Purification, Biochemical Characterization and Bioassay Studies of Chimeric Lectin from *Aponogeton natans* Tubers

A thesis submitted during 2011 to the University of Hyderabad in partial fulfillment of the award of a Ph.D degree in Department of Plant Sciences

By

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CERTIFICATE

This is to certify that this thesis entitled **"Purification, Biological characterization and Bioassay studies of Chimeric lectin from *Aponogeton natans* tubers"** submitted by **Mr. SHASHIKANTH. D** bearing Reg. No. **03LPPH11** in partial fulfillmet of the requirement for the award of **Doctor of Philosophy in Plant Sciences** is a bonafide work carried out by him under my supervision and guidance.

The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

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DECLARATION

I **Shashikanth.D** hereby declare that this thesis entitled **"Purification, Biological characterization and Bioassay studies of Chimeric lectin from *Aponogeton natans* tubers"** submitted by me under the guidance and supervision of **Prof. Dr. Kottapalli Seshagirirao** is bonafied research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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ABBREVIATIONS

APS	Ammoniumperoxodisulfate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Con A	Concanavalin A
CD	Circular dichrosim
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbant assay
et al	et alii (Latin: and others)
FCS	Fetal calf serum
HCl	Hydrogen chloride
HEPES	(N-(2-Hydroxyethyl)-piperizine-N'-(2-ethane sulfonic acid))
kDa	Kilo Dalton
min	Minute
mL	Milli Litre
mM	Milli molar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide Gel electrophoresis
PBS	Phosphate-buffered saline
pH	-log (H^+) concentration
PMSF	Polymethylsulfonylfluoride
pmol	Picomole
pNP	para nitro phenol
pNPP	para nitro phenyl phosphate
PPM	Parts per million

PVDF	Polyvinyldifluoride
rpm	Rotations per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylendiamine
TFA	Trifluroacetic acid
Tris	Tris-(Hydroxymethyl) aminoethane
Tof	Time of flight
UV	Ultraviolet
V/V	Volume/Volume
W/V	Weight/Volume
W/W	Weight/Weight
β-M.E	β–mercaptoethanol
μCi	micro Curie
μg	micro gram
μM	micro molar

CONTENTS

INTRODUCTION

Since the turn of the 19th century, the proteins with sugar specificity have caught the attention of the Bio-medical community. Peter Hermann Stillmark while working on his doctoral thesis in 1888 (which was submitted to university of Dorspat/ now Tartu, Estonia) described the presence of a toxic proteinaceous factor in extracts of castor beans (*Ricinus communus*) which agglutinated erythrocytes. This toxic protein is named ricin after the source. Thus, historically this discovery is considered as a beginning of plant lectins research and this event is internationally recognized as a birth of a new branch of science called "Lectinology". Based on these findings, H.Hellin who was also at Tartu demonstrated the toxic action of a proteinaceous factor from the extracts of jequirity bean (*Abrus precatorius*) which was subsequently named abrin, after the source. Commercial availability of ricin and abrin has prompted Paul Ehrlich in 1890 to establish the fundamental principles of immunology. Ehrlich has proved that the mice could be rendered immune to lethal dose of ricin/abrin by injecting repeated sub lethal doses subcutaneous providing the evidence for the immune specificity. These experiments have also demonstrated the immunological phenomenon and the transfer of humoral immunity from a mother to offspring. The observation that there is some selectivity in the ricin induced hemagglutination of different animals has led to the discovery of human A, B, O blood groups by Karl Landsteiner in 1900. In 1919 James B. Sumner first attempted to obtain a pure hemagglutination by salt precipitation. He isolated Concanavalin A from jack bean (*Canavalia ensiformis*) and crystallized it. In 1936 Sumner and Howel for the first time reported the sugar specificity of hemagglutinins by demonstrating that the hemagglutination by Concanavalin A is inhibited by sucrose. They also suggested the possibility of proteins interaction with the carbohydrates on the surface of erythrocytes. As these toxic proteins are thought to be present only in plants initially they were termed

phytoagglutinins or hemagglutinins. William C. Byod and Karl O. Renkonen in 1940 has independently found that the extracts from lima bean (*Phaseolus limensis*) and tufted vetch (*Vicia cracca*) has specifically agglutinated blood type A erythrocytes but not B or O cells whereas extracts of asparagus pea (*Lotus tetragonolobus*) specifically agglutinated O erythrocytes defining the specificity of hemagglutinins.

The first proof for the presence of sugars on cell surfaces was given by W.M.Watkins and W.J.T.Morgan in 1952 proving that the agglutination of type A red cells by lima beans (*Phaseolus lunatus*) lectin was best inhibited by α-linked N-Acetyl-D-galactosamine. Based on the ability to distinguish between erythrocytes of different blood groups Boyd and Shapleigh (1954) proposed the term lectin (latin *legere* to choose) to hemagglutinins. As the work on lectins progressed lectins were also found in fungi, bacteria, viruses, vertebrates and invertebrates besides plants. The possible applications of the lectins by their known properties grew rapidly followed by the reports of blood group specificity, mitogenecity, antiproliferative nature. The definition of a lectin should fulfill three criteria, a protein which binds to carbohydrates, separated from immunoglobulins and do not biochemically alter the carbohydrates to which they bind. Presently the term lectin is employed to denote all proteins possessing at least one non-catalytic domain, which binds reversibly to a specific mono- or oligosaccharide. Irwin J Goldstein, together with BBL Agarwal, introduced Affinity Chromatography for Lectin purification by exploiting the fact that Concanavalin A reacts with dextrans, they developed an elegant technique for direct isolation of the lectin (Con –A) from jack bean meal: by its specific adsorption on a column of Sephadex (cross linked dextran) and then eluted with glucose solution.

Classification of lectins

Based on their sugar specificity, Goldstein and poretz (1986) classified lectins into following classes:

(1) Mannose/glucose binding lectins.

(2) Galactose/N-Acetylgalactoseamine-binding lectins.

(3) N-acetylglucoseamine binding lectins.

(4) L-Fucose-binding lectins.

(5) Sialic acid binding lectins.

The mannose/glucose-binding lectins comprise a group of agglutinins found mostly in the Fabaceae (Leguminosae) family. The mannose/glucose-binding group of lectins is the mostly studied group. The amino acid sequences of some of the lectins in this group reveal extensive homology and provide new vistas for examining taxonomic and evolutionary relationships.

Galactose/N-Acetylgalactoseamine-binding lectins are the first plant haemagglutinins shown to display human blood group specificity. Galactose-binding toxins were identified in diverse plants including *Ricinus communis* (Euphorbiaceae), *Abrus precatorius* (Fabaceae) and *Viscum album* (Loranthaceae).

N-acetylglucoseamine binding lectins comprise adiverse group of agglutinins that exhibit a primary specificity for their monosaccharide and/or its (β 1-4) linked oligomers (chitin oligosaccharides) and in some instances glucosamine. This group includes lectins from three families of Poaceae, Solanaceaea and Fabaceae.

Fucose-binding lectins were found to be useful serological reagents. In several laboratories the *Ulex europaeus I* is widely used, as it exhibits anti blood group O activity(Liener *et al.*, 1986). Fucose lectins are found in diverse organisms like *Lotus*

tetragonolobus, *Ulex europaeus* (plants), *Anguilla anguilla* (animals) and *Aleuria amantia* (fungus).

The hymolymph and sera of most of the vertebrates are a rich sourse for the Sialic acid-binding proteins. In recent years sialic acid lectins have also been isolated from the bark of Maakia amurensis and different parts of Sambucus sp. also.

Based on the overall structure and the biochemistry of their subunits lectins are divided into four major classes:

Merolectins: Small polypeptide proteins with single carbohydrate binding domain which are incapable of precipitating glycoconjugates.

Hololectins: These are exclusively composed of carbohydrate-binding domains that include lectins with di or multi carbohydrate domains that are either identical or very homologous and bind either the same or structurally similar sugars. They are capable of agglutinating cells or glycoconjugates. Most of the plant lectins belong to this group.

Chimerolectins: Fusion proteins with one or more carbohydrate binding domain with an additional unrelated catalytic domain other than the carbohydrate binding domain. This class has well defined catalytic activity. The later domain with well defined catalytic domain or another biological activity acts independently of the carbohydrate-binding domain.

Superlectins: These are like hololectins with two or more carbohydrate binding domains but differ with their ability to distinguish structurally unrelated sugars.

Based on the carbohydrate recognition domains and evolutionary relationships traced by similarities in the amino acid sequence and structural features lectins are classified into seven families:

1. Chitin-binding lectins
2. Cucurbitaceae phloem lectins

3. Amaranthin lectins

4. Monocot mannose-binding lectins

5. Legume lectins

6. Jacalin related lectins

7. Type 2 RIP

If any lectins do not fit into the above said families they are referred to as unclassified lectins. Cucurbitaceae phloem lectins are now available at atomic level and have been shown to be conserved within each family. This structural conservation is reflects the similarity in regarding specificity among its families [except legume and jacalin related lectins]. Thus, each of these lectin families has its own typical fold and binding site motif and specificity [Van Damme *et al*., 1998; Peumans *et al*., 2000], as shown below:

Chitin-binding lectins: Members of this family binds chitin and lower oligomers of Glc*N*Ac specifically and exhibits sequence and structure similarities to hevein [a 43 amino acid chitin binding merolectin from the latex of rubber tree, *Hevea brasiliensis*]. Not all chitin-binding lectins belong to this family, but only those which have one or more hevein-domain[s] as the chitin binding domain. Hevein-domain is a polypeptide structural unit of about forty amino acids. This family is widespread in many plant species of different taxonomy. Class I chitinases are chimerolectins by definition which have a single *N*-terminal hevein domain linked to the chitinase domain [Beintema, 1994] but most of the Gramineae species like WGA belonging to this class are hololectins [Stinissen *et al*., 1983]. WGA was the first member of this family to be purified and fully characterized. The WGA monomer is built up of four identical hevein domains and two such monomers are non-covalently attached in a head-to-tail manner to form a dimer

having four pairs of hevein domains [one from each monomer]**.** Hence there are eight binding sites in WGA; only four of them were shown by solution, crystal and modeling studies to be of high and roughly equivalent affinities whereas the remaining four are at least 2-fold weaker.

Cucurbitaceae phloem lectins: They have been found only in the phloem exudates of cucurbitaceae species can also bind chitin but structurally not similar to hevein domains. They are dimers composed of about 25 kDa unglycosylated subunits. Like most chitin-binding lectins, they are inhibited only by oligomers of Glc*N*Ac [Sabnis and Hart, 1978; Anantharam *et al*., 1986; Sanadi and Surolia, 1994; Peumans *et al*., 2000].

Amaranthin lectins: These are distinct family of a GalNAc specific lectins composed of are approximately 33-36 kDa homodimeric unglycosylated proteins. They are found only in the seeds of the genus *Amaranthus* species. The prototype member of this lectin family is amaranthin, the lectin from *A. caudatus*, which binds preferentially to T-antigen disaccharide [Rinderle *et al*., 1989]. The crystal structure of amaranthin bound to benzylated T-antigen disaccharide has been solved at 2.2 Å resolutions [Transue *et al*., 1997]. The X-ray diffraction studies of the lectins further confirmed that it's three dimensional structure definitely differs from the other known lectins. The sugar binding activity of amaranthin depends on a complex network of H-bonds and some of them [those involve in binding of the non-reducing part of the disaccharide] are mediated by water. There is no hydrophobic contact involved in the binding process.

Monocot mannose-binding lectins: Historically, the discovery of the first member of the lectin from this family dates back only to 1987 when a lectin with exclusive specificity towards mannose was isolated from the bulbs of snowdrop (*Galanthus nivalis*) [Van Damme *et al*., 1987]. This family consists of a large number of lectins with restricted specificity to mannose and oligomannose, and found only in some of the monocot plant

families. Most of the lectins are homers composed of two or four identical subunits of about 12 KDa, which are synthesized as separate polypeptides. Detailed specificity, cloning and structural studies proved GNA to be different from any previously characterized lectin in its sequence, sugar binding activity and 3-D structure [Shibuya *et al*., 1988; Hester *et al*., 1995]. Thus GNA became the first representative of a new lectin family which has grown rapidly since then by the isolation and characterization of similar lectins from several monocot plant families to form the super family of monocot mannose binding lectins [Barre *et al*., 1996]. The molecular structure of these proteins involves, in most cases including GNA, one, two or four non-covalently associated small protomers [11-14 kDa]. The main secondary structural element of this protomer is β-sheet. It is built up of three tandemly arrayed domains and three mannose binding sites [Peumans *et al*., 2000].

Legume lectins: These are a large group of homologous proteins which are confined to species of the plant family *Leguminosae*. At present legume lectins have been purified from over 70 species belonging to different taxa, most from mature seeds. Therefore, it is the largest group of plant lectins. All legume lectins divalent cations at specific metal binding sites which possess both Mn^{2+} and Ca^{2+} ions held in place by interactions with specific aminoacid residues which are essential for sugar binding activity [Sharon and Lis, 2003]. Demetalisation of legume lectins leads to large changes in their structures and loss of the sugar binding activity. In most cases the protomers are identical or very similar; however, some legume species express two different protomers in their cells which may associate randomly to form different homogeneous and heterogeneous isolectins. The classical example for this is the PHA isolectins, E_4, E_3L_1, E_2L_2, E_1L_3 and L_4, from the common beans, *Phseolus vulgaris* [Feldsted *et al*., 1977].

Based on their structure of their subunits, legume lectins are often artificially subdivided into one-chain and two-chain lectins. However this subdivision is trivial. In some lectins, which are called two-chain legume lectins, e.g., pea lectin [Trowbridge, 1974], the protomer polypeptide is fragmented unequally into light chain [α] and heavy chain [β]. The protomers in all legume lectins have very similar 3-D structure which is built up of two antiparallel β-sheets: one is a six-stranded flat sheet [back face] and the other is a seven-stranded concave sheet [front face]. The β-sheets are interconnected by turns and loops to form a flattened dome. The β-folds of the seven-stranded sheet form the sugar and metals binding sites. The protomers associate noncovalently to form dimers or tetramers. The most common dimerization mode involves side-by-side association of two monomers using their flat β-sheets in a two-fold symmetry plane to form a twelve-stranded β-sheet. As a result, the two binding sites are located at both ends of the dimer. This mode of dimerization in legume lectins is called the canonical dimerization. The 3-D structure of Con A dimer complexed with MeαGlc [Harrop, 1996], clearly depicting the canonical dimerization. Some other modes of dimerization in legume lectins [called noncanonical dimerization] are also known [Elgavish and Shaanan, 2001]. All tetrameric lectins are formed by association of two dimers either by loop interactions using the central parts of the dimers, as in Con A, or by interactions between the outermost strands of the two twelve-stranded β-sheets creating a channel between them, as in SBA. PNA is unusual not only in its noncanonical dimer but also in being an asymmetric tetramer unlike any other homotetramer protein [Banerjee *et al*., 1994, 1996].

In spite of the high sequence homology and very similar 3-D structure among them, legume lectins cover almost all the monosaccharide specificities. This is because few changes in the amino acid sequence and lengths of loops involved in the sugar

binding lead to binding sites of different conformations and hence of different specificities [Sharma and Surolia, 1997].

