shifted to 358 nm due to complete exposure of Trp residues of unfolded protein **(Fig. 4.12).**

Light scattering intensity was also measured from 25-95 °C in time drive module to study its aggregate forming tendency. Generally, due to thermal denaturation, hydrophobic patches get more exposed and protein tends to aggregate. Here, no scattering

was observed for *Di*L9 at even at very high temperature, when already exposed hydrophobic patches on native lectin was present. On decreasing the temperature back to 25 °C, still no aggregation was seen indicating high solubility of the protein (data not shown).

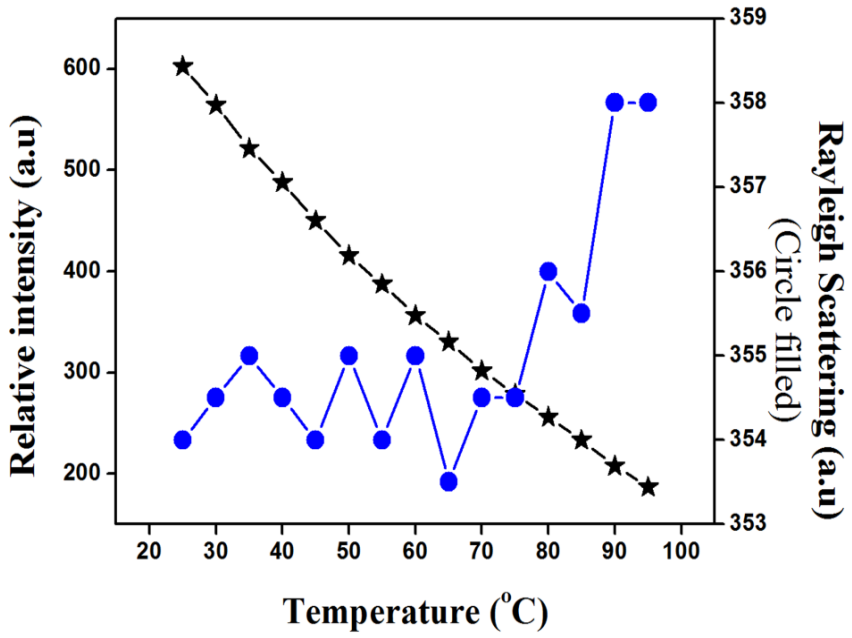


Figure 4.12 Thermal unfolding of *Di*L9, plot of relative intensity and wave length of maximum intensity (λ_{max}) vs temperature (^{o}C).

c) Far-UV CD studies

Structural studies using far-UV CD spectra showed that on exposing *Di*L9 to higher temperatures (25 to 95 ºC), no significant change in secondary structure was observed up to 80 ºC **(Fig. 4.13.A).** On further increasing the temperature, a significant decrease in alpha-helix content with increased intermolecular beta-sheet formation was observed. Such conformational transitions were not detected in fluorescence studies. This

transition change was observed till 95 ºC, a similar observation was also seen in α-synuclein (Uversky *et al.,* 2001). The sigmoidal fit analysis (R^2 = 0.95) of change in MRE at 190 nm gave an estimated T_m value of 74 ºC, a similar value was calculated from hemagglutinating activity as well. The lectin is highly stable with respect to MRE at 208 nm with few changes at 220 nm. Significant decrease in MRE at 190 nm was observed suggesting loss of ordered structure **(Fig. 4.13.A)**. This thermostability feature of *Di*L9 is comparable with stabilities of other reported *Datura* lectins (Desai *et al.*, 1981, Petrescu *et al.*, 1993).

d) FTIR analysis

As we know now that FTIR is the simplest diagnostic tool to study biochemical systems exhibiting apparent peak intensity changes under high temperature, especially those with the OH and C=O stretching vibrations representing hydrogen bonding and dipole–dipole interactions. As shown in the **Fig. 4.13.B,** the increase in temperature resulted in an increase in the peak intensity of absorption (or in other words decreases in transmittance) by *Di*L9. Increased temperature caused the vibrations to increase which eventually resulted in producing large changes in dipole (C=O or O-H stretching). The measured intensity values from the transmittance at wave number 1643/cm and 1533/cm remain unchanged from 25-60 °C implying that temperature had negligible effect on the secondary structure of *Di*L9 (as confirmed by CD studies). At temperatures above 60 °C, the decrease in transmittance intensity was monitored as no loss in activity was observed till 60 °C. Hence, the decrease in intensities at higher temperature clearly indicated the

onset of unfolding event (breaking of hydrogen bonds) of *Di*L9, eventually resulting in the loss of lectin activity as reported earlier.

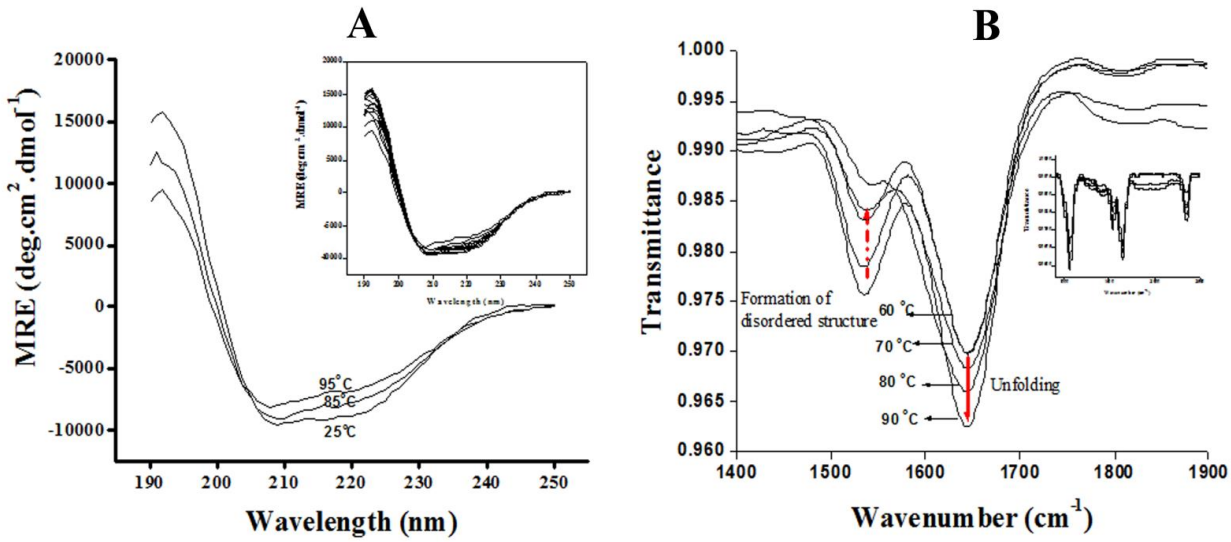


Figure 4.13 *Di*L9 thermostability studies. A) Far-UV CD spectra of *Di*L9 incubated at different temperatures (25-95 ^{o}C) for 5 mins. **B)** FT-IR spectra of *Di*L9 incubated at higher temperatures. The number indicates temperature in ^{o}C.

4.12.3 Chemical denaturation studies of DiL9

a) Effect of chemical denaturants on lectin activity

Chaotropic agents such as urea, GDn-HCl and GDn-SCN have tendency to disrupt the hydrophobic interactions of protein interior that are essential to stabilize native conformation. In our study, urea had no effect on *Di*L9 activity. However, presence of GDn-SCN (2 M) resulted in complete loss of hemagglutination activity of

*Di*L9 but retained 12.5 % of residual activity in the presence of GDn-HCl (6 M) for 24 h **(Fig. 4.14)**. From these observations we conclude that GDn-SCN is the most potent denaturing agent for this lectin. The presence of reducing agents like βME and DTT (2-5 mM) resulted in the complete loss of lectin activity implying presence of disulphide bridges that are playing important role in maintaining the secondary and tertiary structures of the lectin. As reported elsewhere, the *Datura* lectins have unusual amino acid composition of hydroxyproline, glycine and cysteine implying presence of many disulphide bonds (Goldstein & Poretz, 1986) .

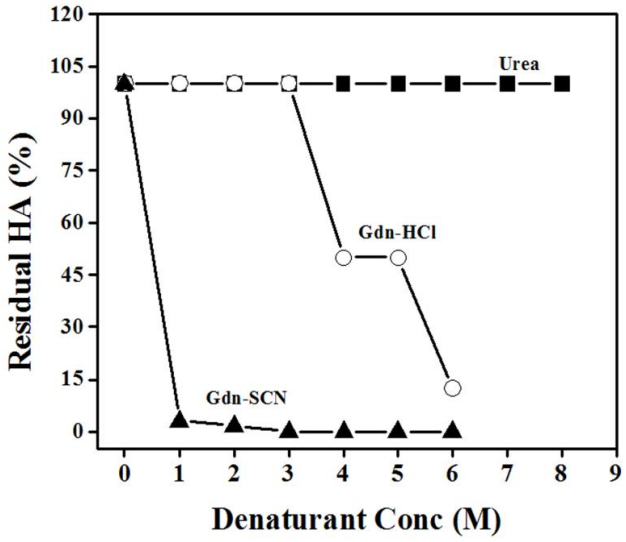


Figure 4.14 Effect of chemical denaturants on *Di*L9 activity. Lectin was incubated with required denaturant concentration in 20mM phosphate buffer, pH 7.4 for 16 h at room temperature.

b) Fluorescence studies

Intrinsic fluorescence

In the presence of 1 M GDn-HCl, red shift of 3 nm was observed with decrease in the intrinsic fluorescence intensity. This might be due to unfolding of the protein structure exposing the last buried Trp residue **(Fig. 4.15.A)**.

Extrinsic fluorescence

ANS binding resulted in 2-fold decrease in fluorescence intensity of *Di*L9 with a blue shift in presence of increasing concentration of GDn-HCl. This might be due to the minor variation occurred in the ionic or hydrophobic interactions in the protein structure, which in turn affected the exposure of the hydrophobic side chains in the presence of GDn-HCl **(Fig. 4.15.B**). Thus, the hydrophobic pockets present in the lectin seem to be very sensitive.

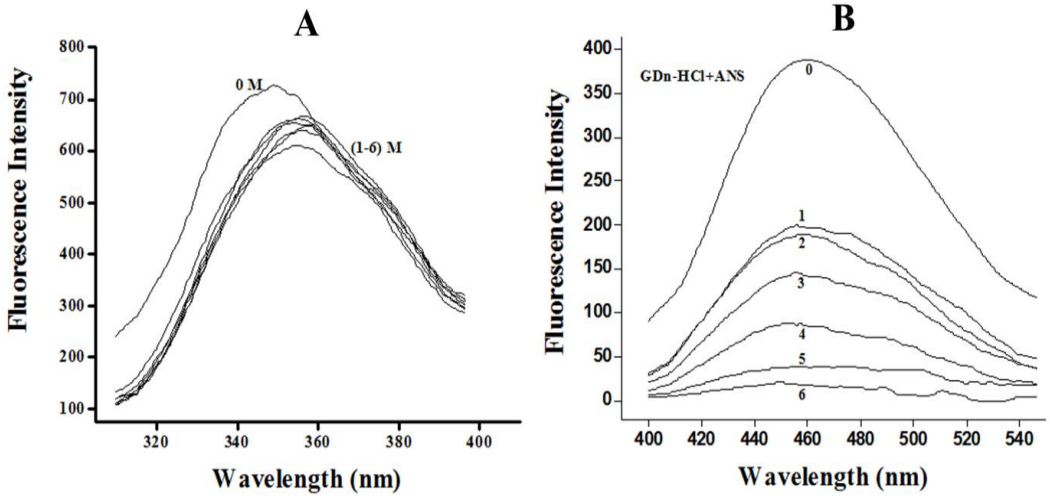


Figure 4.15 GDn-HCl induced unfolding. A) Intrinsic fluorescence spectra of *Di*L9 incubated at different GDn-HCl concentration for 4 h. **(B)** ANS binding to *Di*L9 (0.05 mg/ml) at different concentration of GDn-HCl. The numbers indicate the GDn-HCl concentration.

c) CD analysis

*Di*L9 retained its secondary structure in the presence of 2 M GDn-HCl **(Fig. 4.16.A).** A decrease in the negative MRE at 220 nm was observed implying little perturbation in the secondary structure. In subsequent higher concentrations drastic unfolding of the protein was observed. But to our surprise, during the refolding process by diluting the denaturant concentration, the lectin was found to refold back to native conformation regaining activity. This indicated reversible folding nature of the lectin **(Fig. 4.16.B).**

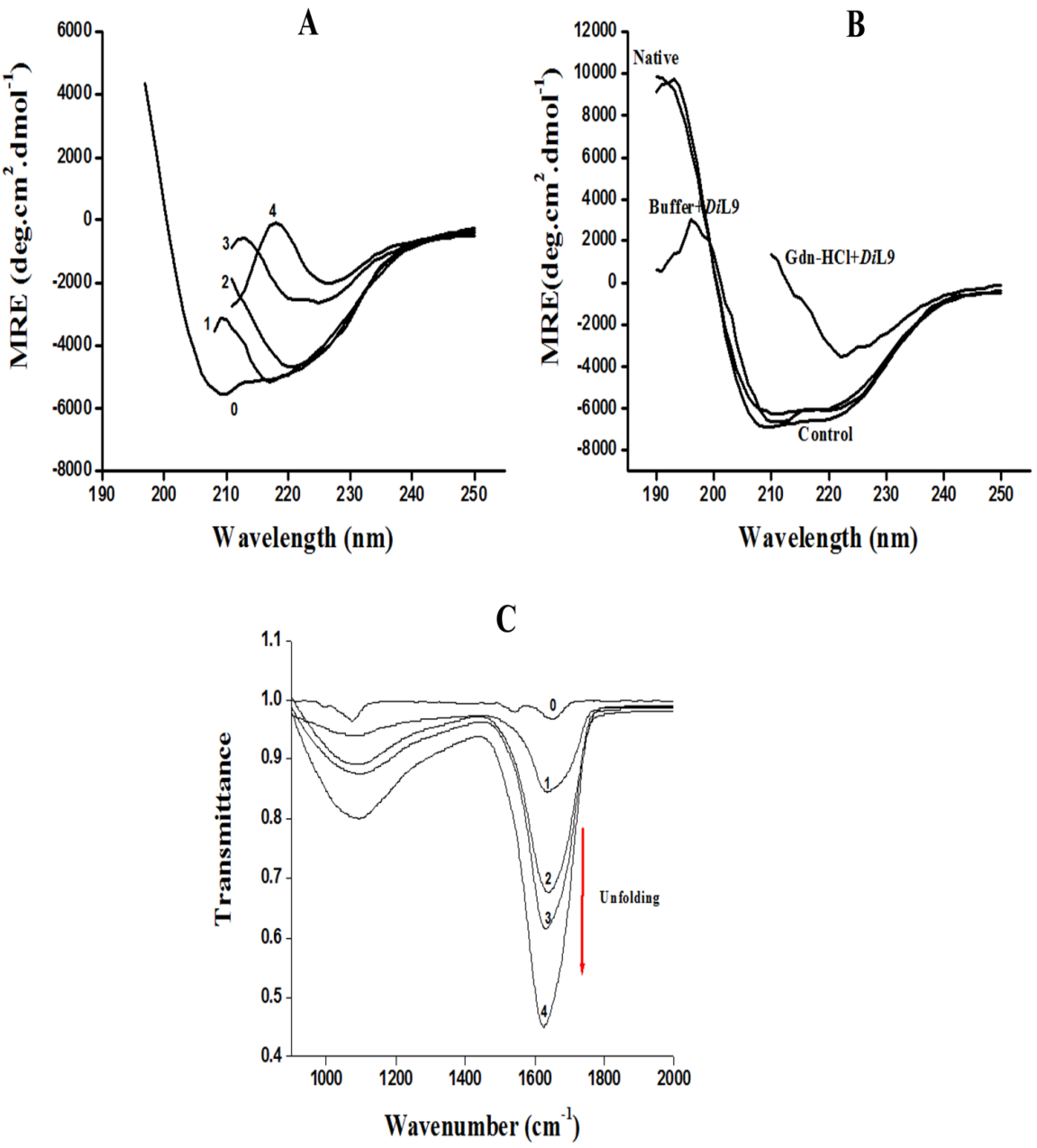


Figure 4.16 Chemical unfolding studies of *Di*L9. A) Far-UV CD MRE spectra of *Di*L9 incubated at different GDn-HCL concentrations for 4 h. **B)** Refolding studies of GDn-HCl denatured lectin incubated for 12 h. **C)** FT-IR spectra of *Di*L9 incubated at different concentrations of chemical denaturants (GDn-HCl) for 4 h. The numbers on the spectra indicate the GDn-HCl concentration.

d) FTIR analysis

Similar to pH and temperature based FTIR studies; here also there was decrease in peak intensity as well as small shift in the wave-number towards smaller number **(Fig. 4.16.C).** As discussed before, with increased denaturant concentration, the lectin undergoes unfolding which results in breaking of hydrogen bonds formed by peptide groups of protein in the solution. These studies have been restricted to 4.0 M GDn-HCl concentration, which is a limitation that cannot be neglected in FT-IR spectroscopy studies.

4.13 DLS analysis

To completely understand the variations in dimensions of protein exposed to different/denaturing conditions is always essential to get a full understanding of its conformation and functions. From the previously discussed data of fluorescence, CD and FTIR studies we have seen that *Di*L9 is quite stable at all denaturing conditions, the DLS studies have also been performed to understand the changes occurring in the protein dimensions during unfolding. Predominantly, all protein tends to fold into compact and tightly arranged globular domains without any void space or water molecules placed in the interior. Mathematically, the volume (V) occupied by the single lectin can be calculated by using the following formula:

$$V = 1.212(nm^3/Da) * 10^{-3} * \text{Molecular weight (Da)} \qquad \textit{Eq. 11}$$

And also,

$$\text{Volume of the sphere} = 4/3\ \Pi\ r^3 \qquad \textit{Eq. 12}$$

The exact molecular weight of *Di*L9 (monomer) is 9653 Da as mentioned before. Using the above equation, the volume of the protein can be estimated as 11.7 nm^3 and radius theoretically determined was 1.4 nm.

It is very important to observe that during unfolding or refolding of the protein, the hydrodynamic radius (R_h) is highly affected with respect to the shape of a protein molecule. To precisely determine the protein shape, usually the sedimentation coefficient or frictional coefficient (*f*) is estimated. But it has been observed that due to uneven contours of protein surface it experiences more hydrodynamic drag as compared to any smooth ellipsoid structure. So to study dimensions of *Di*L9 we have estimated the R_h factor by using Stoke's-Einstein relation (Eq. 8) to understand protein shape and structure. For an elongated or hydrated protein, where a layer of water (frozen) is held by hydrogen or ionic bonds, or frozen by the interaction at the hydrophobic surface, the diameter and hydrodynamic drag of protein will be more as compared to dehydrated protein. Thus, the factor *f* gives insight into both the shape and hydration characteristics. We also know that *f* is inversely proportional to diffusion coefficient (D), which in turn is related to R_h (Eq. 8). The R_h measured experimentally by DLS for native *Di*L9 is 1.5 nm **(Fig.4.17)**. Hence, we find that the calculated value is almost same as the experimentally determined value. Further, DLS was used to study the hydrodynamic properties of the lectin during pH or chaotrope induced denaturation process. The observed hydrodynamic radius at each denaturing condition is listed in **Table 4.7**. Extreme pH was also considered because the lectin activity was stable in a wide range. Thus, the R_h of *Di*L9 at pH 2 and 12 was estimated to be 1.44 nm and 5.33 nm, respectively. At pH 2, the R_h value was near to that of native lectin implying the presence of pH induced molten

globule like structure as previously shown by fluorescence ANS binding studies whereas at alkaline pH, an increase in R_h value implies unfolding of the lectin.