Jacalin related lectins: This family includes all lectins which show high similarity to jacalin, a lectin from jackfruit [*Artocarpus integrifolio*] seeds, in structure and sequence. The jackfruit seeds also contain another lectin very similar to jacalin [jacalin-related lectin] called artocarpin. Both lectins are homotetramers of very similar 3-D crystal structure in which the protomer has a novel fold [called β-prism fold], which consists of three four-stranded β-sheets in three-fold symmetry arrangement [Sankaranarayanan *et al*., 1996; Pratap *et al*., 2002]. Like jackfruit seeds, the black mulberry tree [*Morus nigra*] bark also contains two jacalin-related lectins: one is galactose specific [moringaG] and the other is mannose specific [moringaM] [Van Damme *et al*., 2002]. In fact, jacalin-related lectins are classified into two subfamilies: galactose specific lectins [like jacalin] which are found only in some *Moraceae* species and mannose specific lectins [like artocarpin] which are widespread among flowering plants [Peumans *et al*., 2000].

Jacalin's monomer is synthesized as a preproprotein which, after removal of the signal peptide and a propeptide, is cleaved into two chains, a heavy [α] chain and a light [β] chain. Although it was suggested that carbohydrate specificity of jacalin is probably generated by the cleavage of the single chain polypeptide [Jeyaprakash *et al*., 2003], recent studies indicate that the carbohydrate specificity pattern of recombinant jacalin expressed in *E. coli*, which is not cleaved, is unaltered but its affinity is about 100-fold lower for a battery of sugars as compared to native jacalin [Sahasrabuddhe *et al*., 2004]. Therefore, it appears that post-translational cleavage of the jacalin polypeptide is required for the increase in the affinity for the saccharides that it binds. Artocarpin, the other lectin present in the jack fruit seeds, which is mannose-specific, is not cleaved. Jacalin and artocarpin are promiscuous lectins. Different agglutination/precipitation inhibition assays

of jacalin by sugars showed that in addition to galactose and its derivatives, other unrelated sugars, especially mannose and MeαMan also inhibit the hemagglutination activity of this lectin [Dalmau and Freitas, 1989]. On the other hand, surface plasmon resonance hapten inhibition experiments demonstrated that artocarpin interacts with a wide range of unrelated monosaccharides even though it displays a higher affinity for mannose [Barre *et al*., 2004]. Crystal structure of jacalin complexed with MeαMan at 2.0 Å resolutions has been determined by Bourne *et al*. [1992], who attributed the ability of jacalin to bind monosaccharides with different hydroxyl conformations to its relatively large binding site and the flexibility of the β-prism fold.

Type 2 RIPs: Some of the plant lectins exhibit ribosome-inactivating property that depurinates the rRNA and damages ribosomes and arrests protein synthesis. The Ribosome-inactivating Proteins (RIPs) posses N-glycosidase activity and deadenylate (depurinate) rRNA at a specific position that causes inhibition of protein synthesis (Stripe *et al*, 1992; Barbieri *et al*, 1993). The RIPs are divided into two groups, type 1 and 2. Type-1 RIPs consist of a single polypeptide chain, where as type-2 RIPs composed of two chains: an A (active) chain and a B (binding) chain, held together by a disulphide bridge and hydrophobic bonds. Type 2 RIPs are known to be potent cytotoxic agents. The sugar-binding B chain binds to a (glycoconjugate) receptor on the cell surface, thereby promoting the uptake of the A chain. After its entry into the cell, the A chain catalytically inactivates eukaryotic ribosomes by cleaving the N-glycosidic bond of a single adenosine residue of the large rRNA. Insects seem to react differentially upon feeding type 2 RIPs. Ricin was highly toxic to the coleoptera *Callosobruchus maculates* and *Anthonomus grandis* but had no effect on the Lepidoptera *Spodoptera litoralis* and *Heliothis virescens* (Gatehouse *et al*., 1990). The fact that some insects survive a ricin-containing diet indicates that they either can inactivate the toxin or do not bind the toxin. Another type 2

RIP, namely the lectin from winter aconite *(Eranthis hyemalis)* (Kumar *et al*., 1993), was very toxic to larvae of *Diabrotica undecimpunctata* (a major insect pest of maize). It seems likely, therefore, that type 2 RIPs offer the plant good protection against animals and probably also against some insects. Based on the lethality to mammals the RIPs are classified into two categories i.e. toxic and non-toxic. A number of hypotheses were formulated about possible role of RIPs in nature. It is still not clear what function would be of an enzyme depurinating rRNA, but wide diffusion and abundance of RIPs suggest it should be important to warrant conservation throughout evolution. Obviously, protective effects have been postulated. Experiments during late 1970s on mice and dogs with abrin and ricin were conducted by injecting these toxins through intravenous and observed for clinical, biochemical, and morphological aberrations. In both mice and dogs death occurred within a narrow dose range. Dogs were given toxic doses of ricin and abrin showed weakness, anorexia, apathy, and moderate fever. No signs attributable to the central nervous system were observed. Dogs dying from intoxication expired after 15-40 hours. This study is an example for the potential toxicity of abrin and ricin. Three-dimensional structure of several type 2 RIPs were solved. The crystal structures of ricin and abrin [Rutenber *et al*., 1991; Tahirov *et al*., 1995] show similar overall fold, where the B-chain is built up of tandem repeat of two similar domains and is devoid of extended regular secondary structures but stabilized by four intrachain disulfide bonds, unlike A-chain which consist of eight α-helices and six β-sheets. Modeling studies, on the other hand, on type 2 RIPs from *Sambucus nigra* showed that the structure of their B-chain is very similar to that of ricin and abrin [Van Damme *et al*., 1998].

Plant lectins are generally most abundent in the seeds but they are also widely distributed in other vegetative parts such as roots (*Urtica, Sambucus, Trichosanthes, and Calystegia*), tubers or bulbs (*Solanum, Allium, Tulipa, Iris*), bark (*Sophora, Robinia,*

Maackia, Hevea) or leaves (*Aloe, Lactuca, Vicia unijuga, Viscum album*). The wide distribution of lectins in all the tissues suggests different roles in plants including transport of carbohydrates, packaging and / or mobilization of storage proteins and carbohydrates, cell wall elongation, interaction between plants and microorganisms and defense against the attack of fungi, virus, pest and insects (Van Damme *et al*., 1993; Peumans and Van Damme, 1995; Rudiger, 1998). Lectins isolated chiefly from plants and bacteria are non- immunoglobulin type carbohydrate recognition molecules that are involved in hemagglutination, lymphocyte transformation, inactivation of certain types of tumor cells and precipitation of certain polysaccharides and glycoproteins (Goldstein and Hayes, 1978; Lis and Sharon, 1986). Lectins are being used increasingly to probe the structure of carbohydrates on the surfaces of normal and malignant cells. Lectins not only interact freely with sugars but also with polysaccharides and glycoproteins at their non-reducing terminal glycosyl groups. Lectins are used in wider application such as agglutination of erythrocytes, mitogenic stimulation of lymphocytes, insulin like effect on fat cells, inhibition of fungal, bacterial and viral growth, insecticidal property, anti HIV property and nuclease activity (Kumar *et al*., 1993; Girbes *et al*., 1996; Batelli *et al*., 1997). The main possible physiological function which became of greater importance is defensive role of the lectins. Ever since the discovery of lectins, scientists have been intrigued by their possible roles. As plant lectins can interact with glycoconjugates of other organisms it was understood that they may not play a role in the plant itself, not all plant lectins play a defensive role. Lectins that occur at low concentrations may be involved in specific recognition processes either within or outside the plant. For instance, legume root lectins may be involved in the recognition and/or binding of *Rhizobium* and *Brudyrhizobium* sp. for the purpose of establishing symbiosis. (Diaz *et al*., 1989; Bohlool and Schmidt, 1974). Any lectin-mediated reaction or process resembles antigen-antibody

reactions (lock and key) with the specific binding of the lectin to a glycoconjugate receptor either located within or outside the plant. Therefore, the search for lectin natural receptors is immediately commenced. Lectin receptors can be defined as glycoconjugates that possess a carbohydrate moiety with a structure complementary to that of the binding site of the lectin. Glycoconjugates of different nature (e.g. glycoproteins, glycolipids, and polysaccharides) but with structurally similar carbohydrates can act as receptors for the same lectin. Many plant lectins have a much higher affinity for oligosaccharides, which are not common or totally absent in plants, for instance, chitin-binding plant lectins recognize a carbohydrate that is a typical constituent of the cell wall of fungi and the exoskeleton of invertebrates. Similarly, Sialic acid-binding lectins from elderberry *(Sambucus* sp.) (Shibuya *et al*., 1987) and *Maackia amurensis* (Knibbs *et al*., 1991) bind to a sugar that is absent in plants but is a major carbohydrate component of animal glycoproteins. This gave the indirect evidence for the possible molecular, biochemical, cellular, physiological, and evolutionary arguments indicating that lectins have a role in plant defense. This holds true for all the lectins that bind exclusively to the complex (modified) oligosaccharide side chains of typical animal glycoproteins. This argument is strengthend by their marked stability under unfavorable conditions such as stable over a wide pH range, able to withstand heat, and are resistant to animal and insect proteases. In these respects, they strongly resemble other defense-related proteins such as some pathogenesis related proteins, protease inhibitors, chitinases and glycanases, RIPs, a-amylase inhibitors, antifungal proteins, and thionins.

Food for ecological systems is mostly derived from plant sources which offer an enormous variety of macro and micronutrients necessary for heterotrophs such as microbes and plant-eating organisms including nematodes, insects, various other invertebrates and higher animals. The ultimate source for the survival of animal kingdom

is plants. The exposure of human beings to functionally active lectins are common as lectins are present in the most commonly edible plant foods such as tomato, potato, beans, peas, carrots, soybeans, cherries, blackberries, wheat germ, rice, corn, garlic, peanuts, mushrooms, avocado, beetroot, leek, cabbage, tea, parsley, oregano, spices and nuts, and also in several non-cultivated plant species (Nachbar and Oppenheim, 1980; Liener, 1986; Oliveira *et al*., 2000; Leontowicz *et al*., 2001),. The deleterious effects of dietary lectins on the gut and health have led to a number of outbreaks of food poisoning. For example, Noah *et al*. (1980) reported food poisonings which were attributed to toxins present in uncooked or partially cooked kidney beans (*Phaseolus vulgaris*). In 1981 and 1988 similar outbreaks of kidney bean food poisonings are reported (Bender and Reaidi, 1982) from which no pathogens were isolated but the beans contained abnormally high concentration of PHA. However, problems with other lectin containing foods have not been reported.

Studies on the intrinsic fluorescence properties have been widely used to obtain information about protein structure and conformational changes induced by alteration of environment and/ ligand binding [Lakowicz, 1999; Eftink *et al*., 1981; Grinvald *et al*., 1974]. Intrinsic fluorescence of proteins is dominantly due to the presence of tryptophan residues present in the protein. A valuable feature of this protein fluorescence is the high sensitivity of tryptophan residues to its local environment. Also tryptophan appears to be uniquely sensitive to collisional quenching, due to a tendency of indole to donate electrons while in the excited state. Tryptophan can be quenched by externally added quenchers or by nearby groups in the protein. The intrinsic fluorescence of proteins arises primarily from the side chains of tyrosine and tryptophan residues. By exciting the protein sample at 295 nm or above, where tyrosine residues do not absorb, it is possible to study the fluorescence due to tryptophan alone [Lakowicz, 1999]. Changes in the fluorescence

characteristics of tryptophan residues are used widely to obtain information about conformational transitions in proteins, association of subunits in oligomeric proteins, protein unfolding or ligand binding to proteins [Lakowicz, 1999; Eftink and Ghiron, 1981; Das *et al*., 1981; Kakitani *et al*., 1987]. Hence there are many reports on the study of tryptophan fluorescence. Tryptophan residues present in the active site of protein or on the exterior are relatively easy to study and a large number of studies using chemical modifications and/fluorescence techniques have been published, elucidating the environment and role of these residues in such proteins [Patanjali *et al*., 1984]. However, buried residues are generally much more difficult to study.

Biological Properties

Agglutination activity: lectins are distinguished from other sugar binding molecules by their ability to agglutinate cells. Eventually lectins without agglutination activity are also discovered but majority of the lectins show agglutination activity. Based on this property it was found that some lectins preferentially agglutinate tumour cells as compared to normal cells which has stimulated much interest. Some Gal/GalNAc specific lectins recognize the T-antigen disaccharide Galβ13GalNAc [for example, Puri *et al*., 1992; Sastry *et al*., 1986]. The tumour selectivity of lectins has resulted in attempts to use lectins for targeted drug delivery in chemotherapy [Gabaius and Gabaius, 1991].

Mitogenic Stimulation of Lymphocytes: The triggering of quiescent, nondividing lymphocytes into a state of growth and proliferation is one of the most dramatic effects of the interaction of lectins with the cells which is called mitogenecity. The first mitogenic agent to be described was PHA, the lectin from red kidney bean (*Phaseolus vulgaris*) by Nowell. The discovery of lectin-mediated mitogenesis led to the detection of many other mitogenic lectins, most notably con A , WGA (Wheat germ agglutinin) and some recent

examples include the mitogenic lectins from underground tubers of *Alocasia indica* , *Gonatanthus pumilus* and *Sauromatum guttatum* , seed integument of *Saraca indica* , pulp of *Musa acuminata* , inner shoots of *Allium tuberosum* and the fruiting bodies of *Agrocybe cylindracea* . Mitogenic lectins have been used to assess the immunocompetence of patients suffering from a variety of diseases and investigate the functioning of the immune system under abnormal conditions. Consequently, such mitogenic lectins are invaluable as tools to study the biochemical changes associated with lymphocyte activation and proliferation of various immune cells. Certain lectins can stimulate the triggering of quiescent, nondividing lymphocytes into a state of growth and proliferation. Now many lectins have been recognized to have mitogenecity for the T-cells, B-cells or both. Almost all of these mitogens are inhibited by simple sugars. Lectins can stimulate a large number of polyclones and this greatly facilitates the detection and study of changes associated with proliferation. The exact mechanism of mitogenic stimulation by lectins is not known, but the phenomenon has clinical applications such as in the production of polyclonal antibodies [Kilpatrick, 1991].

Lectins as cryoprotective agents: The leaves of mistletoe contain three Gal/GalNAc specific lectins, two of which are correlated with cryoprotectivity during freezing and thawing of isolated spinach thylakoid membranes [Hincha *et al*., 1997]. Seasonal regulation has been observed with respect to the accumulation of these lectins in the leaves.

Insulinomimetic Activity: As the receptor for insulin is a glycoprotein it is suggested that lectins also bind to the insulin receptor [Lis & Sharon, 1986] and these lectins are able to compete with insulin in binding with fat cells. Con A, wheat germ agglutinin and several other lectins mimic the effects of insulin on adipocytes.

Antifungal activity: Since plant lectins cannot penetrate the cytoplasm of the cells because of the presence of a thick and rigid cell wall, a direct interference with the growth and development of these organisms (i.e. through an alteration of the structure and/or permeability of the membrane or a disturbance of the normal intracellular processes) seems unlikely. However, indirect effects based on the binding of lectins to carbohydrates exposed on the surface of the fungal cell wall are possible. By virtue of their specificity, chitin-binding lectins seemed likely to have a role in the plant's defense against fungi (and insects). The demonstration of WGA inhibited spore germination and hyphal growth of *Trichoderma viride* strongly supported the hypothesis of the antifungal role of the chitin-binding plant lectins, until it was shown that the inhibition of fungal growth was due to contaminating chitinases in the lectin preparation (Schlumbaum *et al*., 1986). More definitive proof followed when it was demonstrated that chitinase-free lectin from by the growth inhibition *Botrytis cinerea, Trickoderma kamatum,* and *Pkycomyces blakesleeanus* by stinging nettle *(Urtica dioica)* lectin (Broekaert *et al*., 1989). The exact mechanism of the nettle lectin has not been elucidated yet, but it was believed that the nettle lectin is involved in the control of the colonization of the rhizomes by endomycorrhiza. Anti-fungal role is certainly not based on a chitinase activity and it does not affect the normal metabolism of the fungal cells. Only the synthesis of the cell wall appears to be affected as a result of disturbed chitin synthesis and/or deposition (Van Parijs *et al*., 1992). In spite of the *in vitro* antifungal activity of the nettle lectin, it is still unknown whether it has any protective activity *in vivo,* since the lectin is not capable of killing germinating spores or mycelium. Such a role is in partia1 agreement with the location of the lectin in rhizomes and seeds. Severa1 other chitin-binding plant proteins, that have to be regarded as lectins, have antifungal properties. The first group is the chitin-binding merolectin, Hevein, a 43-amino acid polypeptide from the latex of the

rubber tree *(Hevea brasrliensis)*, has an antifungal activity comparable to that of the nettle lectin (Van Parijs *et al*., 1991). Other proteins of the same group, e.g. the 30-amino acid chitin-binding polypeptide from seeds of *Amarantkus caudatus*, have more potent antifungal properties but still are unable to kill the fungi (Broekaert *et al*., 1992). The only plant lectins that can be considered fungicidal proteins are the chimerolectins belonging to the class I chitinases. *In vitro* tests with the purified enzymes as well as experiments with transgenic plants have demonstrated that class I chitinases confer resistance against plant pathogenic fungi. However, since the antifungal properties of these proteins reside in their catalytic rather than carbohydrate-binding domain, a detailed description of their protective role falls beyond the scope till this decade (Collinge *et al*., 1993).

Lectins in symbiosis: Several legume seed lectins play a role in rhizobial binding to the plant roots in carbohydrate based signal detection and function in the establishment of symbiosis between nitrogen fixing bacteria. Legume seed lectins bind to carbohydrate moieties on the bacterial surface and either agglutinate the bacteria at a distance away from the root or assist in the initial attachment phase of bacteria, frequently rhizobia, to root epidermal cells. This curious dichotomy in location and function of the lectin can be resolved if lectins are both associated with the plasma membrane and are being secreted from the surface of the root into the secretome. Presuming that the legume in question is growing under nitrogen stress and compatible rhizobia exist in the environment, successful bacterial attachment to root hairs will facilitate infection thread formation, which is required for effective root nodule development. When rhizobia encounter root hairs in the soil, several profound developmental events take place in the infected roots. The invasion into root hair requires a highly specific association between the bacteria and root hair surface. It is assumed that rhizobial attachment to plant roots occurs by the interaction between and rhizobial surface carbohydrates and lectins present in the roots of

legume plants. This is known as 'lectin recognition hypotheses'. Molecular genetic experiments favor this hypothesis. Lectins appear to be involved in the ectomycorrhizal symbioses as well, where they are proposed to function in recognition between the fungus, the source of the lectin, and its host (Giollant *et al*. 1993). It is worth noting that in a symbiosis between two species, there is a complex interplay of various factors, several of which are likely to be involved in the direct modulation of the interface between the two partners. The localization of lectins to this interface and their role in cross-linking glycosylated proteins increases the likelihood that lectins are involved in that reshaping process.

Chitinases

Chitinases catalyze the hydrolytic cleavage of the ß-1, 4-glycoside bond present in biopolymers of N-acetylglucosamine, mainly in chitin. Chitinases are present in various organisms. Depending on the organism of origin, these enzymes have different functions. Bacterial chitinases are mainly involved in nutrition processes. They degrade chitin that delivers carbon and nitrogen to the cells. In yeast and various fungi these enzymes participate in morphogenesis. They take part in remodeling of cell wall structure and daughter cell separation, and also in some pathogenesis processes.

Chitinolytic activity was found in viruses, fish, amphibians, mammals, gymnosperms and angiosperms, despite the fact that chitin is not present in these organisms. In animals and plants, chitinases mainly play a role in the defense of the organism against pathogen attack. They also play an important role in general stress response as well as in growth and development process.

A number of proteins demonstrating chitinolytic activity were identified in plants. It is possible to find them in all organs and plant tissues, in both the apoplast and vacuole. These proteins present a large and diverse group of enzymes; they differ not only in

spatial and temporal localization, but also in their molecule structure and substrate specificity.

Many Chitin-binding proteins and chitinases has been discovered and characterized since long time. The structure of wheat germ agglutinin (WGA) and similar proteins from barley has been reviewed recently. However in S*olanaceae* family lectins that specifically recognize chitin and chitin oligosaccharides were described in Jimson weed *(Datura stramonium)* (Crowley *et al*, 1982), Potato *(Solanum tuberosum)* (Kilpatrick, 1991), Tamarillo (*Cyphomandra betacea)* (Xu *et.al.*1992) and Tomato *(Lycopersicon esculentum)* (Kilpatrick, 1991). Most of these chitin-binding proteins exhibit anti-fungal, anti-microbial and insecticidal activities. A chitin-binding lectin from the rhizomes of *Utrica dioica* is a potent super antigen in mice. A novel chitin-binding lectin isolated from *Viscum album* exhibits cytotoxic properties (Peumans *et al*. 1996).

There are two major classification of chitinases *i.e.* based on function and structure. Based on function chitinases can be divided into two categories:

i) *Exochitinases*: They show activity only for the non-reducing end of the chitin chain. Many plant exochitinases, especially those with a high isoelectric point, exhibit an additional lysozyme or lysozyme-like activity.

ii) *Endochitinases*: They hydrolyze internal ß-1, 4-glycoside bonds.

Chitinases use two different hydrolytic mechanisms:

Substrate assisted catalysis, which leads to retention of conformation at the anomeric carbon of the product Acid catalysis; the reaction of hydrolysis using this mechanism inverts the anomeric carbon.

Classification of Chitinases:

A)Based on structure chitinases have classified into five classes based on their amino acid similarity and the presence of a signal peptide, a hevein (chitin binding) domain, a hinge region, a catalytic domain, and a C-terminal extension (Collinge *et al*., 1993).

Class I chitinases: Class I chitinases have a hevein domain, a hinge region, and a catalytic region, and are mostly basic proteins with the molecular mass between 30 and 36 kDa.

Class II chitinases: Class II chitinases are quite similar to class I chitinases, but they do not have a hevein domain. Their molecular masses are between25 and 30 kDa.

Class III chitinases: Class III chitinases, which have little similarity to class I or II chitinases, are mostly acidic protein without a hevein domain

Class IV chitinases: Class IV chitinases have a hevein domain and their catalytic domain is quite similar to that of class I chitinases, though they lack the four regions of class I chitinases

Class V Chitinases: Class V Chitinases have two hevein domains and their catalytic domain is similar to that of class III chitinases.

B) Plant chitinases are divided into six classes based on their primary structure

Class I chitinases: Class I chitinases have an N-terminal cysteine-rich domain that is thought to be chitin-binding domain (Typical class I chitinases consists of an N-terminal chitin binding hevein domain followed by a chitinase domain and a vacuolar targeting sequence which is cleaved off during processing of the proprotein).

The chitin –binding class I chitinases show stronger specific activity on chitin, a ß-1, 4-linked polymer of GlcNac, and possess significantly stronger invitro antifungal activity than their non-chitin-binding counterpart, the class II chitinases.

Ex: rice, tobacco, potato.

Class II chitinases: Class II chitinases lack the cysteine rich domain but have high amino acid sequence similarity to Class I chitinases

Ex: arabidopsis, barley, tobacco.

Class III chitinases: Class III chitinases have no significant sequence similarity to other types of chitinases, but have a region with weak similarity to prokaryotic chitinases. They have never been reported in any monocot, including rice.

Ex: cucumber, arabidopsis, tobacco, chick pea.

Class IV chitinases: Class IV chitinases also contain a cysteine-rich domain and resemble class I chitinases although they lack four regions of class I chitinase.

Ex: bean, sugar-beet.

Class V Chitinases: Class V chitinase (Chi-V), is similar to bacterial chitinases. In particular, tobacco Chi-V shows 31% and 26% identity *Bacillus circulans* ChiA and *Serratia marcescens* ChiB, respectively. However, the tobacco Chi-V shows endo-chitinase activity whereas the bacterial chitinases have exo-chitinase activity.

Substrates of chitinases

The main substrate of chitinases is chitin oligomers, unbranched chains of ß-1, 4-linked N-acetylglucosamine have a helical conformation. One in six aminosaccharide residues can be devoid of an acetyl group. Deacetylation is a common process involved in the in the chitin-protein interaction. Chitinases can also hydrolyze the lipochitooligosaccharides (Nodulation factors) produced by nitrogen fixing bacteria. The Nod factors consist of the N-acetylglucosamine tetra- or pentamer backbone with an N-linked fatty acid moiety

replacing the N-acetyl group on the non-reducing end. Moreover, the reducing end of the Nod factor aminosaccharide backbone undergoes, depending on the organism of origin, different types of modifications (e.g. acetylation, fucosylation, methylation, sulphurylation).

Chitinases – PR proteins

PR-proteins are defined as proteins that are induced by various stress factors, i.e. draught, salinity, wounding, heavy metals in their environment, endogenous and exogenous elicitor treatment, and plant growth regulators.

Acidic PR proteins are induced by salicylic acid, where as basic PR proteins are induced by ethylene or jasmonic acid.

Based on biological properties, enzyme activity and coding sequence similarities, PR proteins are divided into 14 classes.

Plants produce a large number of defense-related proteins believed to be important in protecting them against pathogen infection. Many of hitherto characterized proteins belong to the group of so-called pathogenesis-related proteins, a heterogeneous group separable into at least six different families and induced in many plants during infection with viral, bacterial, or fungal pathogens (Cutt and Klessig, 1992). Prominent and extensively studied families of pathogenesis-related proteins are chitinases, which have been shown to be capable of inhibiting fungal growth by the degradation of chitin.

Although in the past most interest in plant lectins has focused on lectins in seeds, especially from *Fabaceae* (legumes), evidence accumulated the presence of lectins in vegetative tissues. The root tubers are occurring in large number of plant families belonging to all major taxonomic groupings. Hitherto there are few reports on the root tuber lectins. Amongst monocots, tuber lectins have been isolated one each from *Alocasia*

indicum, Arum maculatum, Colocasia antiquorum, Colacacia esculenta, Dieffenbachia sequina and *Xanthosoma sagittifolia*. It is interesting to note that all the known monocot tuber lectins belong to the family *Araceae*. Recently a tuber Lectin from *Arisaema jacquemontii* with anti-insect and anti-proliferative properties is established by Kaur *et al*, 2006.

Aponogeton are monocots which belong to the family *Aponogetonaceae* and are native to tropical areas of Africa, Asia and Australia. The *Aponogetonaceae* is considered to be allied to the *Potamogetonaceae - Najadaceae* complex of families. Most of the species are used as ornamental plants in aquaria. An *Aponogeton* from Asia will have a single bloom, while those from African heritage will have multiple blooms on the same flower stalk. Many species grow in the temporarily still or floating water and propagate as dormant tuber/bulbs during the drought period. *Aponogeton* flowers are often (although not always) self fertile. It is quite possible to produce viable seeds by gently brushing an inflorescence with a soft brush or even your finger tip to distribute the pollen. If the plant is self fertile, the fruits, called “water berries,” will develop along the flower stalk within a matter of days. In time, the flower stalk will begin to decay and the fruits will float free. Within a day or two the fruits will split open and the seed will drop to the bottom of the pond, where it will quickly germinate. In India *Aponogeton natans, A. microphyllum* and *A. undulatum* species are distributed, and amongst these *Aponogeton natans* is more common.

The present investigation is aimed at purification, biochemical characterization and bioassay studies of chimeric lectin from *Aponogeton natans* tubers.

MATERIALS AND METHODS

Plant Source

Aponogetan natans were collected from the lake of Hyderabad University campus.

Preparation of Protein Extract

10 gm of freshly washed tubers were collected, quenched in liquid nitrogen and was crushed into fine paste using motor and pestle in phosphate buffer saline (PBS) pH-7.2 was added in a ratio of 1:10 (W/V). The sample was centrifuged at 10,000 rpm for 30 min at 4°C, the supernatant was filtered through what man filter paper and the filtered supernatant is designated as crude extract. The crude extract was then subjected to ammonium sulphate precipitation (0-80%) followed by overnight stirring at 4°C, it was then centrifuged at 10,000 rpm for 30 min at 4°C, and the pellet was dissolved in minimum volume of PBS, the sample was then dialyzed against PBS and used for further experiments.

Protein Estimation

Protein was estimated according to Lowery *et al*. (1951) with minor modification. Reagent A was 4 % sodium carbonate in 0.2 N sodium hydroxide, reagent B was 1 % cupric sulphate, reagent C was 2 % sodium potassium tartarate and reagent D was 1 N Folin's reagent. The working reagent mixture of A, B and C in a ratio of 23: 1: 1 was used within 24 hour. 1 ml of the working reagent was added to 1 ml of protein sample and allowed to stand for 10 min. To this, 0.2 ml of reagent D was added rapidly while vortexing the sample. After 30 min the sample, absorbance was recorded at OD 750 nm. BSA (Fraction V) was used as a standard protein (5-50 µg). Protein in the affinity chromatography fractions was detected at OD 280 nm (Peterson, 1983).

Carbohydrate Estimation

Total carbohydrate was estimated by the method of Dubois *et al*., (1956). One ml of the sample was mixed well with 40 µl of 80% phenol and 2.5 ml of sulfuric acid was added rapidly. The steam of the acid was directed against the side of the test tube in order to obtain good mixing. The tubes were allowed to stand for 10 min. After which they were mixed well and cooled to room temperature. The absorbance is measured at 490 nm. D-glucose (5-50 µg) was used as a standard carbohydrate.

Hemagglutination Assay

The hemagglutination activity of lectin was determined with minor modifications according to the method described by Lis and Sharon. The details for the preparation of erythrocytes for the assay are as follows.

Preparation of Alsevere's solution: The Alsevere's solution was prepared by dissolving 2.05 g of Glucose, 0.8 g of Sodium citrate and 0.42 g of Sodium chloride in 80 ml of distilled water. The pH was adjusted to 6.1 with 1% citric acid and the volume was made up to 100 ml with distilled water. The solution was autoclaved, cooled and stored at 4 ºC.