Spectroscopic studies such as fluorescence and CD give information about the physical properties of the molecule as a whole present in the solution, on the other hand R_h deals with the single/individual molecule at a time. Thus, keeping this information in mind the percentage of compactness of a molten globule like state can be calculated as:

Compactness factor = $(R_D-R_M)/(R_D-R_N)$ ***Eq. 13***

where R_D, R_M and R_N are the hydrodynamic radius of denatured, molten globule and native state of a lectin, respectively. Now considering the experimental data obtained **(Table 4.7)**, if R_D, R_M and R_N are taken to be 5.33 nm, 1.44 nm and 1.4 nm, respectively, then compactness factor comes out to be 98 % implying very rigid spherical protein structure. Surprisingly, it was also observed that at lower concentration of the lectin, polydispersity was low around 12 %, but with increase in concentration very high polydispersity was observed, an inept condition for crystallization trials. Similarly, an attempt to study changes in R_h during chemical denaturation was carried out and gave a good measure of increase in protein radii implying its complete unfolding.

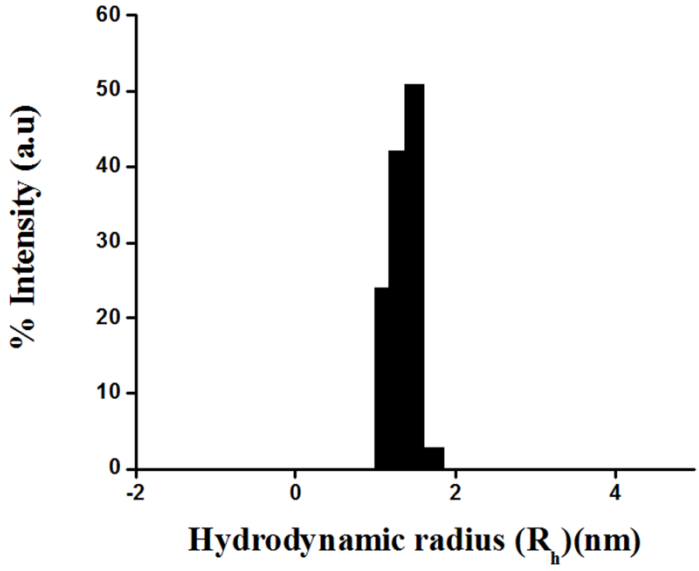


Figure 4.17 Hydrodynamic radius of native *Di*L9. The protein concentration used was 1 mg ml^{-1} in 20 mM PBS.

Table 4.7 Hydrodynamic radii of the lectin (1mg ml^{-1}) at different denaturing conditions

Sl.No.	Condition	*Rh* (nm)
1	Native (20 mM Phosphate buffer, pH 7.4)	1.4
2	pH 2	1.438
3	pH 12	5.33
4	1 M GDn-HCl	3.6
5	2 M GDn-HCl	3.4

4. 14 Mass spectrometry analysis

MALDI TOF-MS/MS was carried out to determine the primary sequence of the lectin. The data obtained gave us the average masses of several peptides after trypsin digestion protocol. We found very few sequence similarities with the lectin database used (Downloaded from Uniprot). For example, *Di*L9 fragments showed similarity with Con A lectin and lectin fragment from *Caragana frutex* and *Penaeus japonicas,* which are totally distinctly related **(Table 4.8).** Due to lack of sufficient homology data for protein sequences in the databases, it was not possible to elucidate primary structure of the lectin by peptide mass fingerprinting. Hence, different techniques need to be tried to determine the primary sequence.

Table 4.8 Analysis of MALDI-MS/MS data using Protein pilot software

Sl.No	Species	Sequence	Accessions	Homology (%)
1.	Lectin fragment from Caragana frutex	DWDPTGDR	tr|Q9ZRN7|Q9ZRN7_9FABA	9
2.	Calcineurin B-like protein 2 OS=Oryza sativa subsp. japonica	GLEDPQVLAR	sp|Q3HRP5|CNBL2_ORYSJ	4.4
3.	C-type lectin from *Penaeus japonicus*	GGVWFDTTMR	tr|I2C0B3|I2C0B3_PENJP	3
4.	REVERSED Concanavalin A-like lectins	EILDYTMGR	RRRRRtr|S3DKM5|S3DKM5_GLAL2	1.2
5.	Coatomer subunit beta OS=Setaria italica	LLCNTGDDVR	tr|K3XE87|K3XE87_SETIT	1

4. 15 Conclusions

In the present work, a chito-specific agglutinin was purified from the seeds of *D. innoxia* belonging to Solanaceae family, using Q sepharose ion exchange and gel filtration chromatography. The purified agglutinin happened to be a monomeric lectin with MW 9 kDa, as estimated from SDS-PAGE and MALDI-TOF/TOF analysis. Hemagglutination inhibition studies interpreted that only chitotriose sugar and glycoproteins could inhibit lectin activity. The fluorimetric binding studies also showed specificity for GlcNAc oligomers, which is an important cancer biomarker moiety. *Di*L9 behaved like cytokinin binding protein implying involvement in some important physiological roles in plant metabolism.

The far-UV CD spectra showed structural features different from other *Datura* lectins, thus, showing presence of diverse lectins in the same plant species. Our study concluded that *Di*L9 is highly stable in the presence of extreme pH range, at high temperature, in the presence of high concentration of chemical denaturants, organic solvents and also showed resistance to proteolytic digestion. The chemical unfolding was found to be reversible on diluting the denaturant concentration, regaining the structural and functional activity. Polydispersity was seen at higher concentration when analyzed using DLS. Though *Datura* lectin in our study forms pH induced molten globule structure, it is still able to retain its sugar binding property as its secondary and tertiary structures remain intact. This property makes *Di*L9 highly suitable as a lectin-mediated drug delivery candidate for use at lower pH condition and also against many therapeutic targets.

Chapter 5

(A) Anti-microbial and Anti-biofilm activity of *Bh*L and *Di*L9 tested against some pathogenic strains

With the rapid increase in resistance of microbes against antibiotics in use, there is urgency to find new anti-microbial compounds. An infection caused by these antibiotic-resistant microorganisms complicates the conventional treatment, resulting in prolonged illness and higher chances of mortality (Barza & Travers, 2002). Acquiring resistance against antibiotics by the microbes is a genetic phenomenon, where these micro organisms acquire antibiotic resistance genes. This can be by importing the genes through transduction, conjugation, and transformation mechanisms (Davies & Davies, 2010), or sometimes due to natural evolution. Another important reason for the mechanism of resistance is the ***biofilm formation***, whereby microbial cells adhere to the surface and encloses itself in the exopolysaccharide matrix (Donlan, 2002).Thus, many efforts are being taken to develop antimicrobial agents from local/natural sources rather than synthetic to avoid high toxicity. This demands search of natural antimicrobial agents with effectiveness in inhibitory action such as plant extracts (Nasar-Abbas & Halkman, 2004), to overcome the drug resistance acquired by microbes over old/new antibiotics used currently in clinical therapy.

Although many reports are available demonstrating the antimicrobial activity present in plant extracts, there is necessity to identify novel compounds with higher inhibitory action against pathogens with high resistance (Recio, 1989, Cragg *et al.*, 1997), with few also reported to show anti-biofilm activity. Among the plant extracts, mostly glycoproteins or lectins are well known to have antimicrobial action, playing important role in controlling various normal and pathological process. As discussed in previous chapters, the lectins bind glycoconjugates present on the surface of bacterial cell, such as

techoic acids, lipopolysaccharides (LPS) and peptidoglycan, acting as potential targets for lectin (Santi-Gadelha *et al.*, 2006).

Biofilm formation and development:

Biofilm formation involves four distinct stages: (1) reversible surface attachment, (2) irreversible attachment, (3) maturation, (4) dispersion (**Fig. 5A.1**).

Initially the bacteria moves towards the surfaces by chemotaxis or Brownian motion, resulting in a reversible bacteria surface attachment, which is mediated by non-covalent interactions like van der Waals forces, electrostatic forces, hydrogen bonding, and Brownian motion forces (Gottenbos *et al.*, 1999), occurring between the attachment surface and the bacterial adhesin proteins. The bacteria start secreting extracellular polymeric substance which helps in final anchoring to the surface, resulting in irreversible attachment. The transition from reversible to irreversible attachment is relatively rapid taking few minutes or less (Palmer *et al.*, 2007). After surface attachment, more planktonic bacteria are recruited, divide and develop the biofilm community, i.e. maturation. During this phase, the differential gene expression can be observed, motility decreases and EPS production increases (Garrett *et al.*, 2008). Once the biofilm gets matured, it becomes dynamic and resistant to any change in pH, temperature, osmolarity or shear forces, which makes its detachment difficult.

Biofilms are known to be hazardous in medical field, since it is frequently found on the surfaces of tissues, biomaterials and medical implants. This results in medical implant failure and often decreases the life times of many indwelling medical devices. Many researchers have shown that the bacteria present in the biofilms are less susceptible to antibiotics (Anwar *et al.*, 1992). In few cases, the only solution is to mechanically

remove the biofilm or implant again, which causes tremendous pain to the patient or is costly (Costerton *et al.*, 1999).

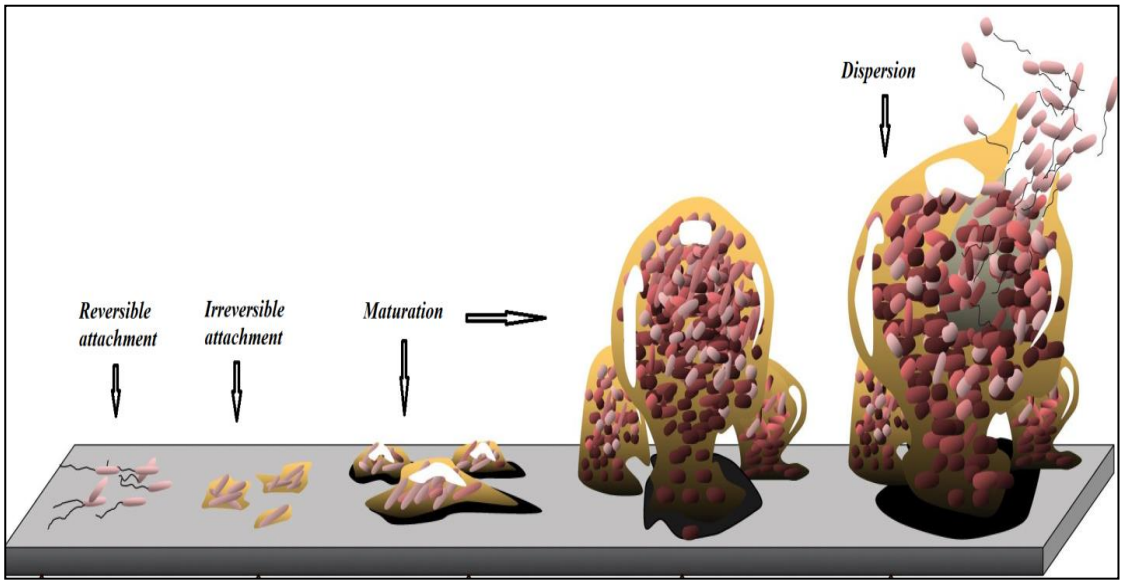


Figure 5A.1 Different stages of biofilm formation *(Image adopted from https://en.wikipedia.org/wiki/Biofilm and modified).*

By understanding the mechanism by which biofilm forms, it can be tackled either by using drugs that penetrate through the exopolysaccharide layer or by inhibiting the bacterial growth and gene expression (for quorum sensing). Thus, in our study we have also investigated the antimicrobial and anti biofilm activity of the purified lectins (*Bh*L and *Di*L9) against various pathogens. They were also checked for their effectiveness against pre-formed biofilms.

5A.1 Antimicrobial activity

As discussed earlier, lectins play defensive role against bacteria, fungi and insects. The growth inhibitory power of lectins depends on the dosage and varies from species to

species. In the present study, purified chito-specific lectins were screened for *in vitro* antibacterial growth inhibition against 10 pathogenic bacteria and their inhibitory power was compared to that of ampicillin, an antibacterial antibiotic. Both the lectins were able to agglutinate both Gram positive and Gram negative bacteria (data not shown). *Di*L9 showed significant antibacterial response against *E. faecalis* and *B. cereus* with 13 and 11 mm of diameter **(Fig. 5A.2)** of inhibition zone, with calculated MIC of 150 and 250 µg ml^{-1}, respectively **(Table 5A.1).** On the other hand, *Bh*L was less effective as compared to *Di*L9. We have also investigated the lectin antibacterial activity against mycobacterium, but found no response. This indicated that these lectins show specificity towards Gram positive/negative bacteria only. This ability of the lectins could be related to their binding the GlcNAc oligomers as already discussed, present on bacterial surfaces. These lectins might have interacted with the GlcNAc, which is a part of the peptidoglycan and murien constituting the cell wall components, and thus interfering with the bacterial growth. Surprisingly, lectin did not show any antifungal activity, even though lectins were chito-specific. *Di*L9 showed some, though not significant, inhibition of growth only against *F. oxysporum.*

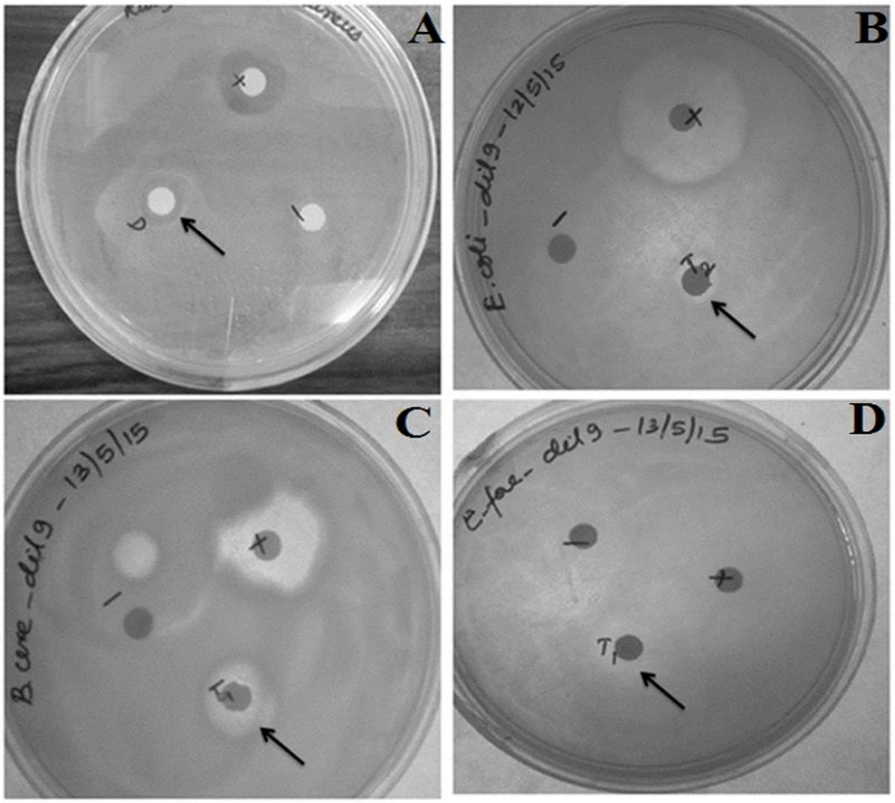


Figure 5A.2 Inhibitory effect of purified *D.innoxia* seed lectin on the *in vitro* growth on nutrient agar plates of **(A)** *S. aureus*, **(B)** *E. coli*, **(C)** *B. cereus* and **(D)** *E. faecalis*. For each plate 5 mm diameter discs were seeded with 10 µl of PBS (-) alone as the negative control, (+) 5 µg of ampicillin and (T_1) purified *Di*L9. Data shown are representative of three repeat assays.

Table. 5A.1 *In vitro* antibacterial activity of Lectin (*Bh*L and *Di*L9)

Sl.No	Pathogen name	Gram staining feature	*Di*L9		*Bh*L	
			Zone of Inhibition (mm)*	MIC (mg ml^{-1})	Zone of Inhibition (mm)*	MIC (mg ml^{-1})
1	*S. aureus*	Gram positive	10	0.325	-	-
2	*B. cereus*	Gram positive	11	0.25	-	-
3	*B. subtilis*	Gram positive	-		7	0.2
4	*B. megaterium*	Gram	-	-	7	0.1

		positive				
5	*E. faecalis*	Gram positive	13	0.15	-	-
6	*E. coli*	Gram negative	7	0.5	-	-
7	*S. typhimurium*	Gram negative	-	-	-	-
8	*P. aeruginosa*	Gram negative	-	-	-	-
9	*P. morganii*	Gram negative	-	-	-	-
10	*K. pneumoniae*	Gram negative	-	-	-	-

**Zone of inhibition (in mm diameter): includes 5mm diameter of disc placed in agar disc diffusion assay.*

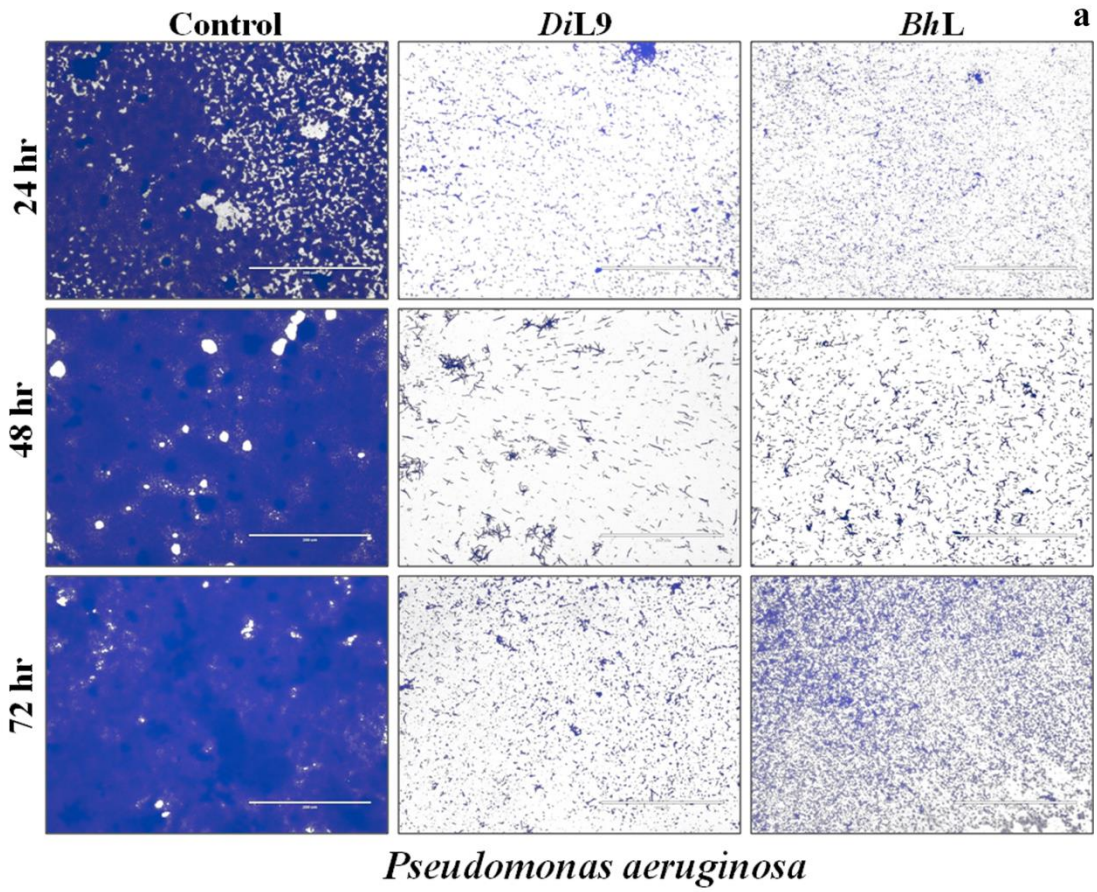
From the **Table 5A.1,** it could be observed that the lectins are more efficient in killing the Gram positive bacteria as compared to Gram negative, this might be because of the difficulty faced by lectins to cross the outer cell wall of Gram negative bacteria to reach the periplasmic space (Nunes Edos *et al.*, 2011). Moreover, Gram positive bacteria has high content of peptidoglycan layer (so more of GlcNAc moiety) in its cell wall providing more interacting sites for GlcNAc-binding lectins, like *Di*L9 and *Bh*L. From these findings, among the two purified lectins, we could suggest that *Di*L9 has more potential antibacterial activity as compared to *Bh*L though less of antifungal activity. Owing to the bacteriostatic effect, *Di*L9 characterized here could be considered for use as an alternative antimicrobial agent. The efficacy of the lectin activity could be enhanced if conjugated with nanoparticles or any other drug.