Preparation of erythrocytes: Venous whole blood was added to an equal volume of Alsevere's solution. The blood suspension can be stored as long as two weeks in the alsevere's solution. The erythrocytes were isolated from the stock blood suspension by centrifugation at room temperature using a table-top centrifuge (1000 x g for 5min). The packed erythrocytes at the 1000 x g considered as 100%. The packed cells were washed with cold saline (0.9 % NaCl) for 3-4 times (5 ml saline for each ml of packed erythrocytes) and finally 4 % cells were made in saline.

Preparation of trypsin treated erythrocytes: The erythrocytes were treated with trypsin on the day of the assay. The 4% erythrocyte suspension was incubated with 0.1 % trypsin

1:250 (1000-1500 BAEE units / mg) for one hour at 37 ºC. The trypsin treated erythrocytes were washed 5-6 times with cold saline to remove the traces of trypsin and 4 % cells were made and used for experiments.

Agglutination assay: 100 µl of the protein sample was serially diluted in a microtitre plate and 100 µl of trypsinized 4 % erythrocytes were added to each well. The agglutination was observed visually after incubation of the plate for one hour at 37 ºC. The highest dilution which showed positive hemagglutination is considered as titre. The amount of protein presents at this dilution represents the minimum quantity of the protein required for agglutination and is defined as one unit. Specific activity is the number of units per mg of protein.

Sugar Inhibition Assay

50 µl of serially diluted carbohydrate solution was mixed with 50 µl protein containing 8 hemagglutination unit in a microtitre plate and incubated at room temperature for 30 minutes, 100 µl of 4 % trypsinized human O^{+ve} erythrocyte suspension was added to the incubated solution and hemagglutination was recorded after incubation of the plate for 1 h at 37ºC. The inhibition concentration of the sugar was recorded as the minimum concentration of sugar required for complete inhibition of 2 hemagglutination units with 2 % of the erythrocytes.

Preparation of Chitin Column

Commercially available chitin flakes (Sigma, USA) were ground to fine powder with the help of commercial blender. The fine powder was packed onto the affinity column; the final bed volume of chitin was 50 ml (14.5 x 75 mm). The column was washed thoroughly with 1 litre of 0.1 M HCl, subsequently with 1 litre of 0.1 M NaOH and finally equilibrated with 10mM PBS.

Purification of Chitin Binding Protein / Chitinase

A) Crude sample (15 ml) was passed through the chitin column. The sample was recycled 2 times. PBS was used for washing and was passed through the column until the OD was up to 0.02 at 280 nm; 3 ml washing fractions were collected. For elution 0.2 M glacial acetic acid was used, 3 ml of elution fractions were collected. OD of each fraction was taken at 280 nm. The fractions were neutralized with 1M Tris, pooled and dialyzed against PBS.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli *et al*. (1970) with minor modifications. The separation of proteins was performed in 12 % resolving gel with 4 % stacking gel. Both the resolving and stacking gel contained 2.4 % bisacrylamide as a cross linker and 0.1 % SDS. The final resolving buffer concentration was 0.375 M Tris-HCl (pH 8.8) in resolving gel and stacking buffer final concentration was 0.125 M Tris-HCl (pH 6.8) in stacking gel. Ammonium persulfate and N, N, N, N-Tetramethyl ethylenediamine (TEMED) was used as polymerizing agents in final concentrations of 0.05 % and 0.1 % respectively. The electrode buffer consists of 0.025 M Tris, 0.192 M Glycine (3.025 gm of Tris, 14.4 gm of Glycine and 1 gm of SDS in one litre of DD H_2O, pH 8.3) and 0.1 % SDS. The samples buffer consists of 0.062 M Tris-HCl, 10 % glycerol, 2 % SDS, 5 % β-mercaptoethanol and 0.001 % bromophenol blue. The samples were incubated for 3 min in boiling water bath with the sample buffer and centrifuged for 5 min at 3,000 rpm. The clear sample solutions were loaded into wells on the gels of 8 x 10 cm dimension, which was polymerized in glass plate fixed to mini- vertical slab gel apparatus. The gels were run at room temperature at 75 and 100 V (direct current) for stacking and resolving gels, respectively. Electrophoresis was carried out until the bromophenol blue dye marker reached about 4–5 mm and the gels were removed.

Native Polyacrylamide Gel Electrophoresis (Native PAGE)

In Native PAGE, β-mercaptoethanol and SDS was not used while preparing the sample buffer, similarly in the preparation of electrode buffer SDS was not used, the resolving gel also did not contain SDS. The samples were added to the sample buffer and centrifuged for 5 min at 3,000 rpm; heating of the sample was avoided. The gels were run at 4°C. Electrophoresis was carried out until the bromophenol blue dye marker reached about 4–5 mm and the gels were removed.

Silver staining

Silver staining was performed according to the method of Blum *et al*. (1987). The gel was fixed for more than 1 h in fixative and washed 3 times with 50 % ethanol for 3 times at every 20 min interval. The washed gel was pre-treated with 0.002 % sodium thiosulphate solution for exactly 1 min and rinsed 3 times in distilled water at every 20 sec. The pre-treated gel was impregnated in 0.2 % silver nitrate containing 0.028 % formaldehyde. The gel was rinsed 2 times with distilled water and developed for the proteins with the solution containing 6 % sodium carbonate, 0.018 % formaldehyde and 0.0004 % sodium thiosulphate. The gel was stopped for the development of protein bands in appropriate intensity with fixative solution for 10 min and was stored in 50 % methanol at 4°C.

Molecular weight determination

The native molecular mass of the lectin was determined using Sephadex G-200, gel filtration column (60 cm x 1.2 cm) equilibrated with 10mM PBS. The column was calibrated with proteins of known molecular weight viz., Phosphorylase b (Mr 90 kDa), Bovine serum albumin (Mr 66 kDa), ovalbumin (45 kDa) and Lysozyme (Mr 14 kDa). 2.0 mL fractions were collected.

Periodic acid-Schiff's staining:

To determine the carbohydrate nature of the protein qualitatively, periodic acid-Schiff's staining was carried out following the method of Zacharius *et al*., (1969).

Preparation of Schiff's reagent: 1g of Basic Fuschin is added to 200 ml of H_2O at 70º C. This was boiled for few minutes cooled and filtered. Temperature is adjusted to 50º C. To this 5ml of HCl and 2gm of Potassium metabisulphate was added and incubated overnight. The solution turns colorless of pale straw yellow. To decolorize completely 0.25g to 0.5g of activated charcoal is added and filtered. The stain solution is stored in stoppered brown bottle and stirred at 4º C. Purified lectin was separated by 12.5% SDS-PAGE and the gel was incubated with 1% periodic acid in 3% acetic acid for one hour. It was washed for one hour with distilled water and stained in Schiff's reagent for 30 min in dark. It was then destained in 10% acetic acid and finally stored in 3% acetic acid.

Temperature stability

The lectin (100 µg/mL concentration) was incubated at different temperatures of 4 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C and at 90 °C for a period of 10 minutes and were brought back to room temperature and their ability to agglutinate erythrocytes was tested.

pH stability

The ANTL (100 µg/mL) was incubated in different pHs of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 (20mM sodium acetate/sodium phosphate and Tris-HCl buffers) for a period of 30 min and the hemagglutination activity was further tested to determine the pH optimum.

Effect of denaturing agents

The ANTL (100 µg/mL) was incubated overnight with different denaturing agents *i.e.,* 6 M Urea, Guanidium Hydrochloride, Lithium chloride, Potassium ferricyanide, periodic acid and tested for hemagglutination activity.

Effect of EDTA

The ANTL lectin (100 µg/mL) was incubated overnight with different concentrations of EDTA and hemagglutination activity is tested.

Insect bioassays

Insect bioassays are performed according to the method of Mirela et.al (2007) with minor modifications. The moths were maintained in plastic boxes with perforated plastic covers at a relative humidity of 65–75% and 28±1 °C, and were fed a standard artificial diet prepared by mixing semi crushed sorghum and vitamins. To examine the effects of ANTL on the development of *Achaea janata* and *Corcyra cephalonica*, larvae up to the fourth instar were fed an artificial diet containing ANTL at concentrations of 0.1%–0.4% (w/w). Ten neonatal larvae were used for each treatment, which was repeated three times for each concentration of ANTL, i.e. 30 larvae per concentration. The weight and number of larvae were determined after incubation for 5 days at 28 °C and a relative humidity of 65–70%. Control artificial diets are without ANTL. Linear regression analysis was used to evaluate the response of *A. janata* and *C. cephalonica* to ANTL. The effective dose for a 50% response (ED_{50}) was defined as the concentration of ACLEC that reduced the larval mass by 50% compared to the control larvae. The lethal dose (LD_{50}) was defined as the concentration of ANTL that reduced the number of larvae by 50% compared to the control larvae fed the artificial diet without ANTL. In the linear regression, X=dose and Y=mean weight or percentage mortality. Larval consumption and faecal output were analyzed on a dry mass basis. The protein content and tryptic activity of the faecal and midgut samples were also determined.

Measurement of nutritional parameters

Several nutritional parameters were used to compare fourth instar larvae fed the control diet and those fed a diet containing 0.4% ANTL. Phosphate buffered saline (0.4%) was

included in the control diet. The larvae, faeces and remaining uneaten food were separated, dried and weighed. The indices of consumption, digestion and food use were calculated as described by Scriber and Slansky Jr (1981):

Efficiency of conversion of ingested food (ECI) estimates the percentage of ingested food that is converted to biomass, and was calculated as:

$$\frac{\text{Biomass gained (mg fresh mass)}}{\text{Food ingested (mg dry mass)}} \times 100$$

Efficiency of conversion of digested food (ECD) estimates the efficiency with which digested food is converted to biomass, and was calculated as:

$$\frac{\text{Biomass gained (mg fresh mass)}}{\text{Food ingested (mg dry mass)} - \text{Faeces (mg dry mass)}} \times 100$$

Approximate digestibility (AD) estimates the amount of ingested food that is digested, and was calculated as:

$$\frac{\text{Food ingested (mg dry mass)} - \text{Faeces (mg dry mass)}}{\text{Biomass gained (mg fresh mass)}} \times 100$$

Metabolic cost (MC) was calculated as: 100−ECD.

Trypsin like enzyme assays

Trypsin-like enzymes of gut extracts and faecal samples from *C. cephalonica* larvae were assayed using the synthetic substrate N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as described by Erlanger *et al*. (1961). For routine assays, BAPNA was used at a final concentration of 1 mM in 1% (v/v) DMSO in a final volume of 1.5 mL at pH 8. Buffer

and enzyme were preincubated at 37 °C for 10 min before adding substrate to start the reaction, which was allowed to proceed for 20 min and then stopped with 200 μL of 30% (v/v) acetic acid. The resulting absorbance was read at 410 nm. Each assay was done in triplicate. The linearity of the relationship between the changes in absorbance with time was checked to ensure that substrate concentrations were not limiting. Substrate and enzyme controls were run to ensure the validity of sample absorbance readings. p-Nitroaniline was used as a standard.

Protease activity of midgut and faecal extracts to ANTL in polyacrylamide gels containing 0.1% gelatin

Proteins from midgut and faecal extracts of *C. cephalonica* larvae were run (without prior boiling or reduction) on SDS-PAGE in 10% gels containing 0.1% gelatin (Michaud *et al*., 1993). Following electrophoresis at 4 °C, the gels were washed with 2.5% Triton X-100 for 2 h with shaking to remove the SDS and then incubated in 0.1 M Tris– HCl, pH 8.0, for 2–3 h. The gels were subsequently stained with Coomassie brilliant blue R-250. Bands of proteolytic activity appeared as clear (white) zones against a blue background.

Fluorescence spectroscopy

All emission spectra were recorded on a Spex Fluoromax-3 fluorescence spectrometer from Jobin-Yvon (Edison, NJ, USA, website: http://www.jobinyvon.com). Slit widths of 3 and 6 nm were used on the excitation and emission monochromators, respectively.

Measurements were performed by irradiating lectin samples (OD280 ≤ 0.1) with 290 nm wavelength light, in order to selectively excite tryptophan residues of the protein and emission spectra were recorded above 310 nm. In fluorescence quenching experiments, small aliquots of 5 M quencher stocks (acrylamide, potassium iodide, or cesium chloride) were added to protein samples and fluorescence spectra were recorded after each

addition. The final quencher concentration attained in each case was 0.5 M. The iodide stock solution contained 0.2 mM sodium thiosulphate to prevent the formation of triiodide (I_3^-). For quenching studies with denatured lectin, the protein was incubated with 6M Guanidium Hydrochloride (Gdn–HCl) overnight at room temperature. For experiments with sugar-bound lectin, the lectin samples and the quencher stocks were made 1mM in chitohexose, a sugar that is specifically recognized by the lectin. Fluorescence intensities were corrected for volume changes before further analysis of the quenching data. All measurements were performed in duplicate at 25 °C and yielded reproducible results. The average values obtained from these are reported.

Circular dichrosim spectroscopy

Circular dichrosim (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan, website: http://www.jascoint.co.jp) equipped with a Peltier thermostat supplied by the manufacturer. Samples were placed in a 2 mm path length rectangular quartz cell. Lectin concentration was 0.8–1.6 μM for measurements in the far UV region (250–190 nm) and 8–15 μM for measurements in the near UV region (300–250 nm). In order to study thermal unfolding of the protein, CD spectra were recorded in the near UV region at different temperatures. In addition, temperature scans were performed between 30 °C and 90 °C at a scan rate of 1°C per minute, by monitoring the ellipticity (Θ) at 280 nm as well as 208 nm, corresponding to the near UV and far UV regions, respectively.

Synthesis of Glycol Chitin

Glycol chitin was obtained by acetylation of glycol chitosan with a minor modification (Trudel *et al*., 1989). 1 gm of glycol chitin (Hi-media, India) was dissolved in 20 ml of 10 % acetic acid by grinding in a motor; the viscous solution was allowed to stand for overnight. 90 ml of methanol was added slowly to the viscous solution with a little

stirring, the solution was filtered through what man filter paper no 4 with vacuum suction. The filtrate was transferred to a beaker and 1.5 ml acetic anhydrite was added with stirring and kept standing for 30 min resulted in the formation of gel. Gel was cut into pieces and the liquid exudates were discarded, the pieces were soaked in methanol and homogenized at high speed for 5 min followed by high speed centrifugation (15,000 rpm) at 4°C. Pellet was re-suspended in equal volume of methanol. Homogenized again at high speed and centrifuged (15,000 rpm) at 4°C. Pellet was dissolved in 100 ml of milli Q water with 0.02 % of sodium azide and stored at 4°C.

Preparation of colloidal chitin (CC):

Colloidal chitin was prepared according to the method of Berger and Reynolds (1988). Ten grams of chitin was slowly dissolved in 400 ml of concentrated HCl with stirring at 4ºC. The mixture was incubated in water bath at 37 ºC until viscosity decreased. To this mixture 4.0 liters of sterile distilled water was added and left overnight at 4 ºC. The supernatant was slowly decanted and the precipitate was collected on a filter paper and washed extensively with sterile distilled water to attain neutral pH. The colloidal chitin was dissolved in 250 ml sterile distilled water prior to use.

Enzymatic Assay

Chitinase activity was checked by gel diffusion assay method following with a minor modification (Zou *et al*., 2002). 0.8 gm of agarose was added to 1 ml of sodium phosphate buffer (pH 6.5) and 99 ml of water, agarose was dissolved by boiling. The dissolved solution was cooled to 50-60°C, to it 10 ml of 1 % glycol chitin was added and the solution was vigorously shaken, the solution was allowed to polymerize in petridish. A cork borer was used to cut wells in the gel, with a volume per well of approximately 30 µl. Excised gel pieces were removed by vacuum with a plastic pipette tip.

Samples of 100 µl each of crude protein extract, purified protein extract, and blank were pipeted into individual wells in the gel plate, which were then covered and sealed with wax film. The gel plates were incubated at 37°C for 16 h. After incubation, gels were stained with 20 ml of freshly prepared (0.1 %) calcofluor white in 500 mM Tris-HCl (pH 8.9) for 15-30 min. After staining, the calcofluor solution was discarded and the gel plates were gently washed with distilled water for 2 h at room temperature. Lytic zones in the gel plates were visualized by UV transilluminator.

Reagents for β-1, 4-N-acetyl glucosamine assay (NAGase):

Reagent A: 6.1 g of dipotassium tetraborate tetrahydrate was dissolved in 100 ml of distilled water.

Reagent B: 1.5 ml of distilled water was added to 11.0 ml of concentrated HCl and made up to 100 ml with glacial acetic acid. 10.0 g of para dimethyl aminobenzaldehyde (DMAB) was dissolved in this mixture. 10 ml of this solution was diluted to 100 ml with glacial acetic acid just before use.

NAGase Assay:

NAGase was assayed colorimetrically as described by Boller and Mauch (1988) using crude filtrate as an enzyme source. The reaction mixture consisted of 0.5 ml 1.5% colloidal chitin, 0.5 ml of cell free culture filterate of *B. subtilis* AF 1 and 0.5 ml of sodium acetate buffer pH 5.2. The reaction mixture was incubated at 37 ºC for 3 h and centrifuged at 3000Xg. Aminosugar estimation (Reisslig *et al*., 1955) was carried out using 0.5 ml of the supernatant by adding 50µl of reagent A and boiled for 3 min. The mixture was immediately cooled to room temperature and 1.5 ml of reagent B was added and incubated at 37 ºC for 45min. The color developed was recorded with appropriate blanks. One unit of enzyme activity was defined as µmole of N-acetyl glucosamine

released ml^{-1} h^{-1}. The specific activity was expressed as units of enzyme activity per mg protein where the protein concentration was determined as described by Bradford (1976).

Chitinase assay in gels

Native PAGE and semi SDS-PAGE (without β-mercaptoethanol) gels were carried out at 4ºC. After semi SDS-PAGE the gels were incubated at 37 ºC for 2 hour in sodium acetate buffer pH 6 containing 1 % (v/v) Triton X-100 to remove SDS. The gels were then washed with distilled water and incubated at 37 ºC in sodium acetate buffer pH 6 for 1 hour. Other gel containing 0.1% (v/v) glycol chitin as a substrate for the enzyme is polymerized. After polymerization, the gel with the substrate incorporated is overlayed on to the gel with the enzyme and incubated overnight. After incubation the gel with the substrate is stained with 0.1 %(w/v) ranipal and observed under uv-transilluminator.

The bands observed in the chitinase activity of the native and semi SDS-PAGE gels are consistent with the bands observed in the native and SDS-PAGE (without β-mercaptoethanol) gels.

Cytotoxic Studies

1. Medium preparation:

RPMI- 1640 Medium: Powdered RPMI- 1640 Medium was dissolved in ultra pure water. 2.2 gms of sodium carbonate was added, pH was adjusted to 7.2 and the volume was made up to 1liter with ultra pure water. This was sterile filtered through 0.22 µ membrane filter using Sartorius filtration unit and stored at 4ºC.

2. Lymphocyte Counting:

Principle: Gentian violet stains the lymphocyte nucleus while dilute acetic acid lyses the RBC.

Turk's solution (0.01% Gentain violet W/V in 3% acetic acid) was added to a small volume of the cell suspension mixed and counted using a Haemocytometer. The average number of the cells in the cell suspension was determined using the formula

$$\text{Number of cells per ml} = \frac{\text{Average number of cells per large square}}{\text{Dilution}} \times 10^4$$

Determination of cell viability:

Principle: Dead cells take up the dye trypan blue while the live cells exclude it, thereby viable cells could be distinguished from non viable dead cells, which are stained

Procedure: A small volume of the cell suspension was diluted approximately in trypan blue solution (0.2% w/v in 0.9 % NaCl). Minimum of 300 cells were counted microscopically using Haemocytometer. The percentage of viable cells were calculated using the formula

$$\% \text{ Viability} = \frac{\text{Number of unstained cells}}{\text{Total number of cells}} \times 100$$

3. Maintenance of cell lines:

SUP T1: it is a Non-Hodgkins T-Cell lymphoma cell line btained from Mc Kessan clinical and biological services, Rockville, USA. The growth medium used for the propagation of the cell line was 90% RPMI-1640 with 10% FCS. Gentamycin was used at a concentration of 50µg/ml. The cell line was grown in a CO_2 incubator with 5% CO_2. The seeding ratio of the cells was 0.2 X 10^6 /ml they were subcultured. Doubling time of the cells was observed to be 36 hrs. Medium was renewed 2 to 3 times per week.

U 266: It is a Myeloma cell line obtained from national center for cell sciences, Pune. The growth medium used for the propagation of the cell line was 90% RPMI-1640 with 10%

FCS Gentamycin was used at a concentration of 50µg/ml. The cell line was grown in a CO_2 incubator with 5% CO_2. The seeding ratio of the cells was 0.2 X 10^6/ml of the medium. When the cells reached a density of 2 X 10^6 cells/ml they were subcultured. Doubling time of the cells as observed to be 55 hrs. Medium was renewed 2 to 3 times per week.

MTT Assay:

Principle: 3-[4, 5-dimethylthiazole-2-yl]-2, 5 dipheyl tetrazolium bromide (MTT) is reduced by mitochondria dehydrogenase enzymes of the living cells Formazan (purple compound). The absorbance of Formazan is measured in a microplate ELISA reader equipped with a 570 and 630nm filter and is proportional to the number of viable cells.

Reagents:

1. 5 mg/ml MTT in PBS
2. 0.01N HCl in 10% SDS

Cultures in 96 welled microtitre plates were centrifuged at 1000 rpm at room temperature and 100µl of supernatant was discarded and 20 µl of MTT was added to each well. The plate was incubated for 4 hrs at 37ºC and 100 µl of acidified SDS was added to wells. The plates were incubated overnight to solubulize the Formazan compound and the absorbance measured at 570-630nm dual wave length mode.

Isolation of mouse and rat Splenic lymphocytes:

Splenic lymphocytes were prepared as described earlier (Zimmerman and kern, 1973)

Complete medium: RPMI-1640medium supplemented with 5% FCS. Mice were killed under mild ether anesthia and spleen was dissected and put in RMPI-1640 medium.

Single cell suspension was prepared as follows:

Spleen was placed on a sterile stainless steel mesh. Connective tissue and fat were removed and the tissue was minced. The tissue was teased into the medium using an arterial forceps fitted with a stainless steel brush. The suspension was allowed to settle for 5 min. large clumps that settled to the bottom were removed. The suspension was centrifuged at 500g for 7 min. The pellet was re-suspended in 8ml RPMI-1640 medium and layered over 3 ml Histopaque (d = 1.077) and centrifuged at 500 g 15 min. The cells at the interface were collected and washed twice with complete medium and suspended in the same medium.

Isolation of human and rabbit peripheral blood lymphocytes (PBL):

PBL were isolated according to the method of Boyum (1964). Venous blood was collected into heparinised tubes (8U/ml) was diluted 1:1 with saline. Diluted blood, 8ml was layered over 3 ml Histopaque and centrifuged at 500g for 20 min. The cells at the interface were collected and washed thrice with complete medium and suspended in the same medium.

Lymphocyte proliferation Assay:

Mitogens, Bacterial DNA, oligodeoxynucleotides

Complete medium: RPMI-1640 supplemented with 5% FCS and 50mg/litre Gentamycin

^{3}H- Thymidine (specific activity 5mCi/m mole)

Scintillation cocktail: 4gms PPO and POPOP in liter of scintillation grade Toluene.

Procedure:

Splenic lymphocytes (2 X $10^{5)}$ in triplicate were cultured with mitogens, DNA, oligodeoxynucleotides in 200 µl of complete medium in 96 well flat-bottomed micro well plates. Cultures were kept at 37ºC in a humidified incubator with 5% $CO_{2.}$ The cultures were pulsed with 1µCi of ^{3}H-Thymidine for the last 24 hrs of the culture period and were

harvested onto glass fiber filter using Skatron automatic cell harvester. The dried filters were transferred into toluene based scintillation cocktail and the radioactivity was measured using Beckman Scintillation Counter. The results obtained were expressed as cpm.10^6 cells.

INSECT CELL TISSUE CULTURE

Cell lines and virus.

Sf9 (*Spodoptera frugiperda*) cell lines (Vaughn *et al*., 1977), which serve as hosts for AcNPV was used for the expression study. Sf9 cells were maintained in complete medium (TNM-FM from sigma) supplemented with 10% FCS and 100 µg/ml antibiotic and antimycotic solution as described by Summer and Smith, 1987.

Preparation of TNM-FH medium:

TNM-FH medium (HINK, 1970) is Grace's insect cell culture medium (Grace, 1962) which is supplemented with lactalbumin hydrolysate and yeastolate. The medium is enriched in all the basic nutrients for the growth of insect cells and it is buffered with sodium phosphate. To make 1 liter of TNM-FH medium, 51.2 gm of Grace's medium was dissolved in 700 ml of distilled water, 350 mg of $NaHCO_3$ was also added and the medium was adjusted to pH 6.2 with autoclaved double distilled water. The medium was filter sterilized by filtering through 0.22 µM filter using sterile filter init in the hood. The filtered medium was kept at room temperature for about 48 hours to check the contamination. After 48 hours, 10% fetal calf serum and 100 µg/ml antibiotic and antimycotic solution were added to make the complete medium.

Sf9 insect cells were maintained at 27 ˚C in complete medium and grown as a monolayer and in suspension culture. Sf9 insect cells double every 24 hours at 27 ˚C. Sf9 cells were maintained in T-25 cm^2 or in T-72 cm^2 tissue culture flasks or in spinner flasks for obtaining monolayer and or suspension cultures respectively. Cells were dislodged by

washing the surface by gentle pipetting (O'Reilly *et al*., 1992). For each subculture, 1-3 million cells were seeded depending on the flask size. Before every splitting or subjection the cells to infection, the viability of the cells were checked by staining with 10% v/v trypan blue (dead cells stain blue). Only cells with greater than 90% viability were used for the expression, freezing and splitting.

RESULTS AND DISCUSSION

The experiments performed with the crude extracts for hemagglutination revealed the presence of the lectin which has shown different specificities towards different blood groups and the agglutination is high with rabbit RBC (Table 1). After confirming the high agglutinable RBC, the sugar inhibition assays were conducted with different sugars. Among the tested sugars the chito-oligomers were found best inhibiting sugars (Table 2). Within the tested chito-oligomers the chitopentose was found best inhibitor and the agglutination of the lectin is only inhibited by oligomers of *N*-acetylglucosamine in the following order of potency: pentasaccharide > tetrasaccharide > trisaccharide > hexasaccharide > disaccharide (Table 3). Simple sugars and glycoproteins did not inhibit the lectin activity suggesting the specificity of the lectin towards the oligomers of *N*-acetylglucosamine. The specific inhibition of hemagglutination by oligomers of N-acetylglucoseamine was the basis for using chitin column as the common affinity matrix for the purification of this monocot lectin. Hence, the chitin affinity chromatography used for the isolation of the lectin from *Aponogeton natans* tuber.

Table 1: Erythrocyte specificity studies of crude protein

Erythrocytes	Specific activity (U/mg)
A+	2
B+	2
AB+	32
O+	8
Rabbit	524590
Rat	2038

* Specific activity is expressed as titre, the reciprocal of maximal dilution of protein that gives visible agglutination with 2% Rabbit erythrocytes.

Table 2: Sugar Inhibition assay with different sugars and oligosaccharides

Sl no	SUGARS	Initial concentration	Final concentration
1	D- GLUCOSAMINE	250mM	X
2	L- FUCOSE	250mM	X
3	β- LACTOSE	250mM	X
4	MANNOSE	250mM	X
5	MANNITOL	250mM	X
6	N-ACETYL GALACTOSAMINE	250mM	X
7	PECTIN	250mM	X
8	D- SORBITOL	250mM	X
9	MALTOSE	250mM	X
10	SUCROSE	250mM	X
11	THYROGLOBIN	250mM	X
12	GLUCOSE	250mM	X
13	FRUCTOSE	250mM	X
14	CELLOBIOSE	250mM	X
15	ARABINOSE	250mM	X
16	MELLIBIOSE	250mM	X
17	FETUIN	250mM	X
18	RAFFINOSE	250mM	X
19	α- LACTOSE	250mM	X
20	LACTOSE	250mM	X
21	GALACTOSE	250mM	X
22	N-ACETYL GLUCOSAMINE	250mM	X
23	CHITOBIOSE	2mM	0.250mM
24	CHITOTRIOSE	2mM	0.065mM
25	CHITOTETROSE	2mM	0.065mM
25	CHITOPENTOSE	2mM	0.015mM
26	CHITOHEXOSE	2mM	0.125mM

X – No inhibition

Experiment was performed with 2% erythrocytes with a final lectin concentration of 10 μg/mL. Only chito-oligosaccharides inhibited the agglutination.

Table 3: Relative Inhibitory potential among tested chitin Oligosaccharides

Sugar	Minimum Concentration for inhibition (mM)	Relative Inhibitory potency (Chitohexose =1.0)
Chitobiose	**0.250**	**0.5**
Chitotriose	**0.065**	**2.0**
Chitotetrose	**0.065**	**2.0**
Chitopentose	**0.015**	**8.3**
Chitohexose	**0.125**	**1.0**

The *Aponogeton natans* tuber lectin (ANTL) was eluted from the column in a single step with 4-fold purification, as a single symmetrical peak with 70.3% yield (Fig 1 & Table 4).