5A.2 Anti-biofilm activity of lectin

Various methods have been employed by many researchers to control the biofilm and biofouling growth on artificial surfaces like medical implants, water pipelines or

dental caries. The antibiofilm compounds inhibit this either by preventing bacterial attachment or interfering with their growth by certain mechanisms of action. Thus, here also we have evaluated the lectin potential to act as anti-biofilm agents against various pathogens known to form biofilms.

To determine the anti-biofilm potentials of lectins used, crystal violet staining method was employed. Both the lectin (*Bh*L and *Di*L9) showed good inhibitory activity against all the bacterial strains tested. Different cultures were treated with lectin and incubated for 24, 48 and 72 h. The microtitre plates were observed under microscope to qualitatively determine the rate of biofilm formation and its subsequent inhibition by lectins. As shown in the **Fig. 5A.3 (i-iii)**, *P. aeruginosa, S. aureus and E. coli* formed good biofilm layer on the polystyrene plates gradually on 72 h of incubation, but on treatment with lectins a significant decrease has been observed. The absorbance of the culture well treated with different lectins at different time intervals exhibited lower values as compared to the untreated wells (control). It is also important to notice that, after 48-72 h of incubation, there is increase in the bacterial growth in the presence of the lectins but no biofilm is formed. This implies that these lectins do not inhibit/kill bacteria but most importantly prevents its adhesion on the surface. Lectin significantly inhibited biofilm formation as observed for other pathogenic bacteria as well as those listed in the **Table 5A.2.**



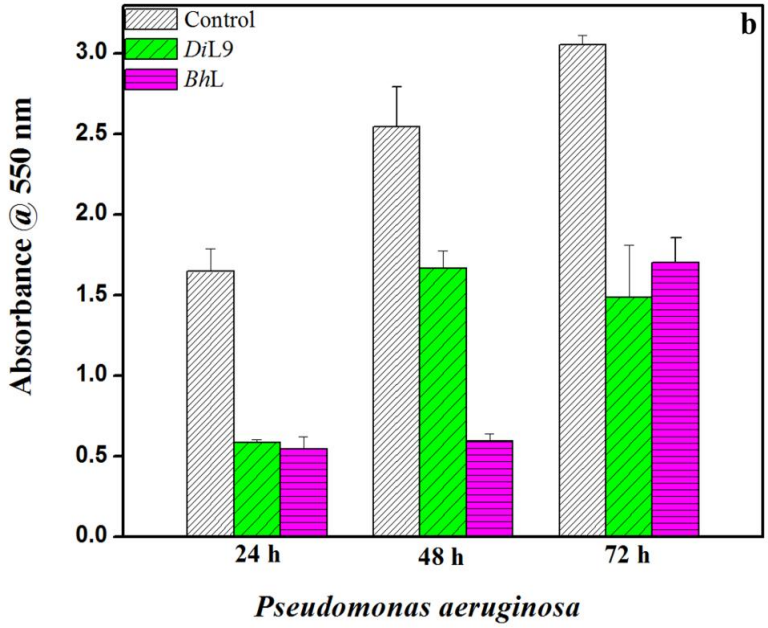
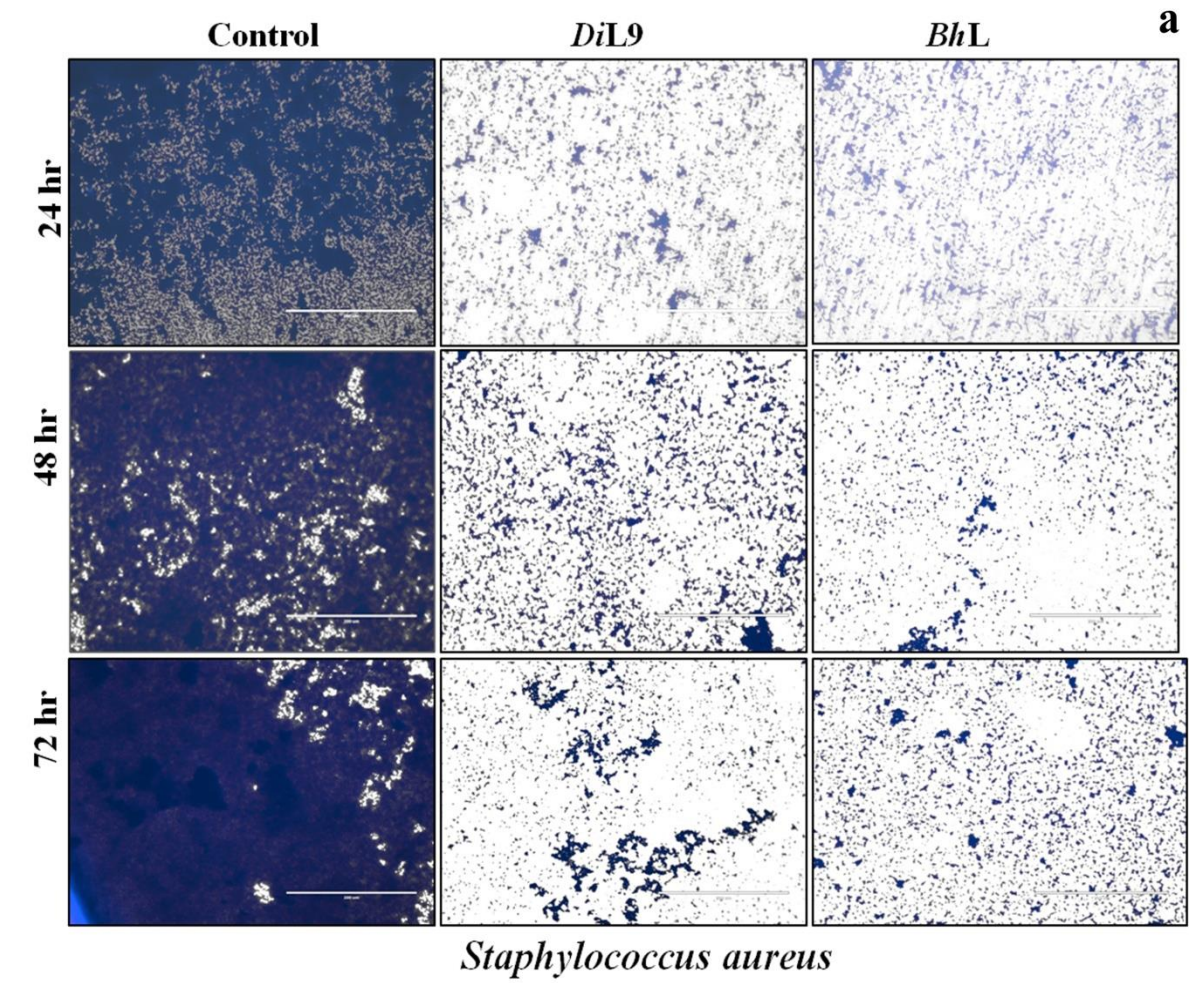


Figure 5A.3 (i) Biofilm formation by *P.aeruginosa.* (a) Microscopic scan showed that with increase in time interval, the biofilm is formed completely on polystyrene microtitre plate. Presence of lectins disrupts the biofilm formation. (b) Bacterial growth absorbance values decreases in the lectin presence.



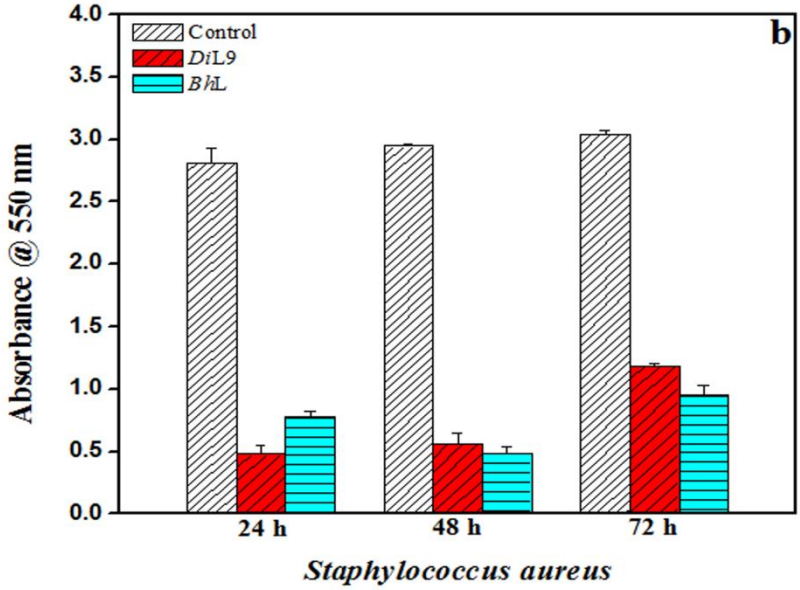
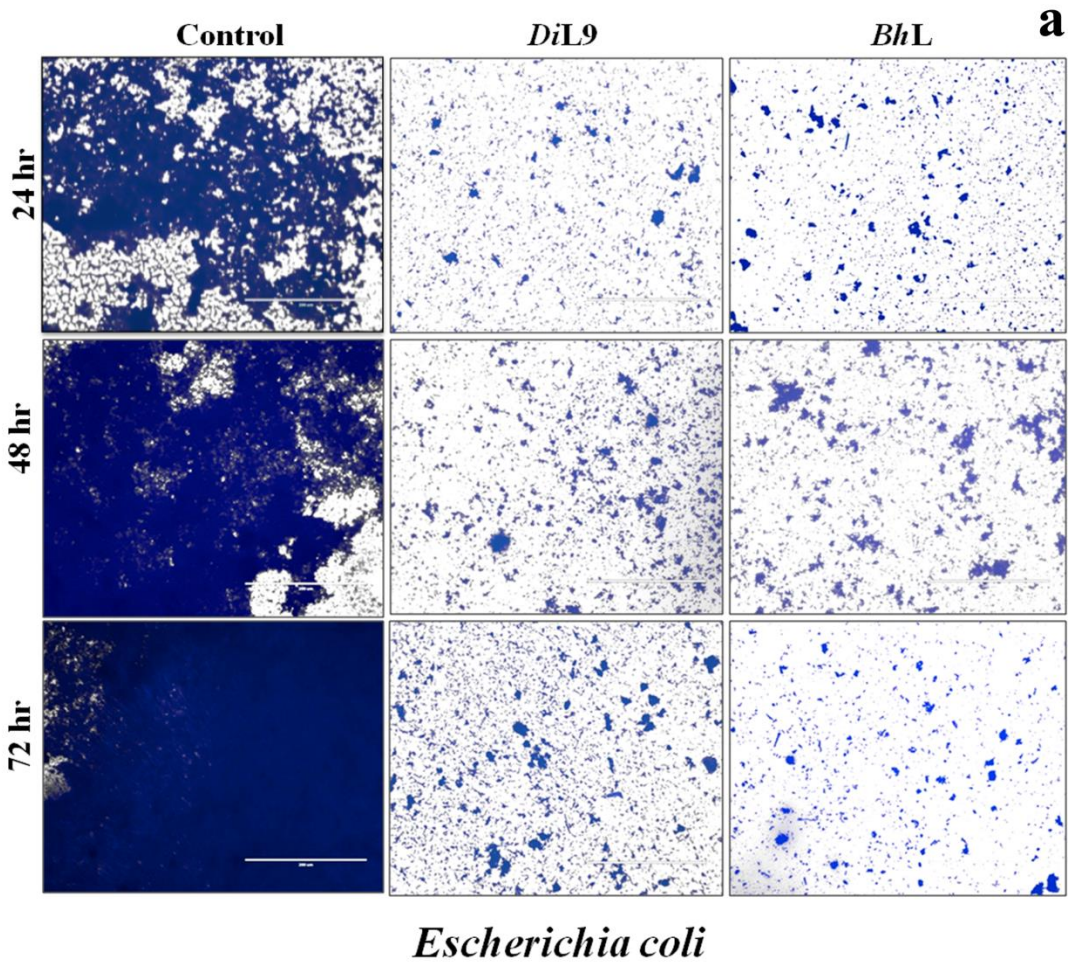


Figure 5A.3 (ii)

Biofilm formation

by *S. aureus*.



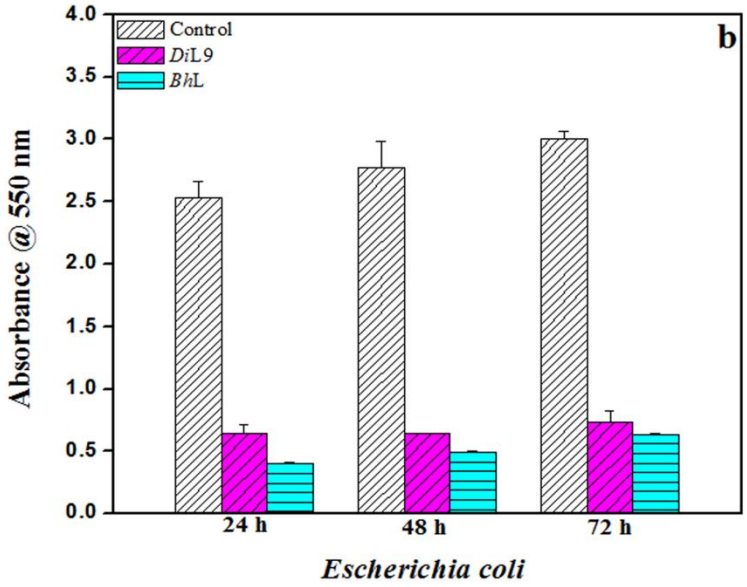


Figure 5A.3 (iii) Biofilm formation by *E.coli*

Table 5A.2 Inhibition of biofilm formation by lectins (*Di*L9 and *Bh*L)

Sl.No.	Pathogen Names	Sample (0.2 mg ml^{-1})	Percentage of inhibition (%)
1.	***Pseudomonas aeruginosa***	Control	0
		*Di*L9	51.3
		*Bh*L	44.3
2.	***Staphylococcus aureus***	Control	0
		*Di*L9	60.8
		*Bh*L	68.7
3.	***Escherichia coli***	Control	0
		*Di*L9	75.6
		*Bh*L	78.8
4.	***Klebsiella pneumoniae***	Control	0
		*Di*L9	82.6
		*Bh*L	80.0
5.	***Bacillus cereus***	Control	0
		*Di*L9	82.9
		*Bh*L	82.2
6.	***Enterococcus faecalis***	Control	0
		*Di*L9	84.1
		*Bh*L	81.0
7.	***Bacillus megaterium***	Control	0
		*Di*L9	62.0
		*Bh*L	33.7
8.	***Bacillus subtilis***	Control	0
		*Di*L9	55.3
		*Bh*L	51.4

5A.3 Scanning electron microscopy study

Figure 5A.4 shows the arrangements of the pathogenic strains (*P. aeruginosa, E.coli* and *S. aureus*) in the development of biofilm in the absence and presence of lectins (*Bh*L and *Di*L9, 0.5 mg ml^{-1}). The observations using electron microscope depicted that the untreated bacterial cells were totally covered by the layer of exopolysaccharides giving smooth appearance after 72 h of incubation. However, in the presence of lectins, the biofilm development appeared to be completely disrupted or absent. The bacterial cells grown in the presence of the lectins appeared in short stretches or freely, with absence of any exopolysaccharide matrix visible, and moreover, resulted in reduced number of cells adhering to the surface. This analysis also revealed that lectin binds to the bacterial cell surface molecules, which might be involved in communicating or maintaining the arrangement and structural integrity of the biofilms.

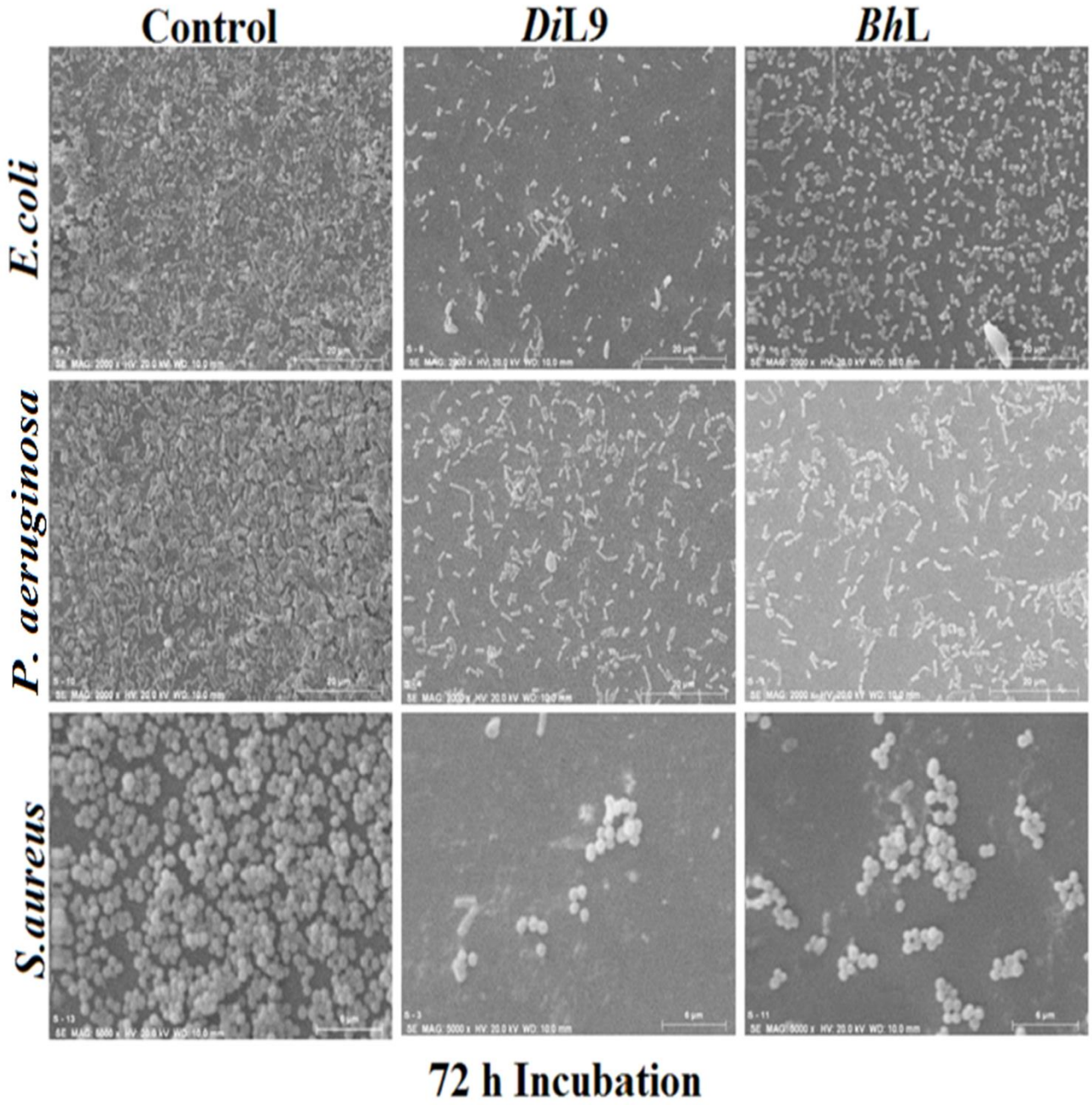


Figure 5A.4 Scanning electron microscopy images of *E. coli*, *P. aeruginosa* and *S. aureus* adhered to the silicon chips, grown in nutrient media for 72 h. Control represents the cell growth in the absence of lectins and *Bh*L and *Di*L9 (0.2 mg ml^{-1}) represents the cell growth in the presence of respective lectin.

5A.4 Effect of lectin on pre-formed biofilms

To confirm the anti-biofilm potential of these lectins, the lectin effect was also determined on the pre-formed biofilm layers. The above mentioned bacterial strains were grown undisturbed for 72 h without any drug treatment. After 3 days, the biofilm monolayer was formed and treated with same concentration of lectins (0.2 mg ml^{-1}). Kanamycin (0.01 mg ml^{-1}) was also used with or without lectin in order to determine its combined effect on biofilm formation. After the lectin treatment, the microtitre plate containing the biofilm was incubated for another 24 to 48 h. Interestingly, it was found that, the presence of lectins in the developed biofilm resulted in gap and patches formation. The 48-72 h old preformed biofilms on treatment with lectins (*Bh*L and *Di*L9) showed large voids formed among the cell aggregates as shown in the **Fig. 5A.5 (i-iii)**.

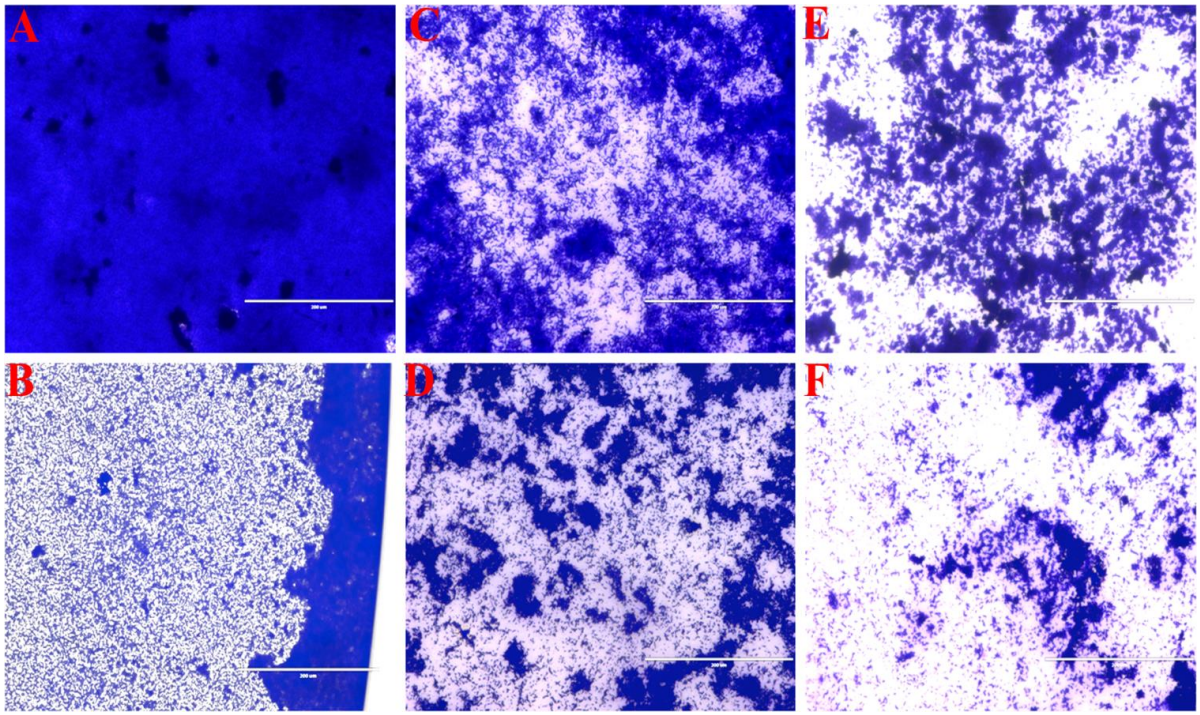


Figure 5A.5 (i) ***Effect of lectins on P.aeruginosa*** **pre-formed biofilm**. **(A)** Untreated control biofilm. Treatment with **(B)** Kanamycin **(C)** *Di*L9 **(D)** *Di*L9+Kanamycin **(E)** *Bh*L and **(F)** *Bh*L+Kanamycin. Drug treatment shows the presence of gaps/or patches formation.

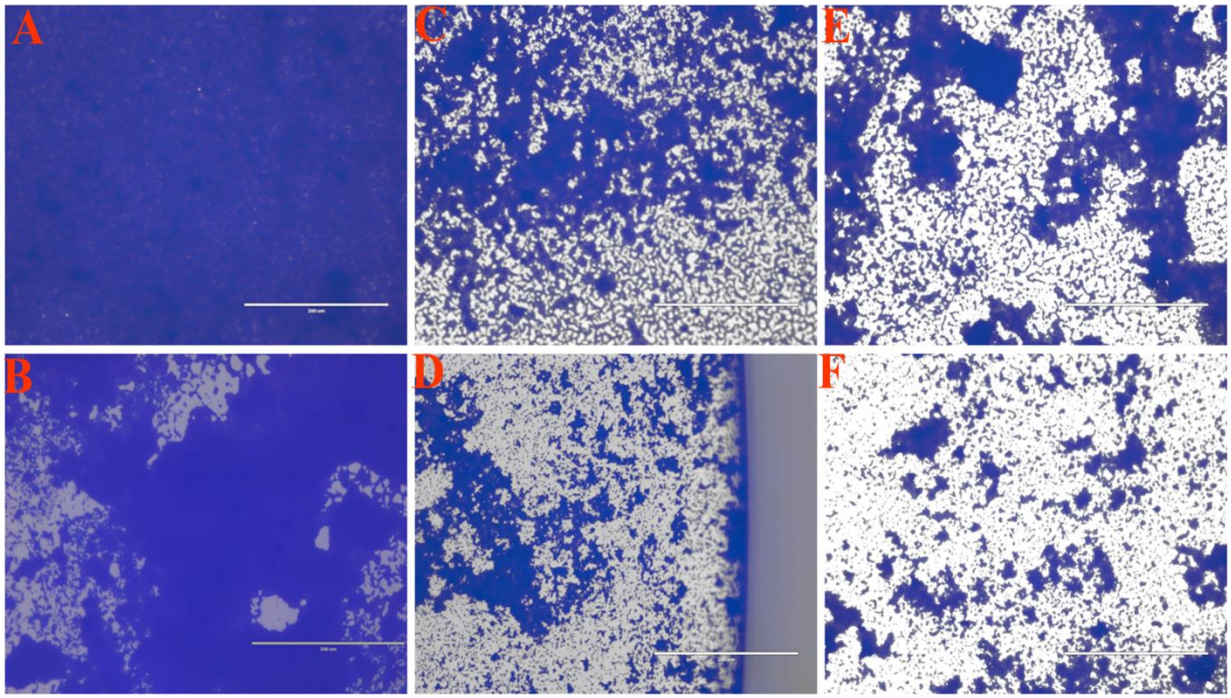


Figure 5A.5 (ii) ***Effect of lectins on E.coli*** **pre-formed biofilm**. **(A)** Untreated control biofilm. Treatment with **(B)** Kanamycin **(C)** *Di*L9 **(D)** *Di*L9+Kanamycin **(E)** *Bh*L and **(F)** *Bh*L+Kanamycin. Drug treatment shows the presence of gaps/or patches formation.

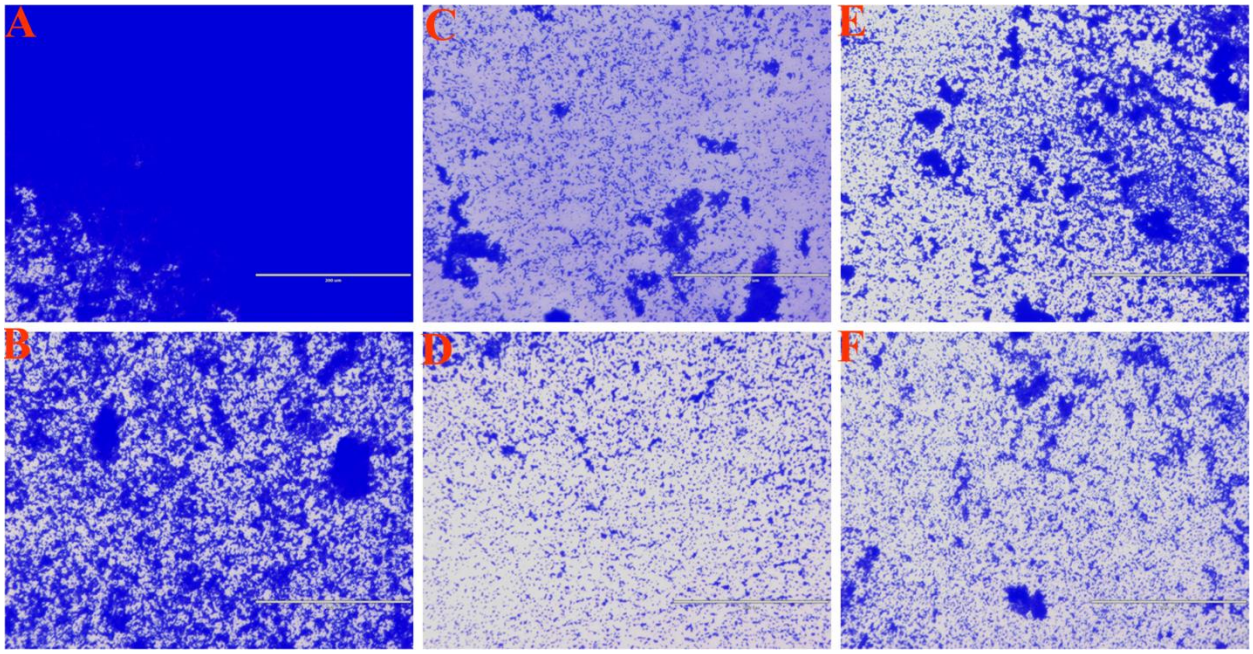


Figure 5A.5 (iii) ***Effect of lectins on S.aureus*** **pre-formed biofilm**. **(A)** Untreated control biofilm. Treatment with **(B)** Kanamycin **(C)** *Di*L9 **(D)** *Di*L9+Kanamycin **(E)** *Bh*L and **(F)** *Bh*L+Kanamycin. Drug treatment shows the presence of gaps/or patches formation.

From the **Fig.5A**, it was very clear that these lectins were able to disrupt the pre-formed biofilm by pathogenic bacteria. Interestingly, synergistic effect was also observed when used in combination with Kanamycin. Both lectins, *Bh*L and *Di*L9 in the presence of Kanamycin showed almost complete removal of biofilm formed earlier, involving anti-adherence and cell lysis.

Thus, we can conclude that lectin treatment is excellent alternative strategy for inhibiting the biofilm formation by various pathogenic strains. Moreover, inhibition by plant lectin is inexpensive and often without side effects. We observed that, the percentage of inhibition for biofilm formation varies with bacterial strains, implying that their cell surface contains a variety of glycoproteins/adhesins. Both *Bh*L and *Di*L9, showed significant difference in inhibiting bacterial adherence, this might be due to difference in sugar affinity as discussed earlier. Thus, both the lectins contribute in disturbing the bacterial cell-cell and cell-surface interactions. The potential of lectins to interfere with bacterial surface lectins/adhesins to adhere to any surface, plant lectins acts as a promising future development of anti-adherence agents.

In the literature, other lectins are also reported, for instance, lectin isolated from *S. tuberosum* inhibits biofilm formation by *P. aeruginosa* at 0.08 mg ml^{-1} concentration (Hasan *et al.*, 2014). Algal lectins were also reported to reduce biofilm formation by *S. aureus* and *S. epidermidis*, without affecting the bacterial growth (Vasconcelos *et al.*, 2014). Certain toxic lectins, purified from *Bothrops jararacussu* snake venom also reported to disrupt *Staphylococcal* biofilms (Klein *et al.*, 2015). Some other studies have also indicated that, the type of biofilm formed is directly related to the type of LPS produced by those bacteria. Since plant lectins could interact with bacterial LPS, the ability of some plant lectins to inhibit biofilm formation may result from lectin-LPS interactions, affecting the adherence of these bacteria and thus influencing biofilm formation.

It is also important to highlight the fact that, these lectins are remarkable in biofilm disruption, but does not affect the growth of the tested bacteria, which in return becomes important feature of compounds that target bacterial virulence. Since, these lectins are not affecting the growth; they will not put any selective pressure on these bacteria leading to develop resistance. Thus, these molecules become the new option as alternative strategy for the control of persistent infections, like those caused by biofilm-forming bacteria (Papa *et al.*, 2013).

5A.5 Conclusion

In this study, we have found that the chito specific lectins, having GlcNAc oligomers specificity, show less inhibitory power for bacterial growth, but showed remarkable anti-biofilm activity. Both the lectins had significant inhibitory effect on several Gram positive and Gram negative biofilms formed. It was suggested that these lectins interfere with the bacterial adhesions protein involved in cell surface attachment, or aggregates the bacterial cells, and hence, act as anti-adherent factors. The SEM analysis also indicated that the bacterial cells grown in the presence of *Bh*L and *Di*L9 lectin showed scattered aggregates as compared to the cells grown in the absence of lectin. Biofilms are known to enhance important virulence factors that enable bacteria to survive antibiotic therapy and the host immune response. *Bh*L and *Di*L9 appear to be promising molecules that can be used against biofilm formation by various bacteria.

Chapter 5

(B) Antineoplastic activity of *Bh*L and *Di*L9 on various cancer cell lines and their anti-angiogenesis activity

Carbohydrates are widely expressed on many biomembranes of living organisms in the form of glycoproteins and glycolipids. They are well catalogued for involvement in many biological functions like cell proliferation, inflammation, cell-cell adhesion and recognition (Varki, 1993, J.P. Zanetta *et al.*, 1994). It is interesting to note that there exists a huge diversity in biological glycosylation levels within the same populations of species or cell types of the same organisms (Gagneux & Varki, 1999). Many researchers have shown that glycosylation structures change at each stage of development or differentiation, where abnormal oligosaccharide expression leads to pathological conditions like cancer (Singhal & Hakomori, 1990).

Cancer in its different forms is the most deadly disease that we encounter in recent times. Many anti-cancer drugs have been formulated by researchers and pharmacology companies, however, due to insufficient specificity or solubility, their bioavailability used to be inadequate. For many decades, the genomic DNA was the main target to combat cancer, but due to resistance acquired by cancer cells that strategy turned ineffective. Thus, designing and delivering drugs at specific cancer site without affecting the pharmacophore has become the most important concern. For this, depending on the glycosylation expressed on cancer cells, the molecules with high specificity and selectivity, like lectins, found/synthesized are capable of recognizing and binding saccharides and eliciting the cell signal transduction are required. Many lectins, especially those of plant origin have been discovered, have been found very specific and could distinguish between normal cells and malignant cells based on expression level of glycosylation associated with metastasis. For example, lectins like Con A which belongs to legume family is known to possess anti-neoplastic activity by eliciting the signaling

cascade to cause cell apoptosis and/ autophagy (Li *et al.*, 2010) and mistletoe lectins (MLs) belonging to Type 2 RIPs are known to induce apoptosis via mitochondria/ death receptor pathways (Kang *et al.*, 2007, Lyu *et al.*, 2002).

Cancer can result by any dysregulation in the cell cycle or apoptosis process. Apoptosis, also called type I programmed cell death (PCD), is characterized by the presence of cytoplasmic and nucleic condensation, fragmentation of nucleic acids, membrane blebbing and phagocytosis (Hengartner, 2000). Many lectins have been reported to block the tumorigenesis cascade, like *Sclerotium rolfsii* (Savanur *et al.*, 2014), leczyme (Tatsuta *et al.*, 2013), *Rhizoctonia bataticola* (Pujari *et al.*, 2013) and Wheat germ agglutinin (WGA) (Aub *et al.*, 1965). Tumor angiogenesis is also majorly responsible for the spread and proliferation of the tumor through metastasis (Folkman, 1995). The best treatment will be based on inhibiting the tumor-induced formation of new blood vessels by Vascular Endothelial Growth Factor (VEGF) binding or targeting tumor induced blood vessels only or using both strategies **(Fig. 5B.1)**.

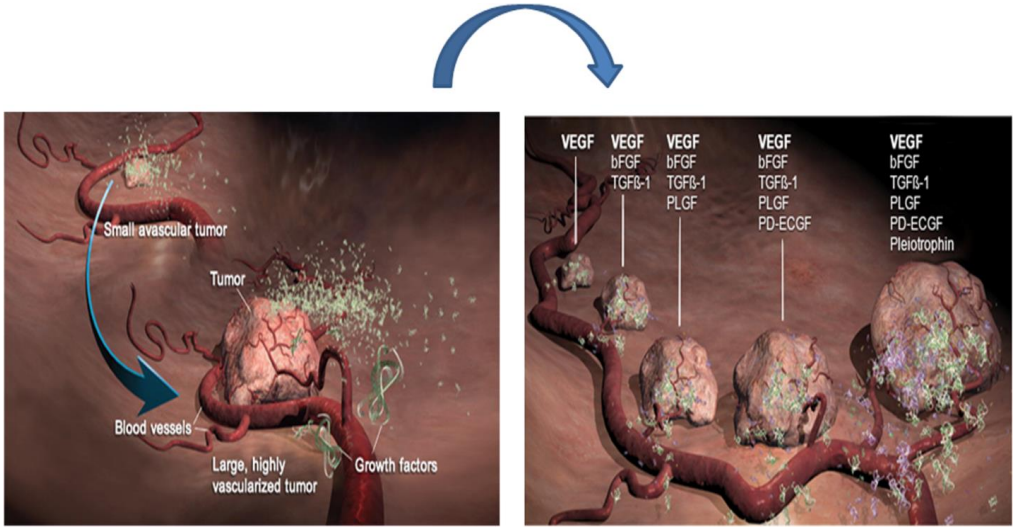


Figure 5B.1 Formation of new blood vessels induced by tumor and its spread. (*Image adopted from biooncology.com)*

Many proteins have been discovered acting as angiogenic enhancers or inhibitors; their expression levels in a system determine the aggressiveness of tumor cells **(Fig. 5B.2)**. However, not many anti-angiogenic drugs are proved effective for long-term survival (Nishida *et al.*, 2006). Hence, there is always need for new therapeutic strategy to fight cancer by combining anti-angiogenic agents with other conventional anti cancer drugs.

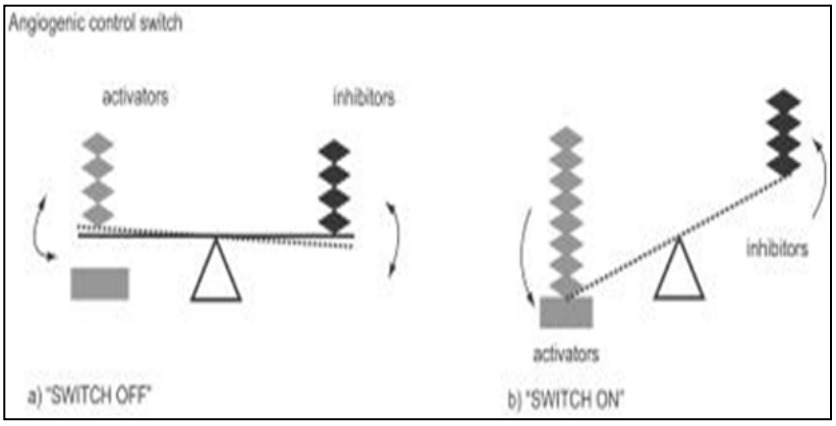


Figure 5B.2. (a) Regulation of Angiogenesis using a balance between activators and inhibitors. **(b)** When a tumor needs its nutrient supply, the level of activators increases with simultaneous decrease in inhibitors, hence, angiogenesis is stimulated (Nishida *et al.*, 2006).