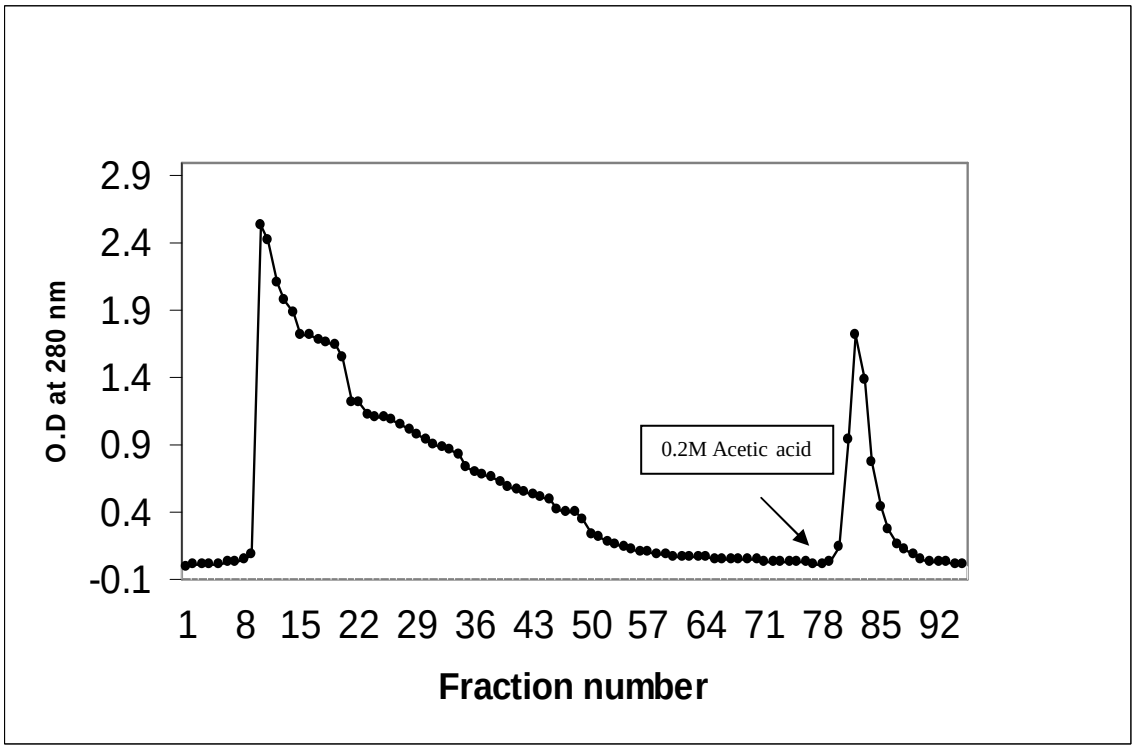


Fig 1: Affinity chromatography of *Aponogeton natans* on chitin Column. The column was equilibrated with 10 mM PBS and 42 mg of protein applied. The bound proteins are eluted with 0.2 M acetic acid at the flow rate of 18 ml/hr. Fractions of 3ml were collected and protein concentration was recorded at 280nm.

Table 4: Purification table of ANTL

Purification step	Protein (mg)	Specific activity* (U/mg)	Total activity (titre x mg)	Yield (%)	Purification factor
Crude	104	524590	54557360	100	1
Purified protein	18	2133333	38399994	70.3	4

* Specific activity is expressed as titre, the reciprocal of maximal dilution of protein that gives visible agglutination with 2% Rabbit erythrocytes.

The total protein content was found to be 2.8 mg/ml, 0.32 mg/ml in crude extract and chitin binding fractions respectively. The lectin amounts to about 11.4% of the total protein of the tubers. The ANTL has resolved into a single band in the native PAGE confirming the purity of the eluted lectin (Fig 2). On SDS-PAGE, ANTL resolved into three bands corresponding to 33,000 Da, 15,000 Da and 14,000 Da in the presence of β-mercaptoethanol (Fig 3). The glycoprotein nature of the lectin is confirmed by periodic acid staining of the gel (Fig 4). The result is consistent with the bands resolved on SDS-PAGE in the presence of β-mercaptoethanol. The molecular weight of the pure protein is found to be 66,000 Da by gel filtration chromatography (Fig 5). The carbohydrate content is found to be 8.2% of the total protein. The lectin agglutinated Red Blood Cells (RBCs) from human, rat and rabbit. It was found to be specific for different human blood groups viz. $A^{(+ve)}$, $B^{(+ve)}$, $O^{(+ve),}$ $AB^{(+ve)}$, rabbit and rat. The specific activity of the lectin is high towards rabbit RBCs 2,133,333 U/mg which is much higher than all known monocot lectins (Table 5). The susceptibility of rabbit erythrocytes and refractory nature of other blood types to agglutination indicated the larger availability of the lectin receptors on the

former RBCs and their minimal presence on the latter. Trypsinization did not alter the results of agglutination indicating that there is no unmasking of receptors on trypsin treatment. The results of heat denaturation showed that ANTL was stable up to 60°C for 10 min and lost its complete activity upon heating at 90°C for 10 min and showed optimum hemagglutination activity at pH 6 (table 6 & 7).

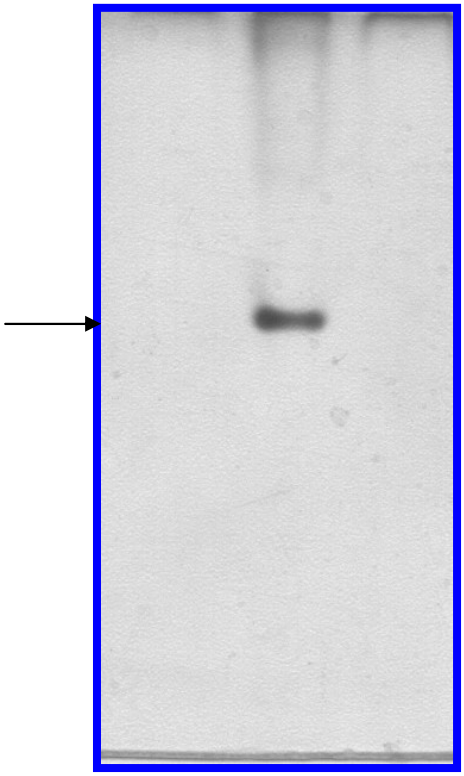


Fig 2: 10% Native PAGE gel of purified lectin eluted from the chitin column. The arrow indicates the position of the single band which shows the homogeneity of the protein

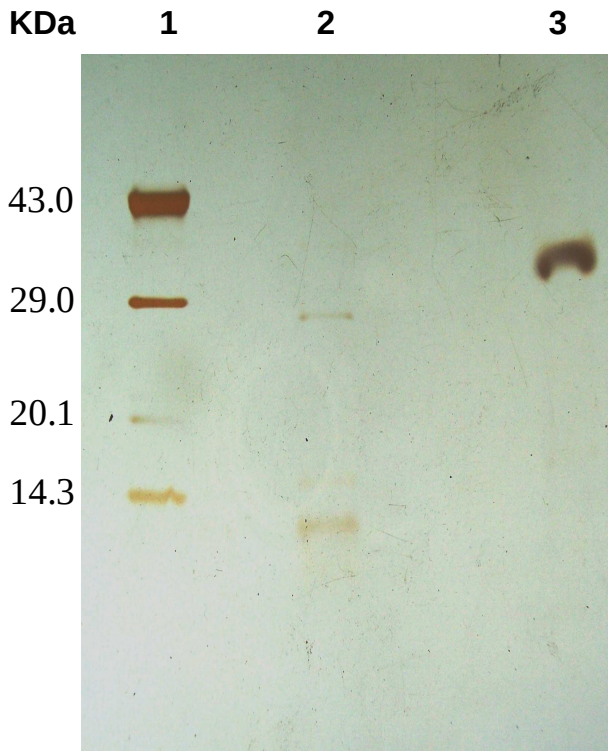


Fig 3: SDS- PAGE analysis of purified protein. Lane 1: Molecular Wt Markers Lane 2: With β-Mercaptoethanol; Lane 3: Without β-Mercaptoethanol. ANTL has resolved into three bands corresponding to 33 KDa, 15 KDa and 14 KDa in the presence of β-mercaptoethanol.

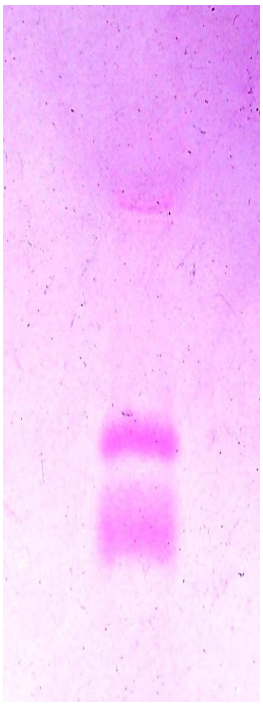


Fig 4: Periodic acid schiff's (PAS) Staining of purified lectin. The bands detected by the PAS which stains glucocompounds are in agreement with the bands obtained on SDS-PAGE with β-Mercaptoethanol

A) B)

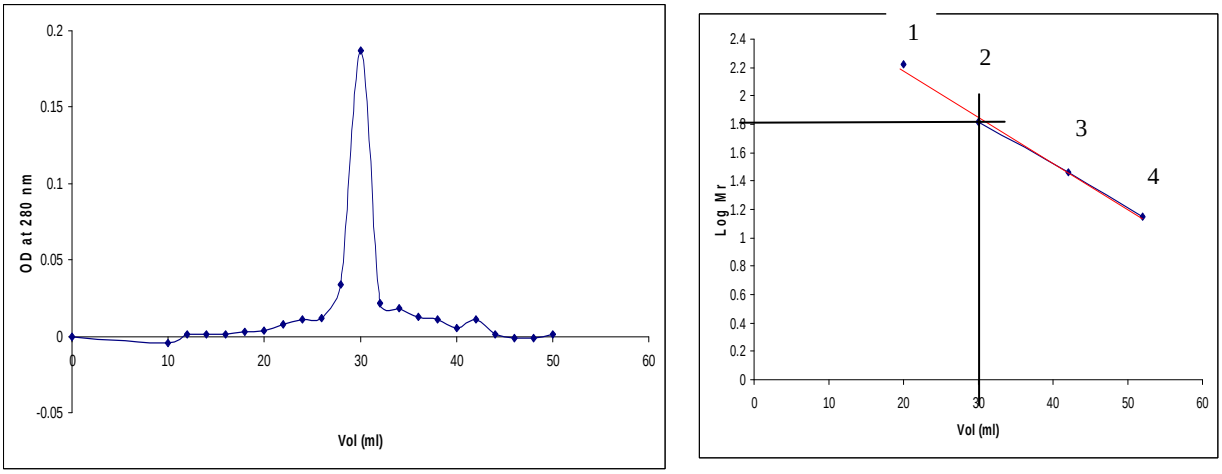


Fig 5: Molecular weight determination by gel filtration (G-200). A) Elution profile of the protein on gel filtration column. B) Shows the proteins of known Molecular weight markers used 1- Phosphorylase b (MW 90 KDa); 2- Bovine serum albumin (MW 66 KDa); 3- Ovalbumin (MW 24 KDa); 4- Lysozyme (MW 14 KDa). Intersection indicates the position of the purified protein (MW 66 KDa) that elutes at the same elution volume as Bovine serum albumin.

Table 5: Erythrocyte specificity studies of purified lectin (ANTL)

Erythrocytes	Specific activity (U/mg)
A+	8
B+	8
AB+	128
O+	32
Rabbit	2133333
Rat	8195

Table 6: Temperature optima studies with rabbit erythrocytes

Temperature (ºC)	Specific activity (units/mg)
30	**2133333**
40	**2133333**
50	**522448**
60	**8195**
70	**32**
80	**16**
90	**8**
100	**0**

Table 7: pH optima studies with rabbit erythrocytes

pH	Specific activity (units/mg)
4	522448
5	2133333
6	2133333
7	522448
8	2051
9	2051
10	2051

The low thermal stability of the lectins is in consonance with the earlier observations about the absence of disulphide bridges and may also be attributed to the low carbohydrate content in the lectin. The lectin was affected by denaturing agents such as Lithium chloride (2M), Potassium ferricyanide (2M), Guanidium hydrochloride (2M), Urea (2M) and Per Iodic acid (2M). As the concentration of the denaturants increased, there is decrease in the agglutination activity (Table 8).

Table 8: Effect of denaturing agents with rabbit erythrocytes

Denaturing agents (2M)	Specific activity (units/mg)
Urea	128
Guanidium chloride	2051
Lithium chloride	2051
Potassium ferricyanide	492
Periodic acid	8195

This decrease in lectin activity may be due to the disruptive effect of the denaturants on hydrogen bonding and hydrophobic interactions which stabilize the three dimensional structure. ANTL does not require metal ions for hemagglutination activity as the activity of the lectin had no effect on the addition of EDTA up to 30 mM (Table 9). Similar behavior has also been observed in the case of members of *Amaryllidaceae* and other monocot lectins with respect to thermal stability, treatment with denaturants and non-requirement of divalent metal ions for their activity.

Table 9: Effect of EDTA with rabbit erythrocytes

EDTA	Specific activity (units/mg)
10mM	2133333
20mM	2133333
30mM	2133333

The cytotoxic effect of ANTL was determined over a range of concentration of (20–100 µg/ml). The cytotoxic studies of the ANTL on insect cell lines (sf9 cells) have revealed that there is no effect of the protein on these cell lines till the concentration of 100µg/ml after 72 hours of incubation (Table 10). Two mammalian cell lines (SUP T1 and U266) were tested for the cytotoxic effect of the lectin. After 72 hrs of incubation SUP T1 cell lines has shown no significant decrease in the proliferation of the cells whereas U266, at the concentration of 50 µg/ml has shown ~50 % inhibition in the proliferation as compared to their respective controls (Table 11 & 12). The variation of proliferation inhibition on different cell-lines may be due to the presence of glycoconjugates varying slightly in their activity, thus leading to different signaling action of lectins. As every lectin has unique fine sugar specificity, there is a need to check a range of lectins against a number of cancer cell-lines. The exact molecular mechanism(s) of the anti-Proliferative effect of plant lectins is not clear at present, although several hypotheses have been put forward which suggests that this effect is associated with the ability of lectins to modulate the growth, differentiation, proliferation, and apoptosis of premature cells in vivo and in vitro. Additional studies are required to understand the exact mechanisms of the antiproliferative effect of plant lectins and future examinations should be focused on the examination of these possibilities in appropriate models of human diseases.

Table 10: Proliferative response of Sf_9 cell lines to ANTL

Conc. of extract (µg/well)	OD at 570-630 nm	% Proliferation
0	0.654 ± 0.02	100
1	0.614± 0.05	94
5	0.601 ± 0.03	92
10	0.629 ± 0.06	97
20	0.617 ± 0.04	94

Values represented are Mean ± SEM of three experiments
Final volume in the well is 200µl.

Sf_9 (*Spodoptera frugiferda*) cell lines which serve as hosts for AcNPV was used for the cytotoxicity study. After 72 hrs of incubation there is no significant decrease in the proliferation as compared to control (without ANTL) till 20 µg/well concentration.

Table 11: Proliferative response of U266 cell lines to ANTL

Conc. of extract (µg/well)	OD at 570-630 nm	% Proliferation
0	0.607 ±0.0 2	100
1	0.422 ± 0.03	69
5	0.311 ± 0.01	51
10	0.238 ± 0.02	39
20	0.207 ± 0.002	34

Values represented are Mean ± SEM of three experiments
Final volume in the well is 200µl.