Pancreatic cancer (PanC)

It is the fourth leading cause of cancer deaths after lung, colorectal, and breast cancer. It is the most deadly type of cancer because of its difficulties in early diagnosis. Currently, many surgical procedures, chemotherapy and radiotherapy or their combinations are available but no improvement in the survival rate of patients is observed. Two types of PanC are known: Exocrine and endocrine tumors. But the symptoms shown in the **Fig. 5B.3** are also likelier to cause cancer because of other pathological conditions which makes its diagnosis difficult.

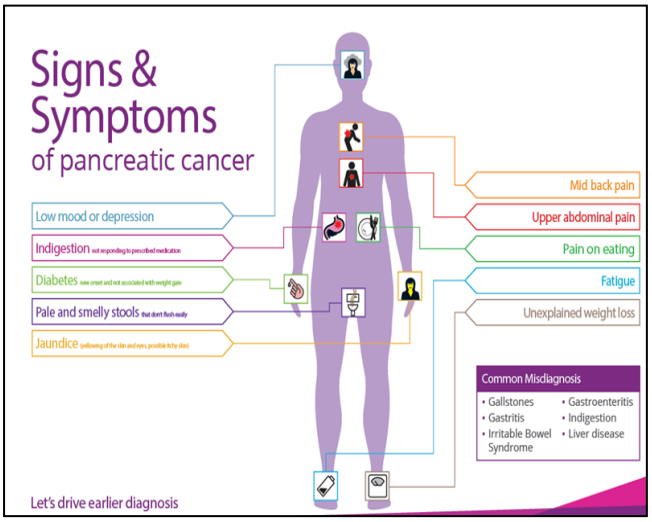


Figure 5B. 3 Signs and symptoms of Pancreatic cancer *(Adopted from www.pancreaticcanceraction.org)*

Causes and Risk factors for PanC

According to American Cancer Society, many factors are involved in chance to get PanC especially exocrine pancreatic cancer. It was also estimated that in the year 2015 PanC accounts for 3 % of the cancers in US and account for 7 % of all cancer deaths. Imaging tests like taking computed tomography scan, magnetic resonance imaging or ultrasound are the basic diagnostic techniques available for its detection. But patients realize the symptoms very late when the tumor has reached its last stage (IV stage) where the survival rate is less than 1 %. Hence, researchers are trying to develop safer and more effective therapies.

This chapter presents report of the investigations carried out to determine the anti-neoplastic activities of two chito-specific lectin, *Benincasa hispida* lectin and *Datura innoxia* lectin described in previous chapters. As already discussed, both these agglutinins are structurally unrelated but they do bind to the same sugar (GlcNAc oligomers) with different affinities. This chapter also describes the methodologies employed to carry out the investigations on various cancer cell lines.

5B.1 Lectin cytotoxicity assay

We investigated the anti-neoplastic activity of these lectins, where both the lectins have affinity for the same sugar but lower affinity is shown by *Di*L9. Using the MTT assay, we observed that *Bh*L showed more pronounced effect on the viability of the cells tested; this might be due to its higher affinity for the glycans present on the cell membranes. **Fig. 5B.4 (A,B)** represents the percentage of cells undergone growth inhibition on lectin treatment. It was interesting to see that both the lectins showed no inhibitory effect towards HUVECs and L929 cells (<30 % inhibition) even at higher doses of lectins, implying their selective effect on cancer cells only.

For each cell lines, the GI_{50} was calculated from the **Fig 5B.4 (A,B)** against each lectin tested as summarized in **Table 5B.1.** GI_{50} of *Bh*L on PMA (phorbol myristate acetate)-differentiated human THP-1 macrophages was estimated at 58 µg ml^{-1} whereas *Di*L9 had no effect on cell viability. This could be due to differences in glycan binding affinity of these lectins towards the cell surface glycans. 50 % of cell growth inhibition was seen in the case of A549, HeLa and PANC-1 cells, but the lowest GI_{50} (*Bh*L: 8.4 µg ml^{-1}; *Di*L9:142 µg ml^{-1}) and GI_{90} value (*Bh*L: 85 µg ml^{-1}; *Di*L9: 468 µg ml^{-1}) was observed in PANC-1 cells. The discrepancy in lectin cytotoxicity may be caused by the divergence of glycoprotein expression on different cell lines. Carboplatin exhibited potent cytotoxicity against all cell lines, as reported. PANC-1 cells were selected for further studies because both the lectins had higher toxicity effect on human pancreatic cancer cells at lower doses. To confirm this observation, we have selected two more different human pancreatic cancer cell lines, namely CFPAC-1 and MIA PaCa-2 (**Fig.**

5B.5). On comparing the data obtained we found that, these lectins showed specificities towards pancreatic cancer cell lines with higher inhibitory effect on PANC-1 cells.

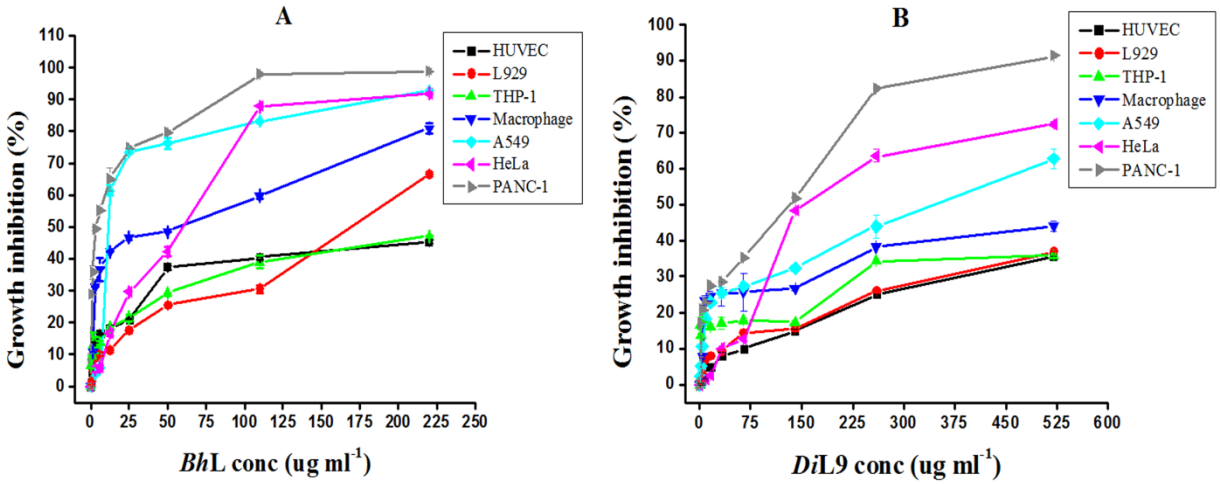


Figure 5B.4 Effect of lectins on proliferation and cell viability. Primary cells and different cancer cell lines were treated with *Bh*L and *Di*L9 (5-600 µg ml^{-1}) and incubated for 48 h. The growth inhibition (%) was measured by MTT assay by considering untreated cells as 100 %. **(A)** *Bh*L **(B)** *Di*L9 treatment on different cell lines.

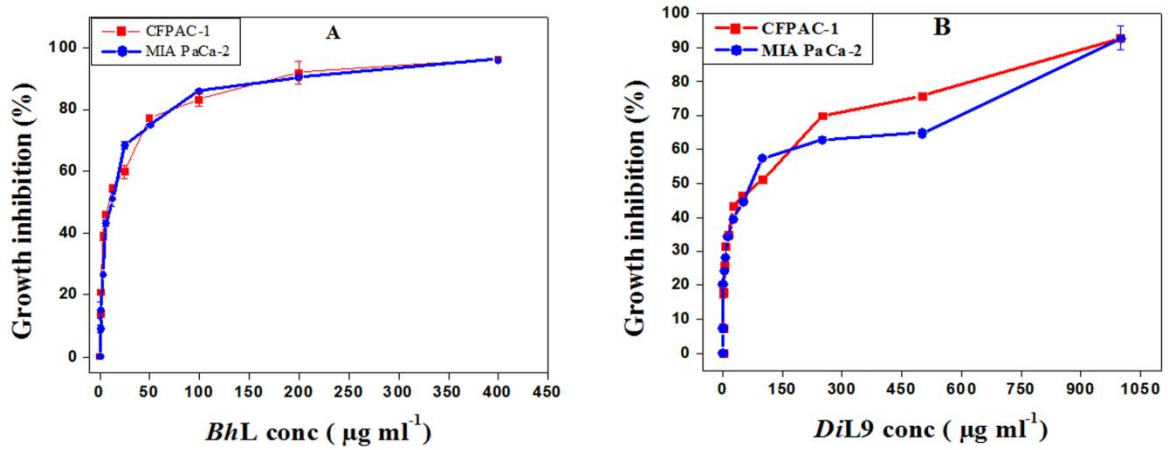


Figure 5B.5 Anti-proliferative activity of *Bh*L and *Di*L9 on CFPAC-1 and MIA PaCa-2 cells: The cancer cell lines were treated with different concentrations of lectins and incubated for 48 h. The growth inhibition (%) was measured.(**A)** Effect of *Bh*L and (**B)** *Di*L9 treatment cell lines.

Table 5B.1 Growth Inhibitory (GI) effect of *Bh*L and *Di*L9 on different cell lines

Cell name	*Bh*L		*Di*L9		[b]Carboplatin	
	[a]GI_{50}	GI_{90}	GI_{50}	GI_{90}	GI_{50}	GI_{90}
[*1]HUVEC	>130	>130	>520	>520	>10	>10
[#2]L929	>130	>130	>520	>520	>10	>10
[##3]THP-1	>208	>208	>1000	>1000	0.1374±0.53	5.8140±0.02
[##4]A549	29.32±0.23	200.54±2.08	344.04±0.56	>520	0.0035±0.71	0.0706±0.60
[##5]HeLa	34.09±0.15	168.45±0.55	146.01±0.35	>520	0.0048±0.36	0.075±0.56
[##6]PANC-1	**8.39±0.49**	**84.98±0.34**	**141.93±0.65**	**468.16±0.32**	**0.8519±0.96**	**5.7150±0.19**
[##7]CFPAC-1	11.32±0.45	172.81±0.25	86.49±0.15	913.32±0.35	0.968±0.46	8.76±0.36
[##8]MIA PaCa-2	13.99±0.4	186.13±0.5	67.92±0.45	943.13±0.5	0.376±0.36	5.0±0.66
[**9]Macrophage	58.52±1.32	>208	>1000	>1000	>10	>10

[a] Growth Inhibition (GI): GI_{50} /GI_{90} (concentration which resulted in 50 % /90 % decrease in cell viability). Expressed in µg ml^{-1}.

[b] Standard anticancer drug and positive control.

*Primary cells: [1]HUVECs- Human Umbilical Vein Endothelial Cells,

[#]Cell Line from mouse origin: [2]L929- areolar and adipose tissue fibroblast Cells,

[##]Human cancer cell lines: [3]THP-1 from acute monocytic leukemia,

[4]A549 from lung adenocarcinoma,

[5]HeLa from cervix adenocarcinoma,

[6]PANC-1 from pancreas carcinoma

[7] CFPAC-1 from pancreatic ductal adenocarcinoma

[8] MIA PaCa-2 from pancreatic epithelial carcinoma

**[9]Macrophage-PMA (phorbol myristate acetate)-differentiated human THP-1 macrophages.

We have also simulated the *in vivo* conditions by growing the cells in the presence of 100 % serum. The effect of lectins on cancer cells in the presence of 100 % serum was also evaluated. To carry out this, the lectins were pre-incubated with serum for 24 h and anti-cancer activity was determined using MTT assay as already discussed. Surprisingly, only 20 % of growth inhibition was observed at a higher concentration of 1mg ml^{-1} (or 30 μM) of *Bh*L, whereas the effect of *Di*L9 was negligible **(Fig. 5B.6)**. This decrease in the activity of the lectins could be due to glycoprotein present in the serum that binds to the lectins and, thus inhibiting its activity (as discussed in the Chapter 3 and 4).

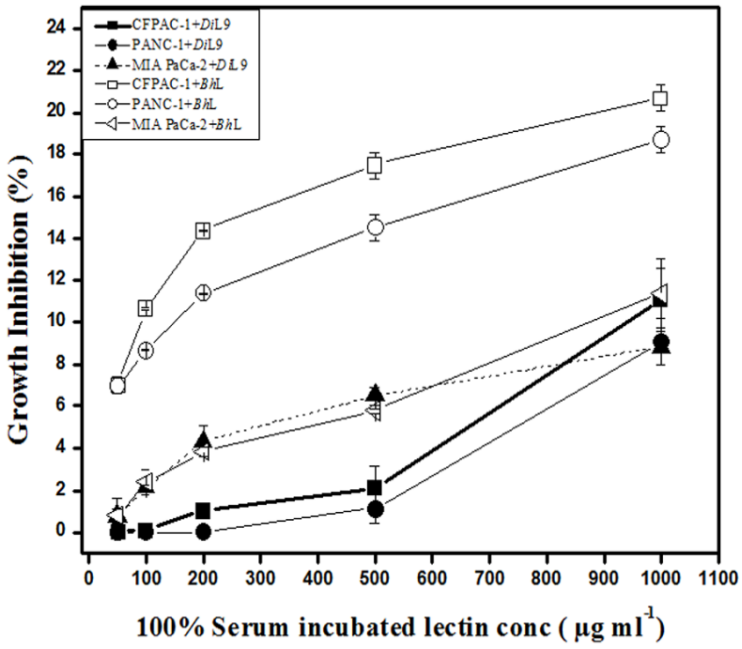


Figure 5B.6 Effect of serum incubated lectin on cancer cells. Both the lectins (*Bh*L and *Di*L9) were incubated with 100 % serum for 24 h. MTT assay was carried out to determine the effect of serum incubated lectin on pancreatic cancer cell lines (PANC-1, CFPAC-1 and MIA PaCa-2).

5B.2 Lectin induced apoptosis in pancreatic cancer cells

To investigate the exact reason behind the cytotoxicity caused by the chito-specific lectins, first we studied the effect of lectin on the cell cycle arrest and then the morphological changes.

The PANC-1 cells were treated with GI_{50} values of lectin for 24 h followed by DAPI staining and HCS analysis, also followed for the rest of the experiments. It is obvious to see that the untreated cells exhibited all the three normal phases G_0/G_1, S and G_2/M of the cell cycle. Carboplatin treatment resulted in S phase arrest whereas both the lectin treated cells showed G_0/G_1 arrest with total of 87 % populations till 24 h **(Fig. 5B.7.A, B)**. It was also interesting to observe that the effect of both the lectins on PANC-1 cells appeared to be similar and time-independent, arresting the cells at G_0/G_1 phase, showing a parallel decrease of the cell population in the S and G_2/M phase to 8 and 2 %, respectively. Similarly, lectin from mulberry leaf was also reported to induce arrest at G_0/G_1 phase of human breast cancer and colon cancer cells (Deepa *et al.*, 2012).

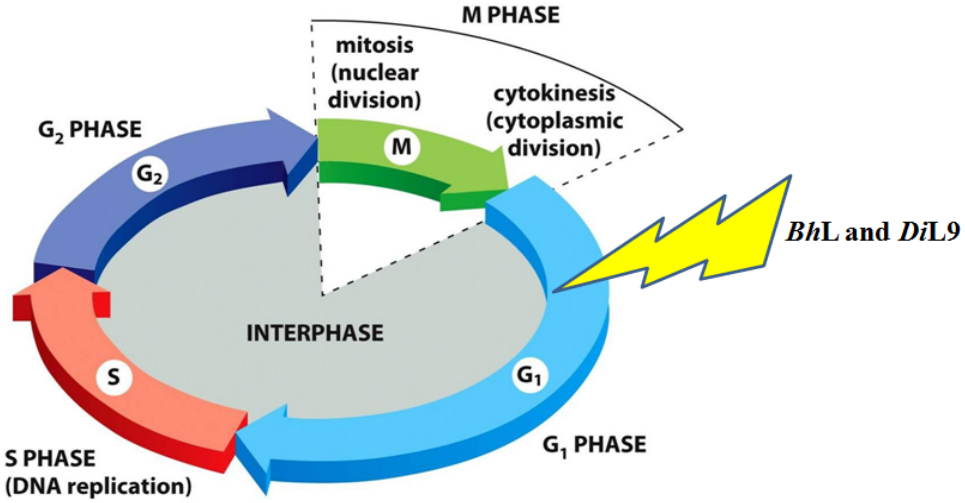


Figure 5B.7 A. Schematics explaining the cell cycle arrest at G_0/G_1 phase by the lectins (*Image Adopted from www.pha.jhu.edu/~ghzheng/old/webct/note71.htm and modified).*

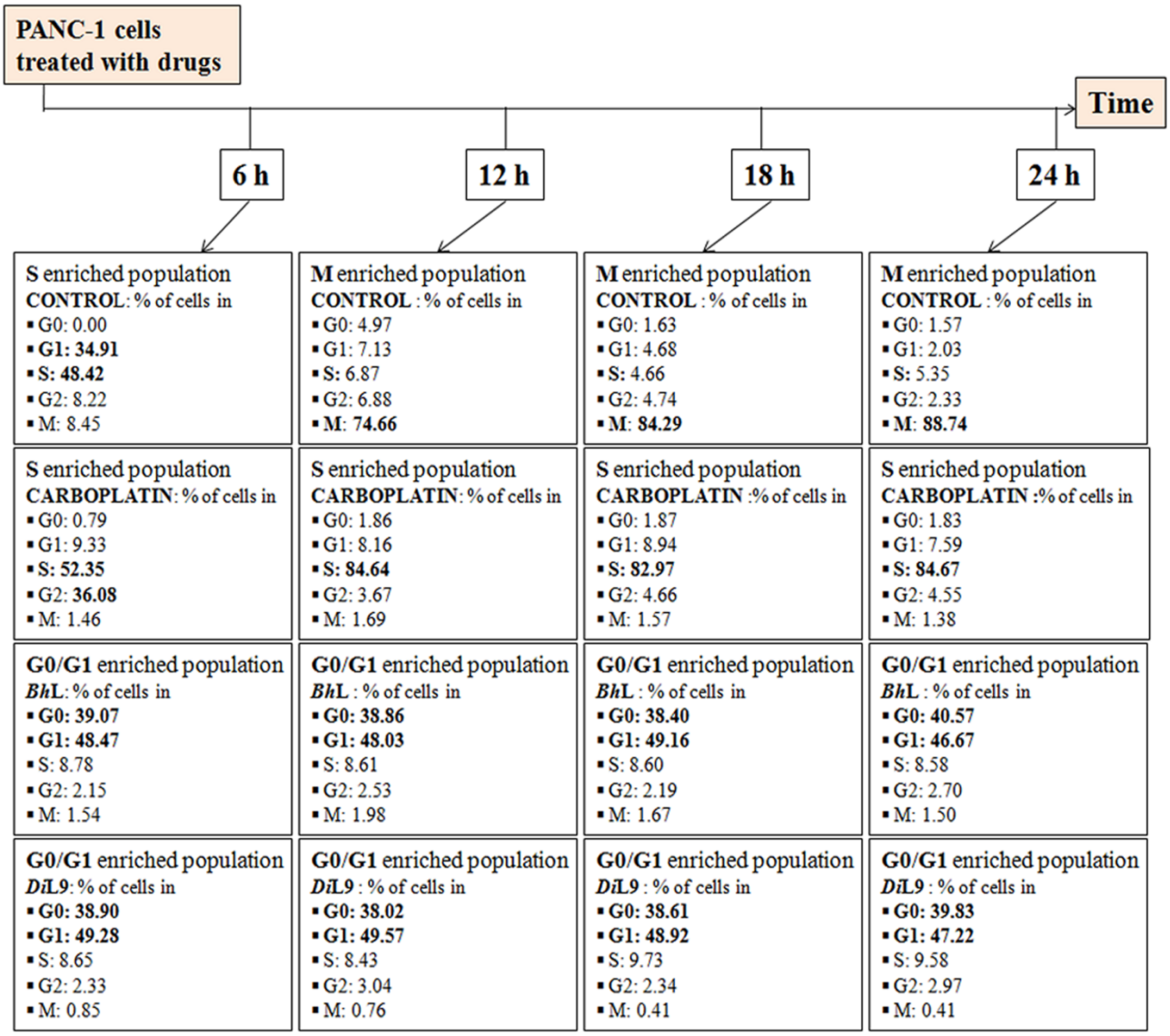


Figure. 5B.7B Effect of *Bh*L and *Di*L9 on cell cycle phases. The progressive cell cycle changes were observed with DAPI staining after 6, 12, 18 and 24 h on lectin (*Bh*L: 8.4 µg ml^{-1} and *Di*L9: 142 µg ml^{-1}) treatment and calculating the number of cells using HCS software. The darkened numbers indicate the percentage of cells arrested in different phases of cell cycle.