It is a Myeloma cell line. The seeding ratio of the cells was 0.2 x 10^6 /ml. Medium was renewed 2 to 3 times per week. After 72 hrs of incubation there is 50% decrease in the proliferation as compared to control (without ANTL) at 5µg/well concentration.

Table 12: Proliferative response of SUP-T_1 cell line to ANTL

Conc. of extract (µg/well)	OD at 570-630 nm	% Proliferation
0	0.848 ±0.002	100
1	0.821 ± 0.04	97
5	0.795 ± 0.03	93
10	0.788 ± 0.03	92
20	0.787 ± 0.02	92

Values represented are Mean ± SEM of three experiments
Final volume in the well is 200µl.

It is a Non-Hodgkins T-Cell lymphoma cell line The seeding ratio of the cells was 0.2 x 10^6 /ml. Medium was renewed 2 to 3 times per week. After 72 hrs of incubation there is no significant decrease in the proliferation as compared to control (without ANTL) till 20 µg/well concentration.

As many of the lectins from *Euphorbiaceae*, *Leguminosae and Gramineae* serve as potent mitogens in normal splenic lymphocytes, ANTL was checked for the mitogenic activity in normal murine and human splenic lymphocytes. ANTL showed potent mitogenic response towards murine and human peripheral blood mononuclear cells as evidenced by lymph proliferation of the lectin into the cultures. The relative mitogenic stimulation of ANTL towards murine and human lymphocytes was almost double than that of Con A, a well-known standard plant mitogen. The optimum proliferation dose of ANTL was 5 µg/ml both in case of murine and human lymphocytes (Table 13 & 14).

Table 13: Proliferative response of murine lymphocytes to ANTL

Conc. of extract (µg/well)	^{3}H-thymidine incorporated into DNA (cpm/10^{6} cells)
0	**2893 ± 214**
1	**140343 ± 5562**
5	**94481 ± 5114**
10	**65216 ± 1274**
20	**49601 ± 537**

Values represented are Mean ± SEM of three experiments
Final volume in the well is 200µl.

Table 14: Proliferative response of human lymphocytes to ANTL

Conc. of extract (µg/well)	^{3}H-thymidine incorporated into DNA (cpm/10^{6} cells)
0	**4030 ± 230**
1	**94940 ± 5235**
5	**112080 ± 6820**
10	**75224 ± 1584**
20	**36908 ± 322**

Values represented are Mean ± SEM of three experiments
Final volume in the well is 200µl.

The optimum dose is required for mitogenic studies as there is inhibition of mitogenesis at higher lectin concentrations, for example the toxic action by supraoptimal concentrations of Con A has been reported to cause a decrease in mitogenic response (jagmohan singh et.al., 2005) . Thus mitogenic lectins can be of significant use in increased understanding of the relationship between chromosomal abnormality and human diseases, which will tremendously help the diagnosis. Besides other cells, lymphocytes have been the usual target cells for mitogenic assays, and the study of lectin–lymphocyte interaction can result in substantial contribution of elucidating the mechanism of lymphocyte activation and its control, thereby contributing to our understanding of cell growth and development. The mitogenic response of murine and human lymphocytes by ANTL was inhibited in a concentration dependent manner in the presence of chitohexose (figure not shown). The inhibition of hemagglutination and mitogenicity of the lectin in the presence of chitohexose suggest that lectin is responsible for these properties by binding to the cell membrane via receptor site(s) on the lectin, which is recognized by chitohexose like structure on the cells. The serially increasing concentrations of chitohexose proportionately decreased the available sugar binding sites on lectin molecules causing reduced binding of lectin to the murine and human lymphocytes. FITC-conjugated lectin studies revealed that the lectin is bound to all the cells but the exact mechanism with which it is inducing responses in some cell lines is not clearly understood (Fig 6 & 7).

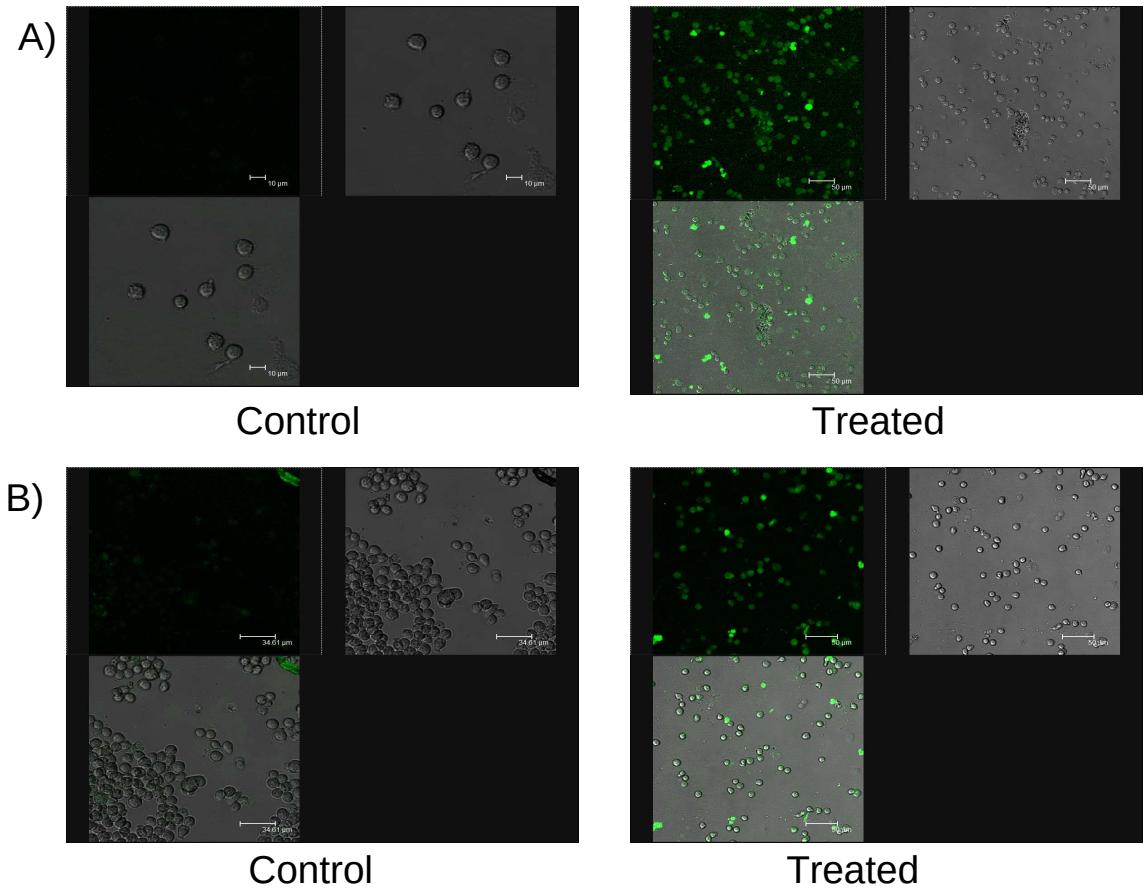


Fig 6: Interaction of FITC-conjugated ANTL with Human Cancer Cells A) SUP-T_1 T-cell lymphoma B) U266 Myolema cell lines. The cells (U266 & SUP T1 cells) (0.1 ml, 5 x 10^6) were incubated with FITC (fluoroscein isothicyanate) conjugated to ANTL (0.1 ml) for 15 min at room temperature. The percentage of florescent cells was determined for the two sets of lymphocytes under Confocal Scanning Electron Microscope.

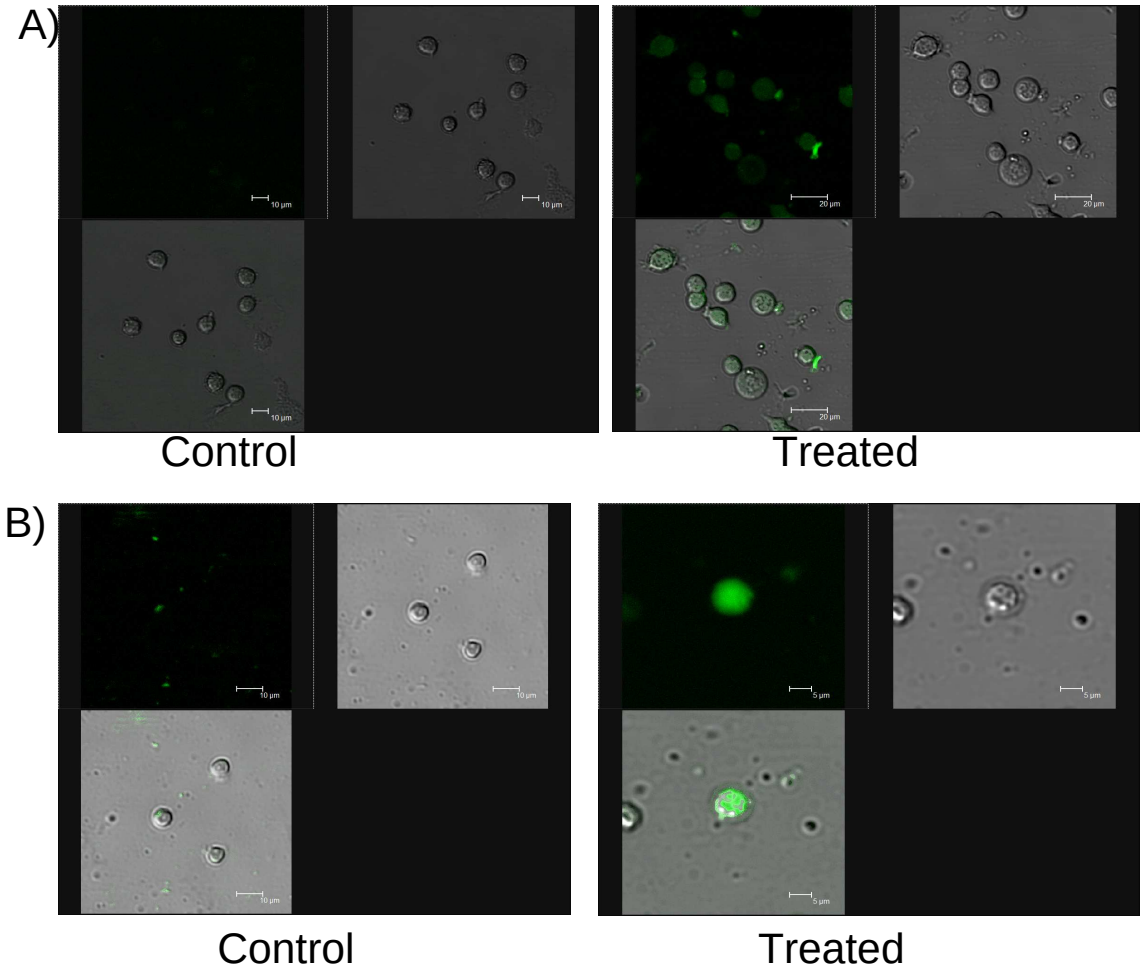


Fig 7: Interaction of FITC-conjugated ANTL with normal A) Murine splenic lymphocytes B) Human peripheral blood lymphocytes. The normal murine splenic, human peripheral blood lymphocytes (0.1 ml, 5 x 10^6) were incubated with FITC (fluoroscein isothicyanate) conjugated to ANTL (0.1 ml) for 15 min at room temperature. The percentage of florescent cells was determined for the two sets of lymphocytes under Confocal Scanning Electron Microscope.

Fluorescence and circular dichroism spectroscopic studies were carried out for ANTL. Excitation wavelength was fixed at 290 nm to selectively excite tryptophan residues and the emission spectrum was recorded in the range of 310 to 450 nm. The concentration of the sample was 0.32 mg/ml. The spectra were obtained after incubating the samples in pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 (20mM sodium acetate/phosphate and Tris-HCl for 40 min (Fig 8)

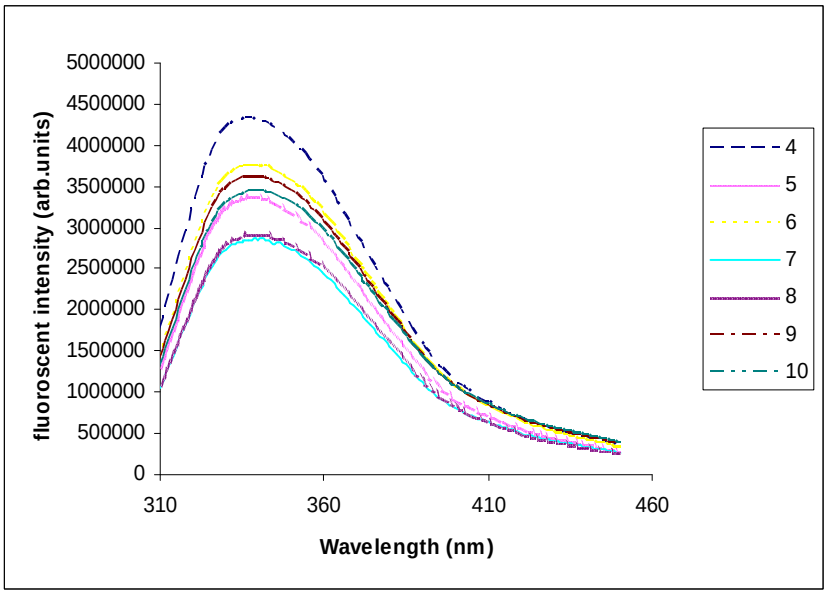


Fig 8: Steady state tryptophan fluorescence spectroscopy. ANTL(0.32 mg/ml) was incubated at different pH for 40 min and emission spectra was recorded in the range of 310-450 nm fixing excitation at 290 nm.

As the pH from 7 was increased or decreased there is an increase in the fluorescence intensity. This could be due to the conformational changes in the structure of the protein and the subsequent exposure/closure of buried tryptophan (Trp) residues. The intrinsic inflorescence of the ANTL was quenched by a neutral quencher (acrylamide), an anionic quencher (iodide ion) and a cationic quencher (cesium ion) in the native, denaturing and ligand bound conditions to investigate the microenvironment of the tryptophan residues (Fig 9, 10 & 11).