5B.3 Cell morphology studies

Any cell morphological changes can provide a better method for recognizing the apoptosis process. Lectin treated PANC-1 cells showed complete nuclei disintegration

which formed into small condensed apoptotic bodies, as observed on AO staining **(Fig. 5B.8A).** The number of cells undergone apoptosis was quantified using Annexin V-FITC staining. Sometimes due to external factors like toxins, most of the cells undergo necrosis. So, in order to differentiate between viable, apoptotic and necrotic cells, Annexin V-FITC staining was coupled with PI staining also. During investigation, we found that lectin treated PANC-1 cells showed a time-dependent increase in Annexin V-FITC positive cells from 12 % at 24 h to approximately 20 % at 48 h and 50 % at 72 h achieved by both the lectins (*Bh*L and *Di*L9), as shown in the graph **(Fig. 5B.8B)**. This indicated that these two lectins induced apoptosis in PANC-1 cells. Even after 72 h exposure of lectin treatment to cells, there were no PI-positive cells. This confirmed that major cause of PANC-1 cell death by these chito-specific lectins is apoptosis activation and not through necrocytosis **(Fig.5B.8C)**. The other pancreatic cancer cell lines, CFPAC-1 and MIA PaCa-2 also showed similar observations on incubating with lectins for 72 h, confirming its selectivity and specificity towards pancreatic cancer cells only **(Fig. 5B. 9)**.

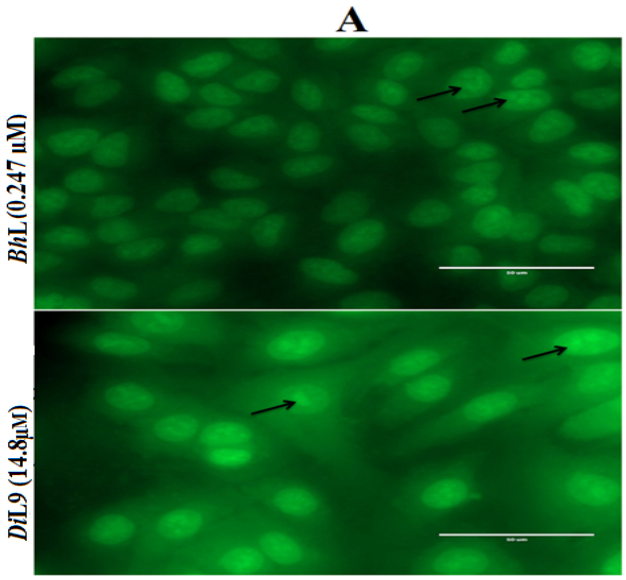


Figure 5B. 8 (A) *Bh*L and *Di*L9-induced apoptosis in PANC-1 cells. Acridine orange staining of lectin stimulated PANC-1 cells. (*Bh*L: 0.247 µM/8.4 µg/ml and *Di*L9: 14.8 µM/142 µg/ml). The arrows indicate apoptotic bodies formed inside the lectin treated cells.

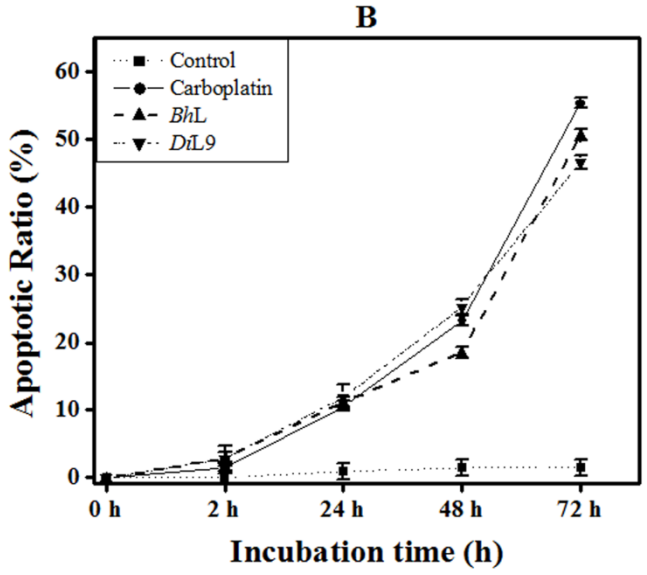
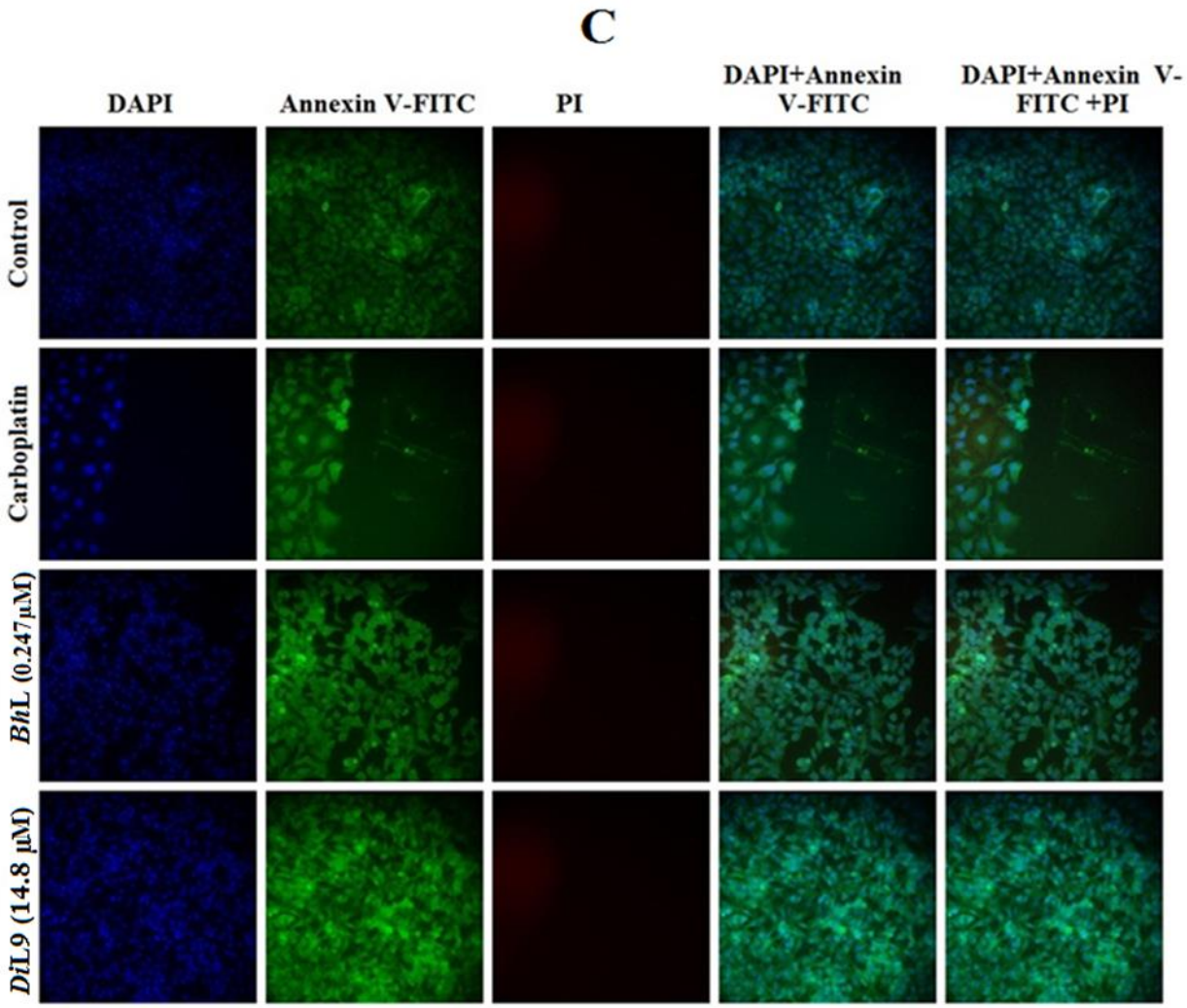


Figure 5B. 8 *Bh*L and *Di*L9-induced apoptosis in PANC-1 cells. (B) The graph represents percentage of cells undergoing apoptosis over a period of time. **(C)** The overlay represents the cells that have undergone apoptosis (Annexin V-FITC positive, green) or necrosis (PI positive cells, red) after 72 h of incubation.



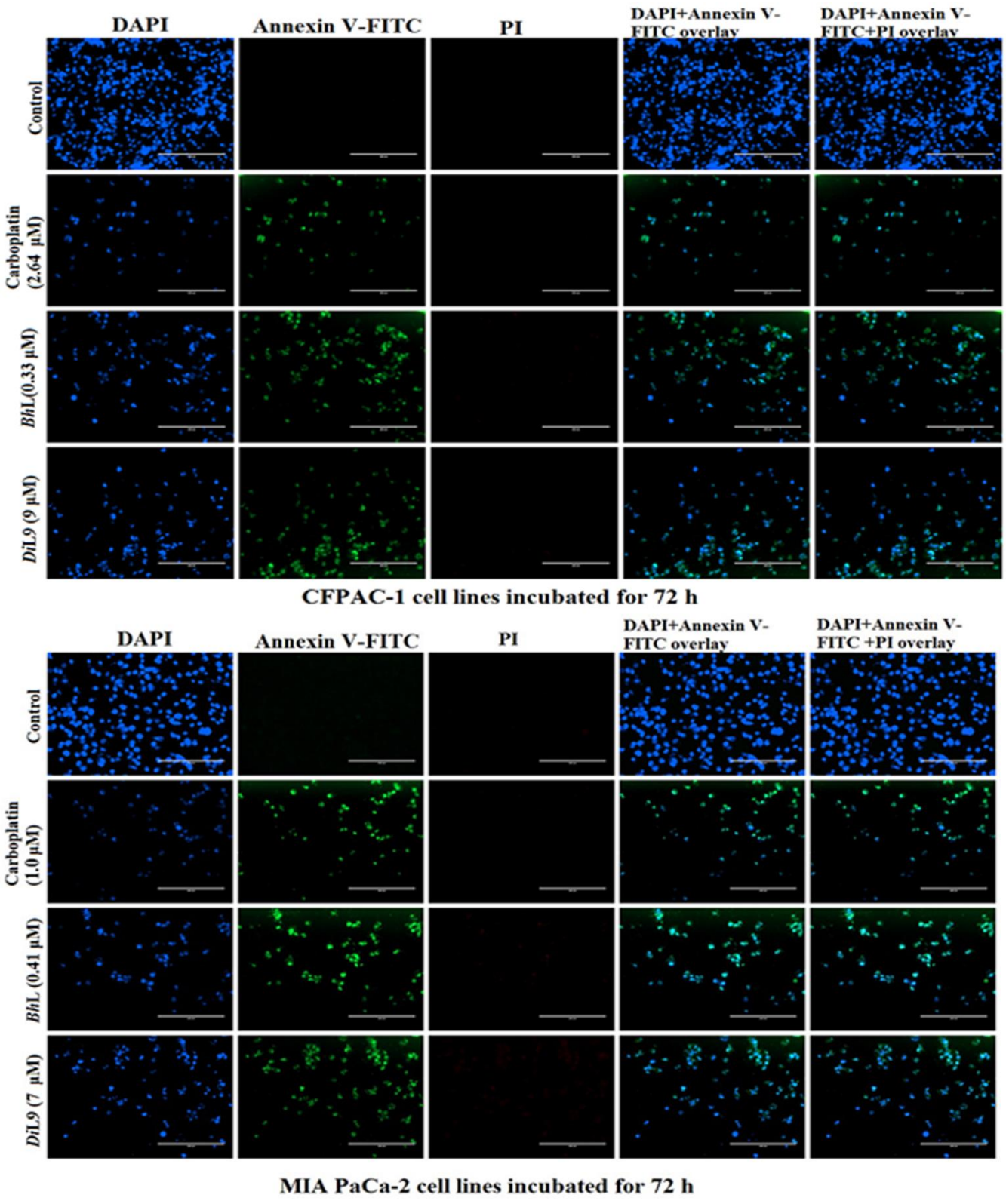


Figure 5B. 9. Apoptotic effects of *Bh*L and *Di*L9 on CFPAC-1 and MIA PaCa -2 cells. The human pancreatic cells were incubated with or without lectins (*Bh*L and *Di*L9, GI_{50} conc.) for 72 h. The cells were stained with DAPI, Annexin V-FITC and PI. The overlay represents the cells

that have undergone apoptosis (Annexin V-FITC positive, green) or necrosis (PI positive cells, red).

5B.4 Perturbation of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondria is the source of energy which supports life under aerobic conditions, but it is now known that it can also trigger cell death by making the cell apoptotic (Gottlieb, 2000). As mentioned before, the release of Ca^{2+} and Cytochrome c is associated with mitochondrial membrane disruption, a key event in initiating apoptosis by forming the apoptosome that is needed to activate downstream caspases. A complete structural damage to mitochondria may be required to achieve rapid release of cytochrome c or Ca^{2+}. So here in this work, we have tried to link the relationship between disruption of mitochondrial membrane potential (MMP) and apoptotic fate of the cell.

In order to evaluate the integrity of mitochondrial membranes, the lectin (GI_{50})-treated pancreatic cells were incubated for 4, 8 and 12 h and stained with Mitotracker dye. During observations, we found that there is gradual decrease in the fluorescent intensity of Mitotracker dye staining implying loss of $\Delta\psi_m$ in a time-dependent manner as shown in the **Fig. 5B.10 (A-C).**

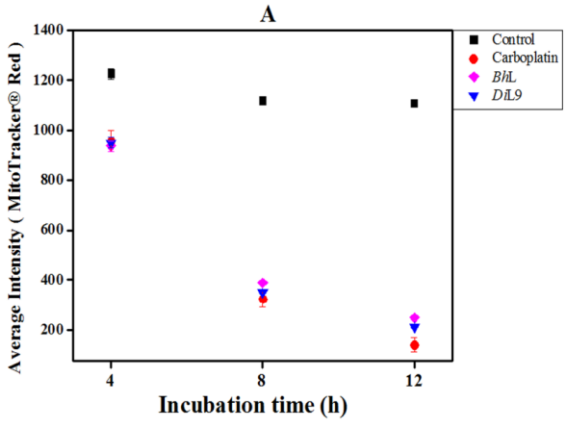


Figure 5B.10 (A) Alteration of mitochondrial membrane potential. Quantification of fluorescence intensity of mitochondrial depolarized cells on lectin treatment (*Bh*L: 8.4 µg ml^{-1} and *Di*L9: 142 µg ml^{-1}).

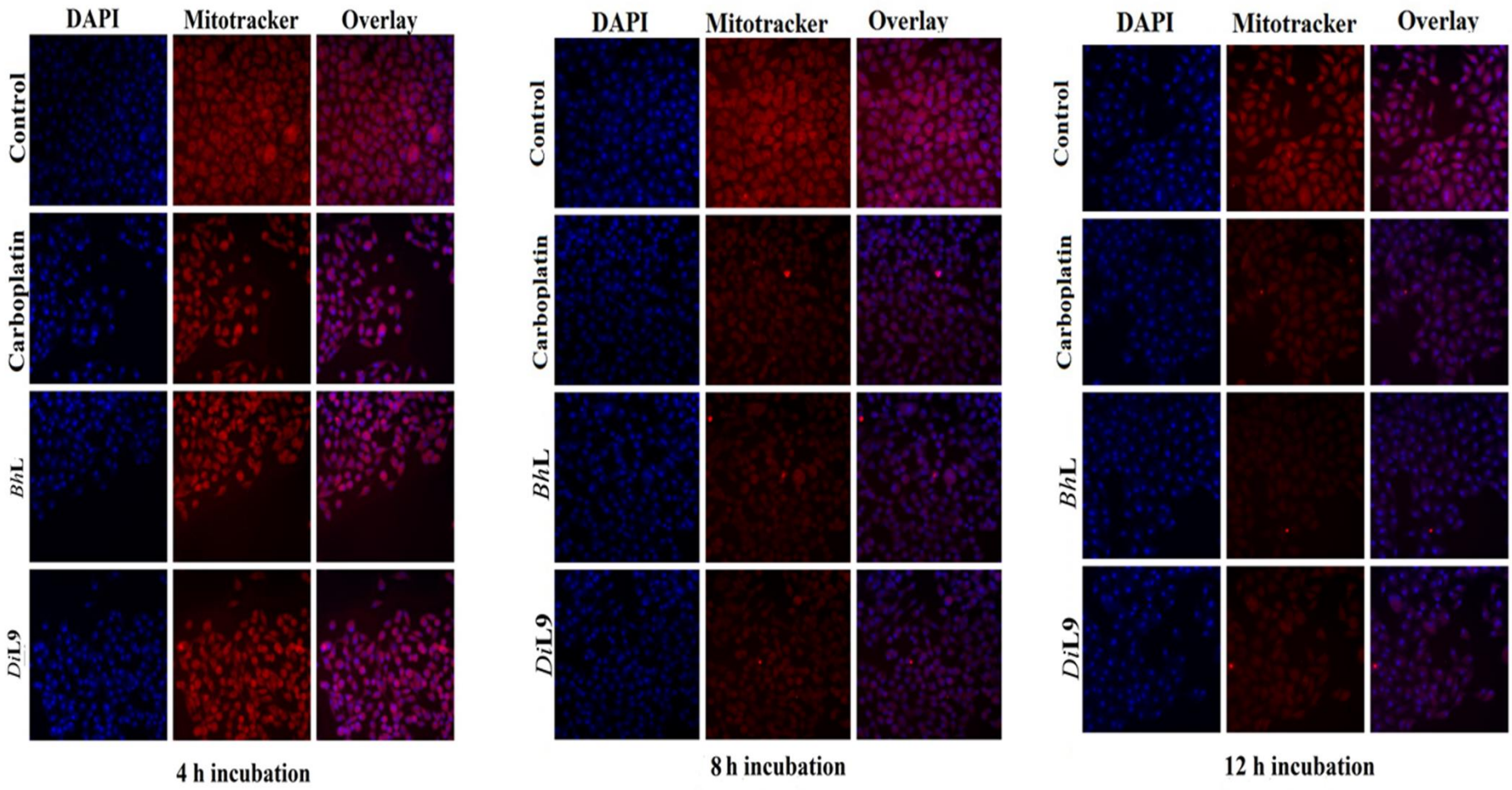


Figure 5B.10 (B) Alteration of mitochondrial transmembrane potential. Lectin (*Bh*L: 0.247 µM and *Di*L9: 14.8 µM) treated cells were incubated for 4, 8 and 12 h and stained with Mito Tracker Red (0.1 µmol l^{-1}) for 15 min at 37 °C. The overlay represents the fluorescence intensity of cells bound with Mitotracker Red and DAPI staining, recorded by LSCM, Magnification 20X objective (scale, 100 µm). Loss in red intensity represents the loss in mitochondrial membrane potential in a time dependent manner.

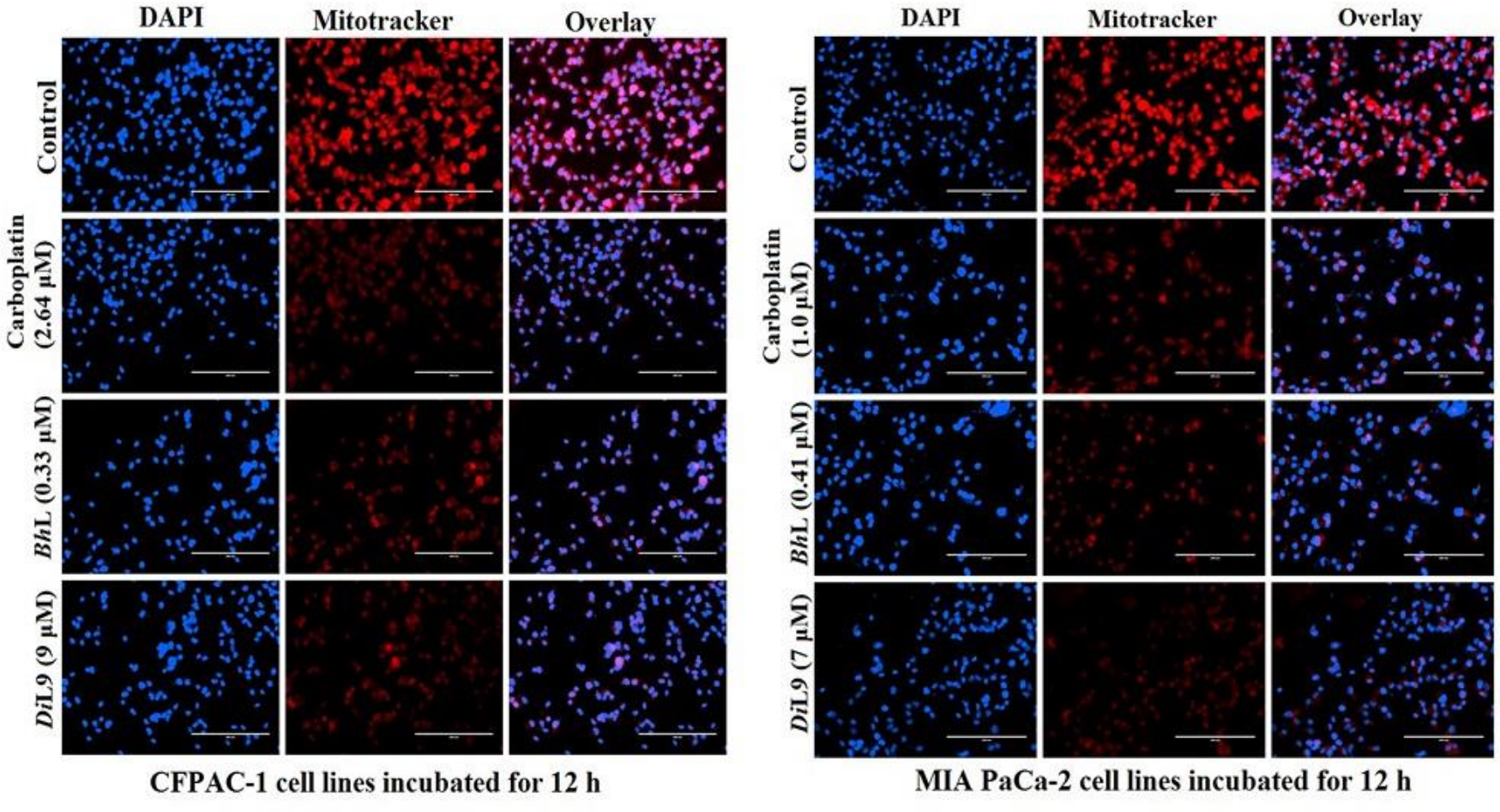


Figure 5B.10 (C) Alteration of mitochondrial transmembrane potential. Lectin stimulated cells CFPAC-1 and MIA PaCa -2 cells were incubated for 12 h and stained with Mito Tracker Red (0.1 µmol l^{-1}) for 15 min at 37 °C. The overlay represents the fluorescence intensity of cells bound with Mitotracker Red and DAPI staining, recorded by LSCM, Magnification 20 X objective (scale, 100 µm). Loss in red intensity represents the loss in mitochondrial membrane potential

With disruption in the MMP, there will be increase in the calcium ions in the cell cytoplasm. The effect of lectins in modulating apoptosis by elevating the $[Ca^{2+}]_i$ in PANC-1 cells was also demonstrated by using calcium sensitive dye (Fluo-4 AM). On incubation of the lectin exposed cells at different time intervals, showed gradual increase in the number of cells releasing calcium in a time-dependent manner. It was measured as 93.6 % (*Bh*L), 96.2 % (*Di*L9) and 95.8 % (Carboplatin), where the untreated cells showed only 0.9 % even after 12 h incubation **(Fig. 5B.11 A, B)**. Hence, these results indicated that cytotoxicity of *Bh*L and *Di*L9 is induced through mitochondrial membrane disruption releasing calcium ions, finally activating the downstream pro-apoptotic signals.

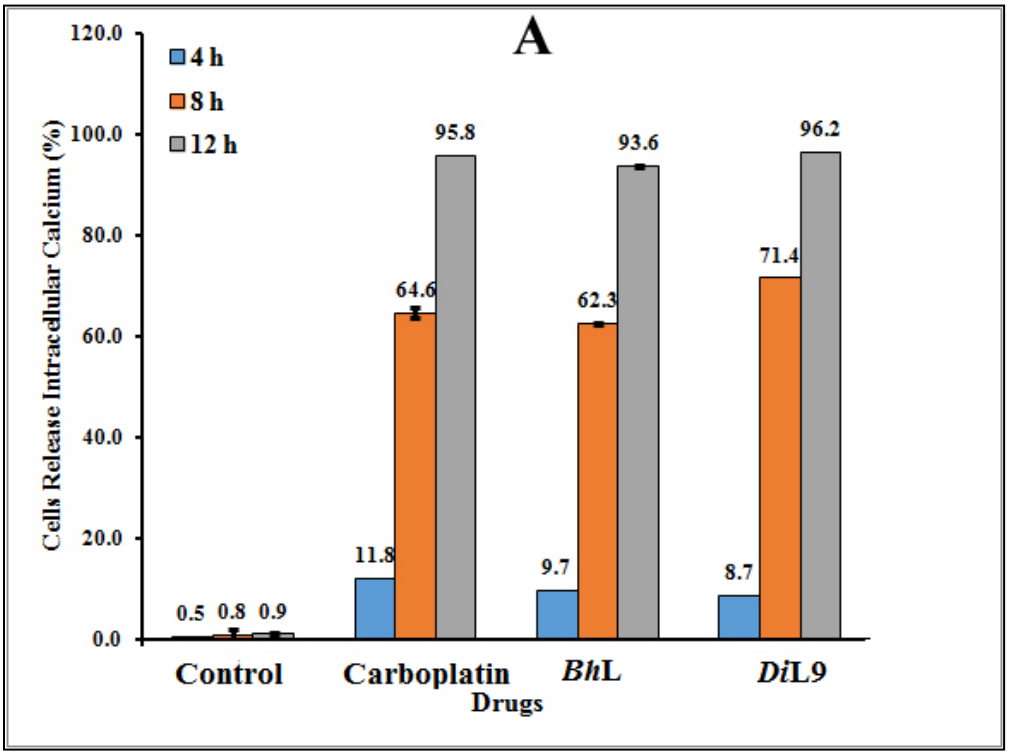


Figure 5B. 11 (A) Increase in intracellular $[Ca^{2+}]_i$ release in lectin stimulated PANC-1 cells. PANC-1 cells were treated with lectins (*Bh*L: 8.4 µg ml^{-1} and *Di*L9: 142 µg ml^{-1}) for 4, 8 and 12 h, stained with Fluo-4/AM (4 µM, green) and DAPI (blue). The graphs depict the percentage of cells releasing calcium over a period of time.

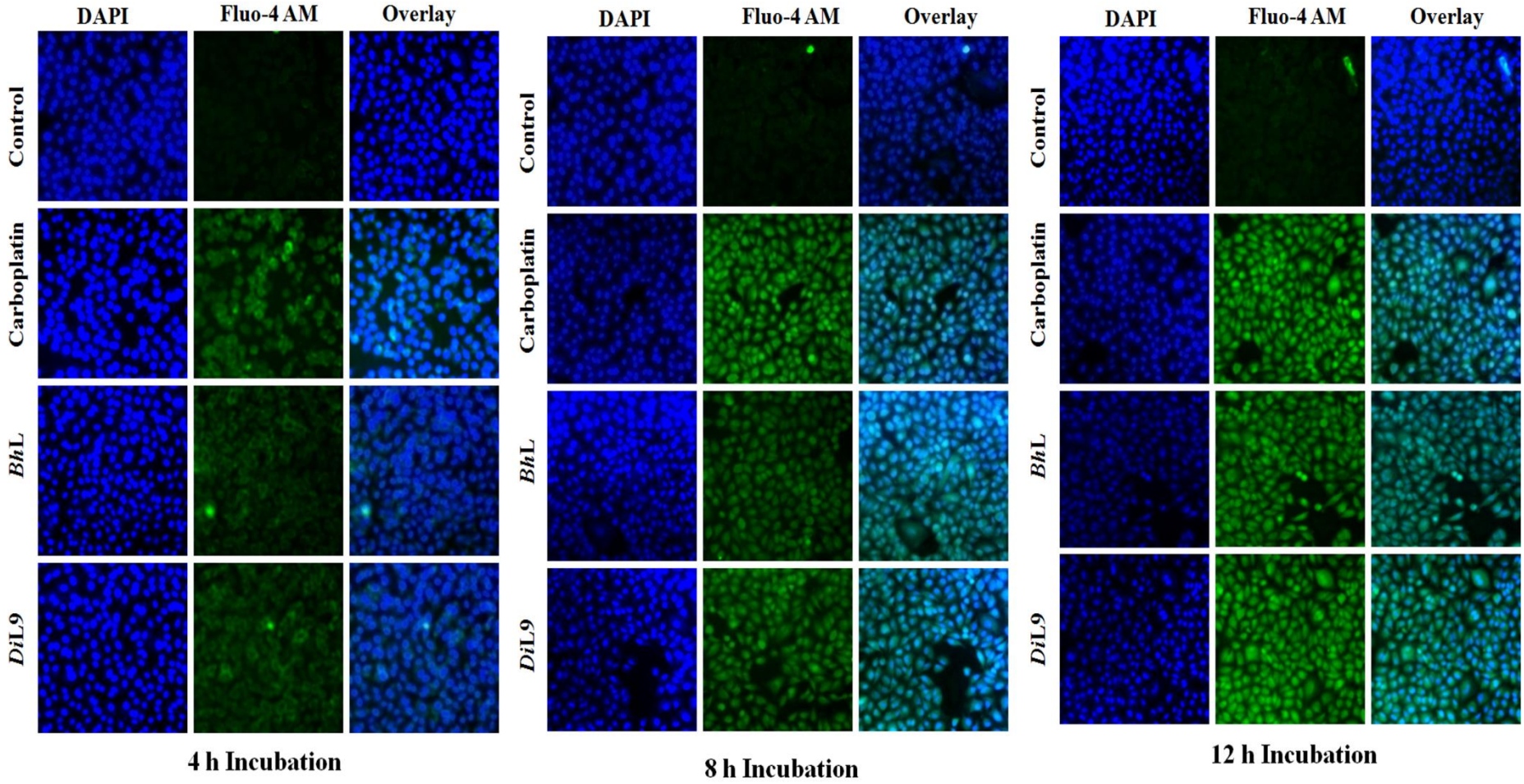


Figure. 5B.11 (B) Increase in intracellular [Ca^{2+}]i release of lectin stimulated PANC-1 cells. PANC-1 cells were treated with lectins for 4, 8 and 12 h, stained with Fluo-4/AM (4 µM, green) and DAPI (blue). This represents the overlay of confocal microscopy images of fluorescence intensity of cells bound with Fluo-4/AM (green) releasing calcium after 4, 8and 12 h of incubation.

5B.5 Activation of intrinsic apoptotic pathway

With the disruption of the MMP, to analyze the details of lectin-induced pancreatic cancer cell death, the effect of *Bh*L and *Di*L9 on activation of initiator caspases was evaluated. Activation of caspase-8 and -9 determines the pathway destined for the apoptotic induction. The time course profiles showed a drastic difference in the caspase-8 and -9 activities. We observed no change in caspase-8 activity even after 36 h of lectin treatment whereas caspase-9 activity increased gradually over a period of time when compared that with untreated cells **(Fig. 5B.12)**. To confirm this observation, we investigated the caspase-8 and -9 activation patterns in the presence of caspase inhibitors being specific to each. Following the kit manufacturer's instructions, caspase-8/9 inhibitor was added to the cell cultures to completely inhibit the enzyme activity preventing the apoptotic induction. Interestingly, pretreatment with caspase-8 inhibitor could not prevent the cell death whereas the caspase-9 inhibitor could abolish the cell death. These results clearly suggested that only caspase-9 was activated by both the lectins. On further investigation, a time-dependent increase in caspase-3 proteolytic activity was also observed, caused by *Bh*L and *Di*L9 with 5-fold and 3-fold increase, respectively, after 48 h of incubation **(Fig. 5B.12)**. Pretreatment of caspase-3 inhibitor, Ac-DEVD-CHO could also prevent lectin-induced cell apoptosis. Thus, we can clearly say that the caspase-9 activity detected after 3 h was followed by activation of caspase-3 at 12 h. Both the lectins behaved similarly in action and found similar to carboplatin response (Han *et al.*, 2009, Lin *et al.*, 2010).

The aforementioned findings indicate that the lectins, *Bh*L and *Di*L9 induced mitochondrial dependent intrinsic pathway of caspase-dependent apoptosis in all pancreatic cancer cell lines.

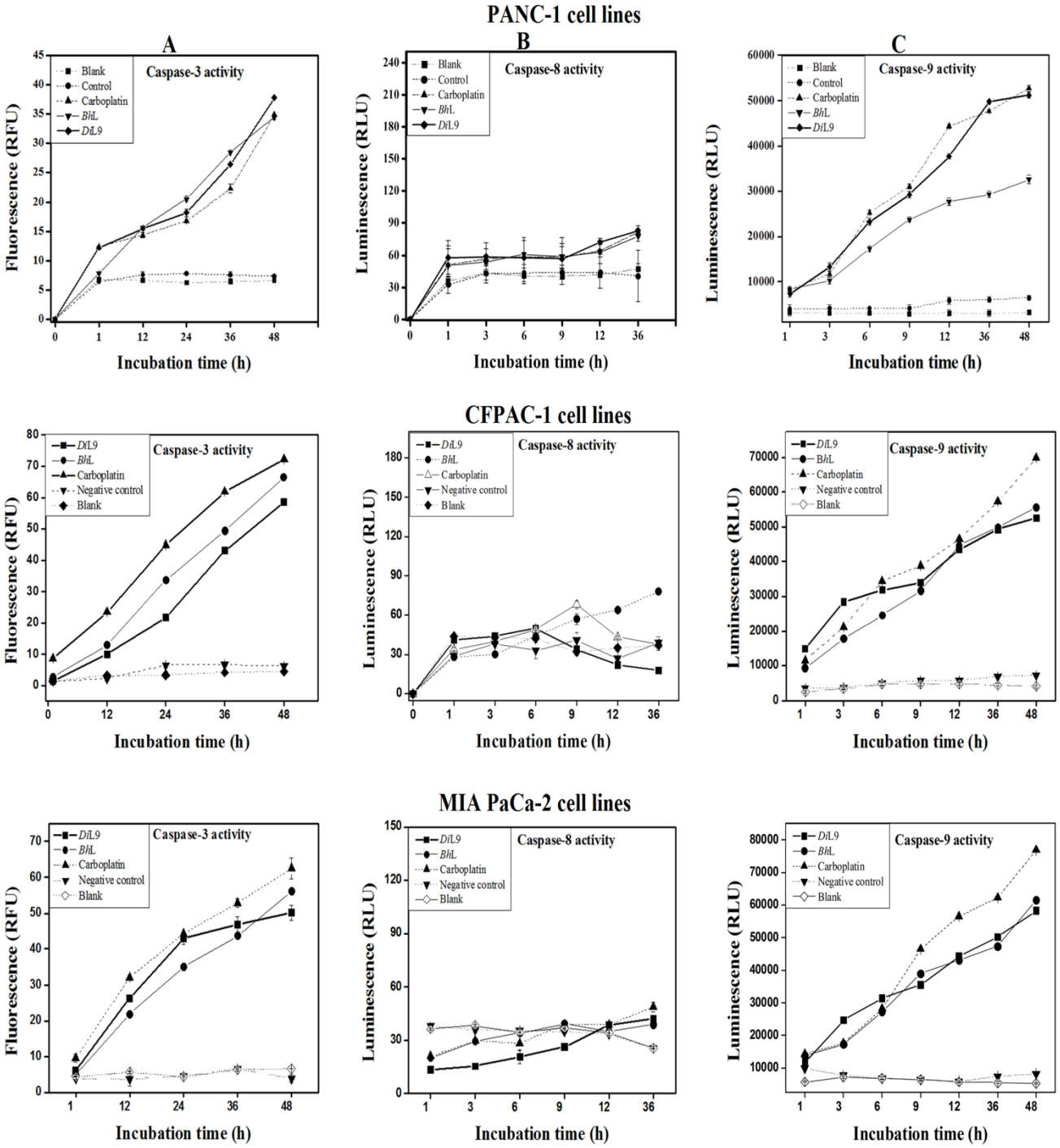


Figure 5B.12 Effect of *Bh*L and *Di*L9 on Caspases activation. The lectin (*Bh*L: 8.4 µg ml^{-1} and *Di*L9: 142 µg ml^{-1}) treated PANC-1, CFPAC-1 and MIA PaCa-2 cells were incubated for different time periods and activities of caspases were assessed by fluorimetric assay. Activities of **(A)** Caspase-3, **(B)** Caspase-8 and **(C)** Caspase-9 were measured with respect to untreated cells.

5B.6 Lectins showing anti-angiogenesis activity

Angiogenesis, as mentioned before is also responsible for the growth of solid tumors and making them metastasized by providing adequate blood supply (Auerbach *et al.*, 2003). Many cancer chemotherapeutics strategized mainly by inhibiting the cell growth, proliferation or inducing apoptosis; and the blocking of angiogenic activators provides a novel therapeutic target to inhibit tumor spread. Few examples of molecules are known which can inhibit angiogenesis *in vitro* and *in vivo* models, like genistein (Sarkar & Li, 2002) and curcumin (Thaloor *et al.*, 1998). Recently, resveratrol, a phytoalexin present in grapes has been reported to possess anti-angiogenic activity by suppressing the FGF-2 and VEGF-induced neovascularization *in vivo* (Brakenhielm *et al.*, 2001). Currently, in many laboratories natural and synthetic angiogenic inhibitors like snake venom peptides called disintegrins (McLane *et al.*, 2004) and lebectins (Pilorget *et al.*, 2007) are also studied. The discovery of such agents being non-toxic in nature, having multiple biological activities and effectively acting at various stages of angiogenesis cascade could be of great medical relevance.

In this study, we have analyzed the effect of two purified lectins (*Bh*L and *Di*L9) on angiogenesis *in vitro* on HUVEC cells. These cells undergo rapid re-organization in the presence of matrigel, ceases further proliferation and initiates capillary like tubule formation in the presence of large vessel endothelial supplement (LVES) and VEGF and in its absence, cells tend to proliferate only. To evaluate the lectin effect, these cells were treated with lectins for 24 h. During the experiments, we found that both the lectins behaved similarly by inhibiting the tubulogenesis process. Previously, we have shown that both the lectins do not inhibit the growth of HUVECs as evaluated by MTT assay but could inhibit endothelial tubulogenesis *in vitro* **(Fig.**

5B.13). It can be suggested that these lectins might be blocking the receptor required for VEGF or other angiogenic factors binding and eventually disrupting the tubule formation.

This is the first report of chito-specific lectins possessing anti-angiogenic activity at such low concentrations. Previous reports showed that *V. album* extracts inhibited angiogenesis by killing the endothelial cells (Duong Van Huyen *et al.*, 2002) and ConA targets anti-angiogenesis pathway at 25 µg ml^{-1} (Liu *et al.*, 2009, Li *et al.*, 2011). Our studies showed that *Bh*L at 8 µg ml^{-1} (247 µM) showed much more effective response. Till now, brain tumor therapies are most risky, where we suggest these lectins as anti-angiogenic agents to be the hope for such cases. According to our present results, we suggest that *Bh*L and *Di*L9 lectins could be used with high efficiency for the inhibition of the brain angiogenic process without any side effects and warrants further investigations like in *vivo* studies.

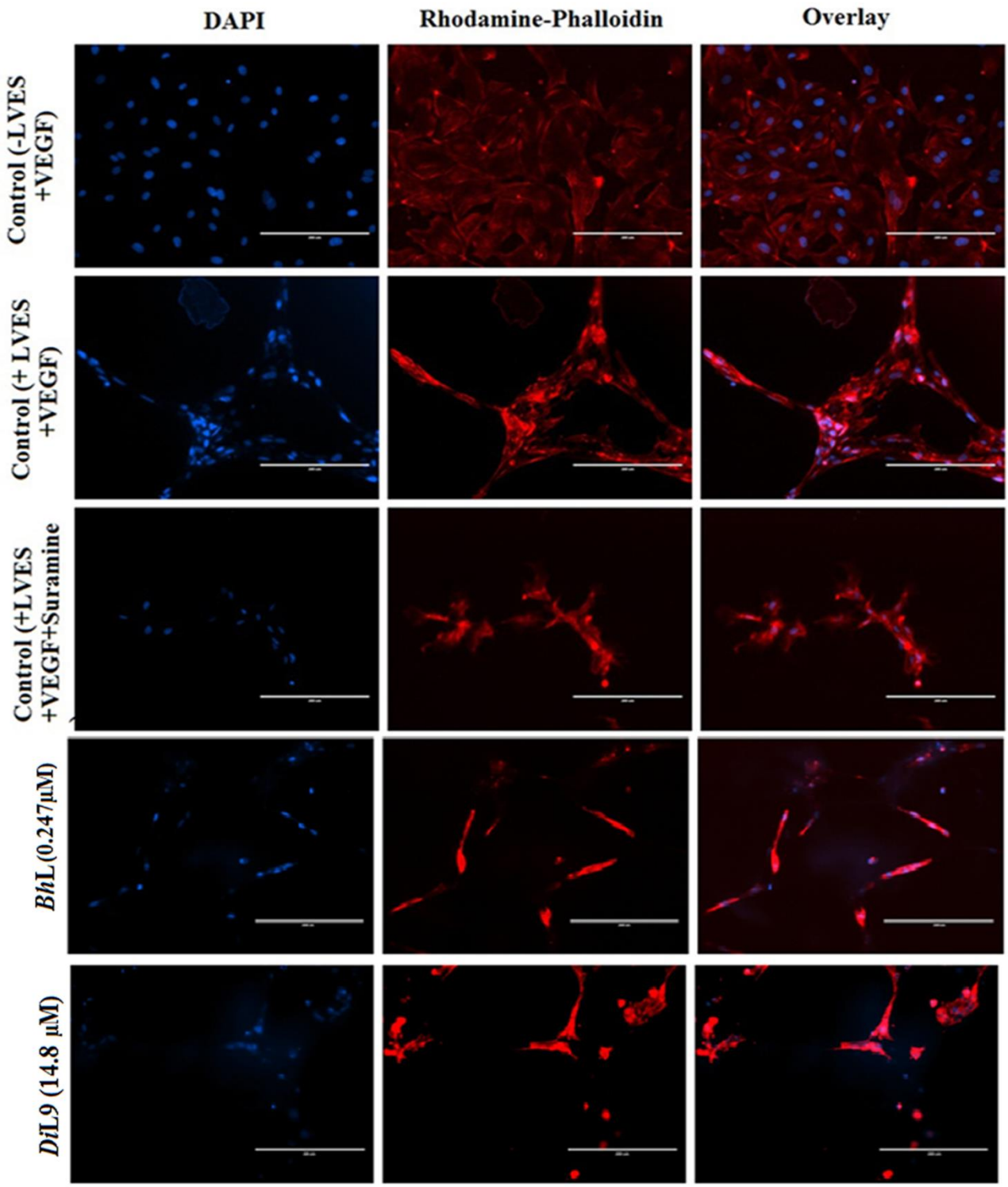


Figure 5B.13 Inhibition of angiogenesis by *Bh*L and *Di*L9. HUVECs were plated on Matrigel (± LVES+VEGF) precoated 96-well plates as control. The cells were treated with lectins. The tubes were stained with rhodamine conjugated phalloidin (red) and nuclei with DAPI (blue). Anti-angiogenic activity is determined by the breakage in the tubule formation.

To summarize, many of the anticancer drugs have been isolated or modified from natural resources (Cragg & Newman, 2005). Due to the property of selectivity and being specific in nature, lectins have gained much more attention from scientific researchers for cancer therapeutics (De Mejía, 2005). In this chapter, we have investigated the anti-proliferative property of two chito-specific lectins, *Bh*L and *Di*L9, purified from different plant families. These lectins showed similar dose-dependent cell cytotoxicity effect against human neoplastic cell lines, with minimum GI_{50} values for pancreatic cancer cells. Among the two lectins, *Bh*L showed more inhibitory effect as compared to *Di*L9. As already reported mucin like glycans are over-expressed by many tumors (Yu, 2007). This clearly suggests that the lectin binding to the tumor glycoproteins/receptor present appears to be the mandatory steps in causing cell cytotoxicity. Considering the overexpression of mucin glycoproteins (MUC1), it has been investigated that mainly pancreatic cancer cell line exhibits a unique pattern of MUC1 glycoforms (McCarter *et al.*, 2015). Therefore, binding of these lectins to PANC-1, CFPAC-1 and MIA PaCa-2 selectively could provide more accurate analyses of tumor glycans and could provide more insight for the pancreatic cancer therapy. For normal cell lines like HUVECs and L929, *Bh*L and *Di*L9 were not cytotoxic even at higher concentrations (> 200 µg ml^{-1}), implying its exclusive specificity for cancerous cells.

In order to develop lectin-based anti-cancer therapy, it is obligatory to understand the underlying mechanism involved in human tumor cell death induced by lectins (Liu *et al.*, 2010). The proposed underlying mechanism of these lectin-induced (*Bh*L and *Di*L9) pancreatic cell death is depicted in **Fig. 5B.14**. Investigations like MTT assay of lectin's antiproliferative effect on tumor cells, morphological changes, activation of caspases, release of calcium ions and mitochondrial membrane depolarization studies were conducted. Programmed cell death is a

process that eradicates uncontrolled cells. So the agents targeting apoptotic pathway, specifically in tumor cells, are the most potential ones for antitumor therapy (Reed, 2003). Unlike previously reported lectins ConA and WGA (Chang *et al.*, 2007, Suen *et al.*, 2000, Gastman *et al.*, 2004), *Bh*L was found to be more potent because of its ability to induce cell death at a very low concentration and activating early intrinsic apoptotic cascade in the pancreatic cancer cells.

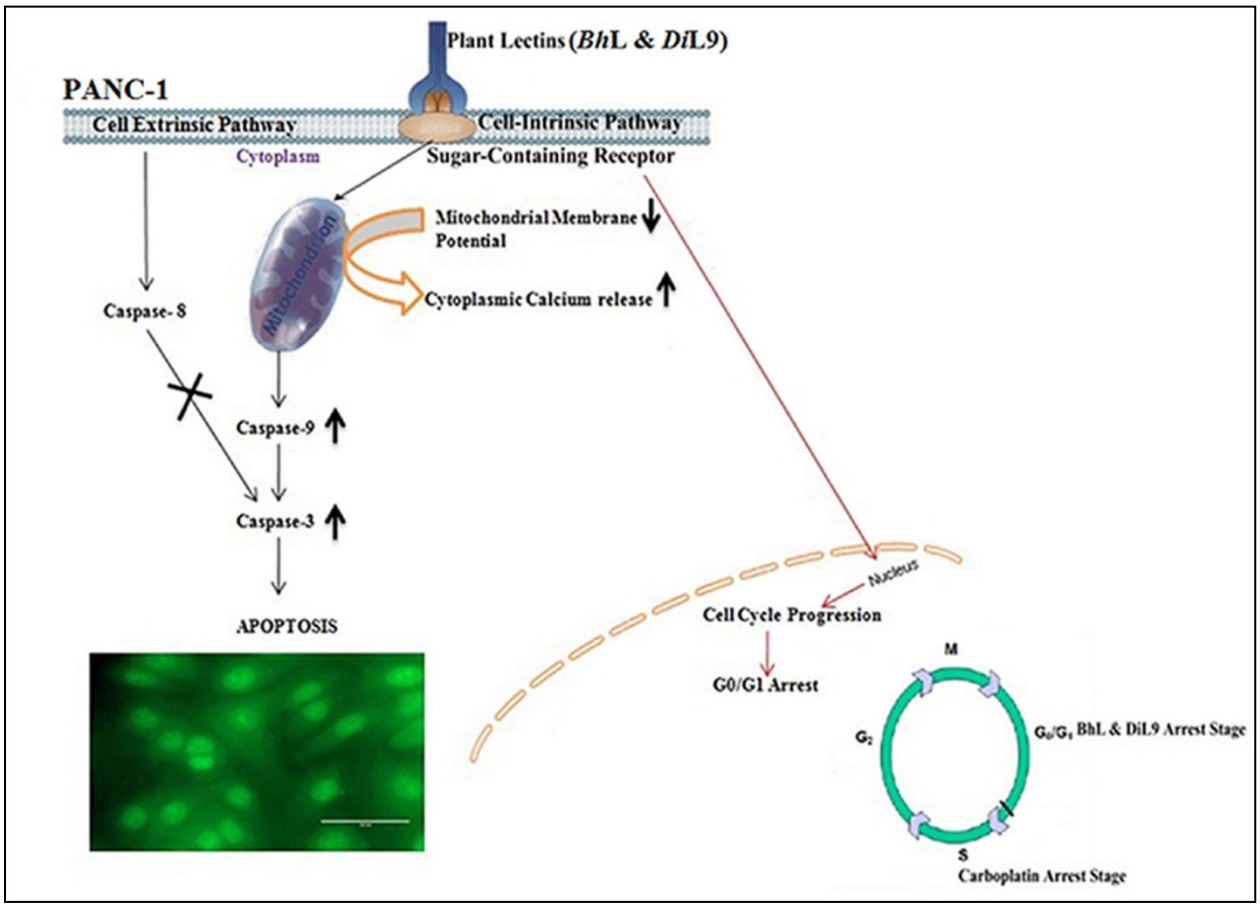


Figure 5B.14 Proposed model for mechanism of apoptosis induced by *Bh*L and *Di*L9 in pancreatic cancer cells. Lectins bind to the glycoprotein receptors of tumorcells and triggers mitochondrial membrane depolarization cascade. The apoptotic signal was amplified by activation of Caspase-9 and -3 leading to final cell death.

5B.7 Aspects and future prospects

Researchers have performed *in vivo* studies using mistletoe lectins on different cancer animal models and had reported significant decrease in tumor size rand growth on intratumoral injection of the lectins (Marvibaigi *et al.*, 2014). Due to heterogeneity in tested animal models used for investigations, or difference in administration route and sample size; many i*n vivo* investigations gives inconsistent results on the lectins' ability to inhibit cancer cell growth. Considering the wide range of *Bh*L and *Di*L9, being non-toxic to normal cells, intratumoral administration might be a promising alternative therapy for pancreatic cancer patients. Intra dermal or subcutaneous administration seems to be unsuccessful because of the presence of serum glycoproteins in the blood circulation system as discussed earlier. Further qualitative *in vivo* and clinical trials evaluating the effect of lectins on pancreatic cancer patients must be carried out addressing safety parameters, standard dosage, and appropriate endpoint measures. These studies should take into account the patients sample size, drug dosage range, and methodological design preventing failures happened in the cases of previously tried lectins.

5B. 8 Conclusion

We report for the first time that two chito-specific lectins, *Bh*L and *Di*L9 possessed remarkable antiproliferative activity against pancreatic cancer cells by apoptotic induction of cell death. Among these two lectins, *Bh*L gave much more effective cytotoxic response as compared to *Di*L9. The underlying apoptotic mechanism was found to be the intrinsic pathway causing depolarization of MMP and increase in intracellular Ca^{2+} leading to activation of executioner caspases (Caspases-9 and -3). Interestingly, both the lectins also inhibited endothelial tubulogenesis. Our results would open exploration of dietary molecules like plant lectins as

potential novel candidates for pharmaceutical exploitation. Hence, the successful therapy will be based on the selective elimination of the abnormal cell without disturbing the function of the normal cells.

Chapter 6

Summary and Conclusion

The classification and applications of lectin have been reviewed in detail. Lectins are ubiquitously distributed in nature and present in all living beings. Stillmark had discovered the castor bean lectin in 1888 and thereafter various applications of lectins for human welfare were identified. Since carbohydrates play crucial role in many biological processes ranging from cell-cell communication, cell matrix interactions, and are traced during the altered expression of cancerous cells, lectins which recognize carbohydrates also assumed importance in biology. In the present investigations, two plant lectins belonging to different families but with similar sugar specificity were identified and characterized. The first plant lectin, *Bh*L was isolated from *Benincasa hispida* belonging to Cucurbitaceae family, whereas the second lectin *Di*L9 was purified from *Datura innoxia*, belonging to Solanaceae family. Both the lectins showed specificity towards chito-oligosaccharides and their hemagglutination activity inhibited by complex glycoproteins such as fetuin, thyroglobulin, fibrinogen, mucin, serum and corresponding glycopepetides.

The structural and functional characterization of the first lectin investigated in this research, *Bh*L, was carried out using biophysical and biochemical methods. The lectin was purified using chitin affinity and gel filtration chromatography techniques. *Bh*L was identified as a dimer of molecular weight 34 kDa and fluorescence spectroscopic studies accounted for the presence of six Trp residues belonging to Class II conformer. The lectin was found to be very stable in a broad pH range, high temperature, in the presence of high concentrations of chemical denaturants and also in the presence of organic solvents. Rayleigh scattering experiments showed that the lectin underwent irreversible thermal unfolding and aggregation. However, surface of native lectin molecule is not

hydrophobic as evidenced by the absence of ANS binding. CD analysis suggested structure with high β-sheet content, similar to other Cucurbitaceae lectins. *Bh*L was also found to be metal independent for its hemagglutination activity. In the presence of βME, *Bh*L retained its activity implying absence of any disulphide bonds. Binding of adenine by *Bh*L indicated the importance of this lectin in plant physiological mechanism.

The second lectin studied here, *Di*L9, was purified from dried seeds of *Datura innoxia* using ion-exchange and gel filtration chromatography. The lectin was found to be of MW 9 kDa and also displayed chito-oligosaccharide specificity. Similar to *Bh*L, this lectin also showed unusual stability in solution. Fluorescence spectroscopic studies indicated the presence of four Trp residues belonging to Class III conformer. *Di*L9 retained hemagglutination activity at higher temperatures upto 85 °C, over a broad pH range, in the presence of chemical denaturants and resistant to proteolysis, at the same time it lost complete activity in the presence of βME, implying presence of disulphide bonds that play important role in maintaining the secondary and tertiary structure of the protein. At pH 2.0, the lectin showed characteristics of molten globule state retaining full activity and showing increase in ANS binding. Rayleigh scattering studies showed reversible thermal folding and no aggregation However, DLS analysis confirmed polydispersity in solution at higher protein concentration.

Further, it is interesting to note that both the lectins show anti-biofilm activity on Gram positive and Gram negative bacteria. These lectins were able to inhibit the adhesion of bacteria to any surface either by blocking the bacterial adhesion proteins or aggregating the cells. These findings would be crucial in the development of medical and dental implants as they are easily colonized by biofilm-forming pathogenic bacteria.

Moreover, we are also reporting for the first time these two lectins, *Bh*L and *Di*L9, possess remarkable antiproliferative activity against pancreatic cancer cells. These chito-specific lectins were able to induce apoptotic pathway via disrupting the mitochondrial membrane and activating caspases leading to the death of pancreatic cancer cells. Surprisingly, both the lectins also inhibited new blood vessel formation by endothelial cells *in vitro*. Thus, our investigations on *Bh*L and *Di*L9 had opened exploration of these molecules as potential candidates for pharmaceutical exploitation for the treatment, prevention and diagnosis of chronic diseases like cancer.

Few reports form clinical studies are available where pure lectins have been used directly and have found results promising. Additional research, including interaction studies of lectin when binding to the cell surface receptors, mechanism of action at molecular level, and more of clinical trials should help researchers to examine and elucidate the therapeutic effects, nutritional benefits, and toxicity caused by lectins. The use of genomics and proteomics analysis can also help in further elaborating the structure-function relation of these lectins and interactions with genes and encoded proteins. The effectiveness of this type of molecules can be enhanced if conjugated with nanoparticles, increasing their bioavailability. Nevertheless, the gap between observations made from *in vitro* studies and *in vivo* experiments always found to be problematic. Thus, testing on a chosen animal model which is closely related to humans would be a critical stage in the successful development of any therapeutic drugs based on the proteins studied in this research.

In conclusion, our research elaborated in this thesis had identified and characterized two chito-specific highly stable plant lectins showing anti-microbial, anti-

biofilm and anti-angiogenic and anti-proliferative effects against cancer cells. The scope for further research on these lectins involves structure determination to understand the molecular mechanism of their function and animal based experiments and clinical trials to consolidate their potential for applications.

Appendix-I

Reagents for SDS-PAGE

Reagents

1. Acrylamide	: 30.0%
N,N' methylene bis acrylamide	: 0.8%
Distilled water to make	:100 ml

2. Resolving gel buffer

Tris-HCl, pH 8.8	: 3.0 M

3. Stacking gel buffer

Tris-HCl, pH 6.8	: 1.0 M

4. Ammonium per sulphate (APS)

freshly prepared	: 100 mg
Distilled water to make 1 ml	

5. Sodium dodecyl sulphate	: 10 g
Distilled water to make 100 ml	

6. Running or reservoir buffer, pH 8.3

Tris base	: 3.03 g
Glycine	:14.4 g
Sodium dodecyl sulphate	: 1.0 g
Distilled water to make	: 1000 ml

7. Sample buffer

Glycerol	:1.6 ml
Tris-HCl pH 6.8	: 1.0 ml
2-mercapto ethanol	:0.4 ml

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List of Publications

- **Ruby Singh**, Laxman Nawale, Dhiman Sarkar, C. G. Suresh. Two Chitotriose-Specific Lectins Show Anti-Angiogenesis, Induces Caspase-9-Mediated Apoptosis and Early Arrest of Pancreatic Tumor Cell Cycle. ***Plos one***. 2016 Jan 21;11(1):e0146110. doi: 10.1371/journal.pone.0146110.

- **Ruby Singh**, Sushama M. Gaikwad and C. G. Suresh. A Chito-specific, Adenine Binding Agglutinin from *Benincasa hispida* Shows High Structural and Functional Stability. ***International Journal of Biochemistry Research & Review***. *Vol 9(4):1-14.*

- **Ruby Singh** and C. G. Suresh. Purification and Characterization of a Small Chito-specific Lectin from *Datura innoxia* Seeds Possessing Anti-microbial Properties. ***International Journal of Biochemistry Research & Review.*** *Vol 9(2):1-17.*

- **Ruby Singh**, Sushama M. Gaikwad and C. G. Suresh. Structural characterization of a novel cytokinin binding agglutinin from *Datura innoxia* seeds and its acid-induced molten globule state (Communicated).