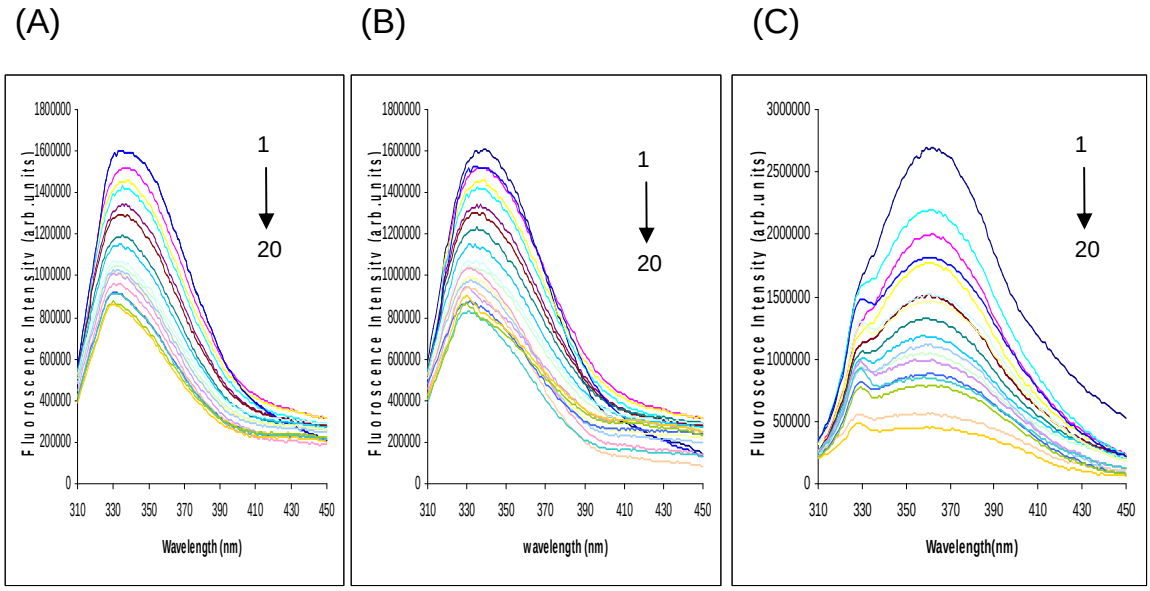


Fig 9: Fluorescence spectra of ANTL in the absence and in the presence of Acrylamide (A) Under native conditions; (B) under sugar bound conditions (C) under denaturing conditions (6MGdn–HCl). Spectrum 1 corresponds to the lectin alone and spectra 2–20 correspond to the lectin in the presence of increasing concentrations of acrylamide. The final concentration of the quencher in both A, B and C is 0.5 M.

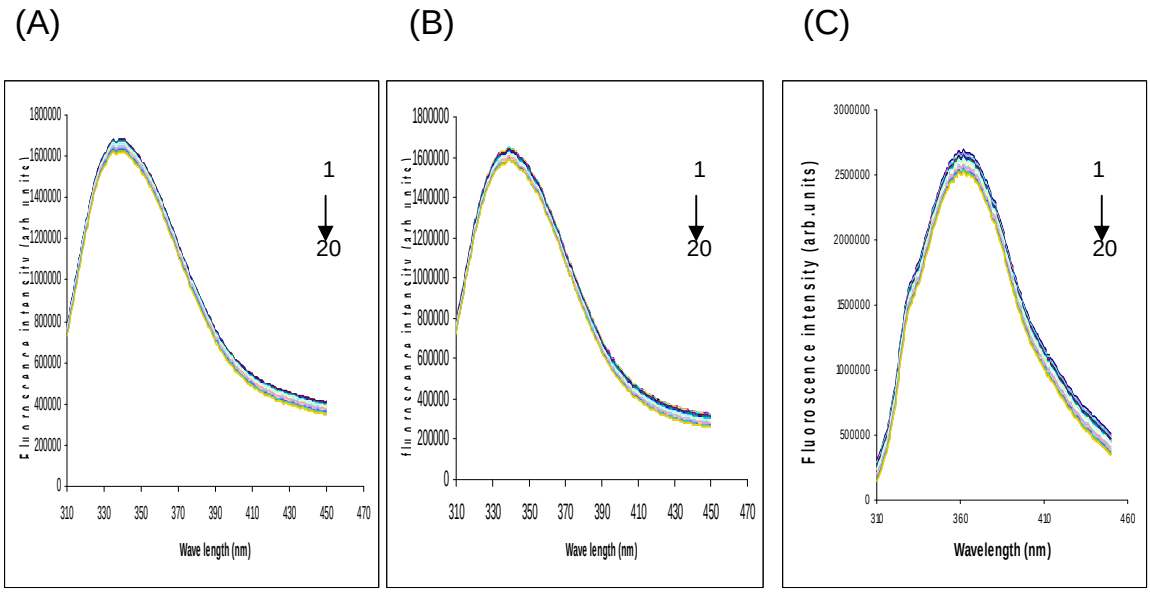


Fig 10: Fluorescence spectra of ANTL in the absence and in the presence of Cesium (A) Under native conditions; (B) under sugar bound conditions (C) under denaturing conditions (6MGdn–HCl). Spectrum 1 corresponds to the lectin alone and spectra 2–20 correspond to the lectin in the presence of increasing concentrations of acrylamide. The final concentration of the quencher in both A, B and C is 0.5 M.

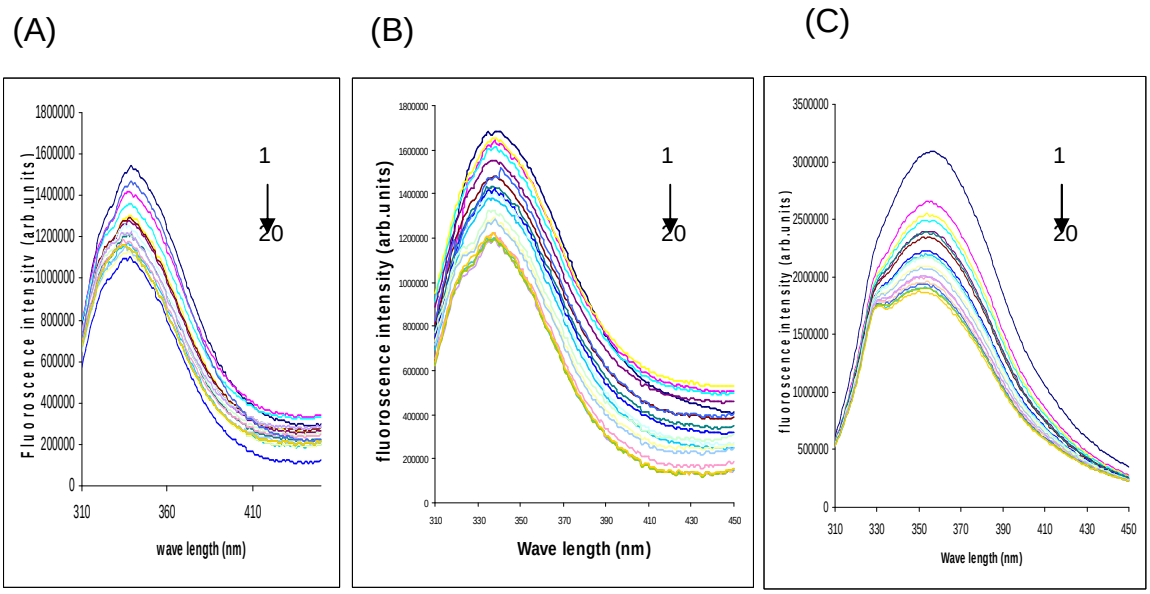


Fig 11: Fluorescence spectra of ANTL in the absence and in the presence of Iodide (A) Under native conditions; (B) under sugar bound conditions (C) under denaturing conditions (6MGdn–HCl). Spectrum 1 corresponds to the lectin alone and spectra 2–20 correspond to the lectin in the presence of increasing concentrations of acrylamide. The final concentration of the quencher in both A, B and C is 0.5 M.

The most effective quenching is brought about by acrylamide with 46.9 % quenching of the total fluorescence of the native protein at a quencher concentration of 0.5 M. I^- and Cs+ could quench only 26.6 % and 2.5 % respectively, at the same concentration. Low quenching of the charged quenchers indicates that most of the fluorescent tryptophan residues in ANTL are buried in the hydrophobic core of the protein and are in a significantly non-polar environment. This is also consistent with the emission maximum of the protein at 334 nm, which is indicative of a significantly non polar environment around the indole side chains of Trp residues. The very low quenching of Cs^+ with the charged ions appears to be due to the inability of this quencher to access the fluorophores which indicates the presence of positively charged residues in the vicinity of some of the exposed/partially exposed tryptophan residues, that repel the positively charged cesium ion, but allow the neutral acrylamide and the negatively charged iodide ion to approach the indole moieties of the tryptophan residues in their neighborhood. As the highest quenching is observed with acrylamide which has larger ionic radius than cesium (1.88 Å) and iodide (2.2 Å) ions the size of the quenchers doesn't seem to be playing a role in effective quenching whereas Cs^+ which is the smallest of the three quenchers, exhibited the lowest extent of quenching. Presence of positively charged residues near tryptophan residues was also suggested for several cucurbitaceae seed lectins based on the results from fluorescence quenching studies with neutral and ionic quenchers [Sultan *et al*., 2005; Komath *et al*., 1999; Kenoth *et al*., 2003]. Besides, the inherently low quenching efficiency of Cs^+ may also suggest the positively charged residues in the vicinity are partly responsible for the lower quenching observed with it. . In the primary structure of the polypeptide chain it appears that the charged residues are proximal to the tryptophan residues by virtue of their presence near the Trp residues as complete unfolding of the polypeptide chains of the protein does not

seem to render all the tryptophan residues accessible to these two quenchers. The red shift in the fluorescence spectrum upon denaturation of the lectin with Gdn. HCl indicates that the unfolding results in a significant increase in the exposure of the tryptophan residues to the aqueous environment. Also the accessibility of the tryptophan residues to the quenchers (Acrylamide, I-, Cs+) increases upon denaturation by exposing their Trp residues. Denaturation with 6 M Gdn-HCl results in the increased quenching percentage to 84.7 %, 47.5 % and 7.1 % with acrylamide, iodide ion and cesium ion, respectively which are in excellent agreement with the unfolding of the protein (Table 15).

Table 15: Fluoroscence quenching obtained with different quenchers

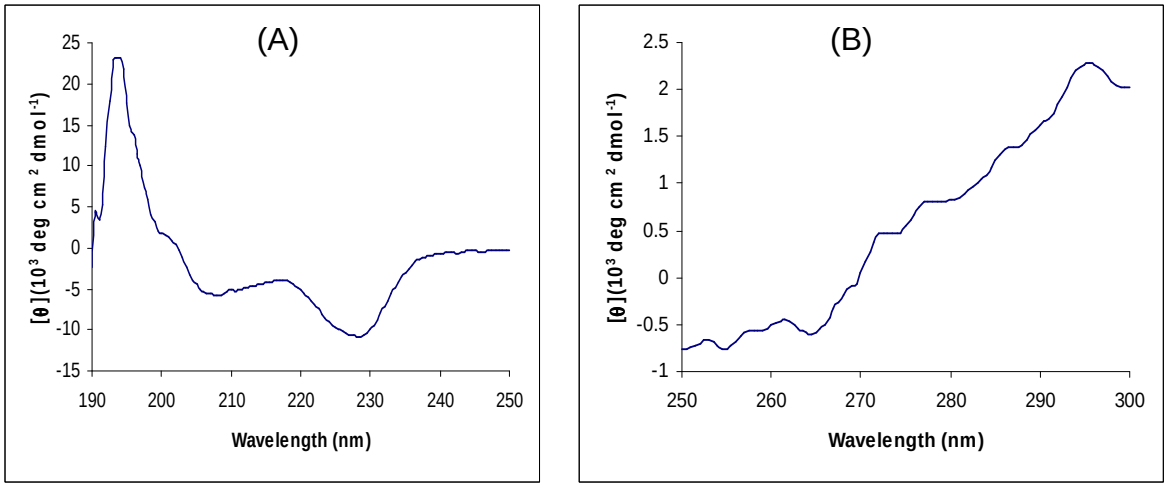
	Quenching %		
Quencher	**Native**	**With 400 µM chitohexose**	**In 6M Gdn-HCl**
Acrylamide	**46.9 (±0.7)**	**47.4 (±1.4)**	**84.7 (±0.3)**
Iodide ion	**26.6 (±1.5)**	**28.4 (±1.3)**	**41.5 (±0.5)**
Cesium ion	**2.5 (±0.5)**	**3.1 (±0.8)**	**7.1 (±0.1)**

The final quencher concentration in each case was 0.5 M. Values are averages from three independent experiments with the estimated errors given in parentheses. Quenching is found to be effective with Acrylamide(neutral quencher) followed by iodide and cesium ions(negatively and positively charged quenchers) suggesting that tryptophan residues in ANTL are buried in the hydrophobic core of the protein and are in a significantly non-polar environment

The far UV CD spectrum of ANTL revealed that the secondary structure of the lectin is helical consisting of 53% α-helix, 21% β-sheet, 9% β-turns and 16% unordered structures as analyzed by CDSSTR. Thermal unfolding of ANTL investigated by monitoring CD signals, showed a sharp transition around 70 °C both in the far UV region (208 nm) and the near UV region (280 nm). Binding of the ligand (Chitohexose) to the protein probably leads to a slight increase in the accessibility of some of the residues to

acrylamide by tightening the protein structure a bit; some of the residues that are partially accessible to I^- become more exposed, resulting in a somewhat higher extent of quenching being observed with it (Fig 12). The results of CD spectral studies indicated that the secondary and tertiary structures of ANTL are not significantly altered by ligand binding which are consistant with the fluorescence studies. The far UV CD spectrum of native ANTL, exhibits two minima centered around 208 nm and 228 nm, suggesting the presence of helical structure along with other secondary structural elements. Ligand binding induces insignificant changes in the secondary and tertiary structures of the protein as the spectra obtained in the presence of ligand is nearly indistinguishable from that of native protein alone. For a detailed quantitative analysis and specific assessment of the secondary and teritiary structure of the protein the routines available with DICHROWEB have been used. The near UV CD spectrum of ANTL is characterized by the presence of a maximum around 280 nm and several peaks of somewhat lesser intensity between 297 nm and 275 nm. These features are likely to arise due to contributions from the side chains of tryptophan and tyrosine residues, which absorb in the 270– 300 nm region. Here as well, ligand binding does not seem to perturb these signals to any detectable level. The unfolding temperature of ~70 °C indicates that ANTL is a rather stable protein (Fig 13 & 14). Most lectins, particularly seeds and tubers investigated so far have been found to be generally stable to thermal denaturation with the midpoint of transition being found between 56 and 92 °C for different lectins [Srinivas *et al*., 2001]. However, lectins were found to adopt different unfolding mechanisms. For example, the tetrameric Con A undergoes a two-state unfolding transition (folded tetramer↔unfolded monomer) with the midpoint of the transition at 87–92 °C, whereas peanut agglutinin, another tetrameric legume lectin exhibits a more complex unfolding process, with two separate transitions with midpoints at 56–61 °C (corresponding to

dissociation of the tetramer into monomers) and 63 °C (corresponding to unfolding of the monomers), respectively [Schwartz *et al*., 1993; Reddy *et al*., 1999]. Although the studies reported here appear to suggest that thermal unfolding of ANTL involves a two-state transition as found with Con A, further experiments are required to draw firm conclusions on this.



(C)

Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
0.39	0.14	0.11	0.10	0.09	0.16	0.99

Fig 12: Circular dichroic spectra of ANTL (A) Far UV region. (B) Near UV region. The spectra were recorded at 25 °C. C) The far U.V spectrum analyzed by using the CDSSTR program is shown below. Helix 1 and Helix 2 corresponds to regular and distorted α helical structures, Strand 1 & Strand 2 corresponds to regular and distorted β sheet structures. CD spectral studies indicate that ANTL is predominantly helical with higher content of α-helix (53 %) than β- sheet (21 %), β-turns (9%) and unordered structures (16%).

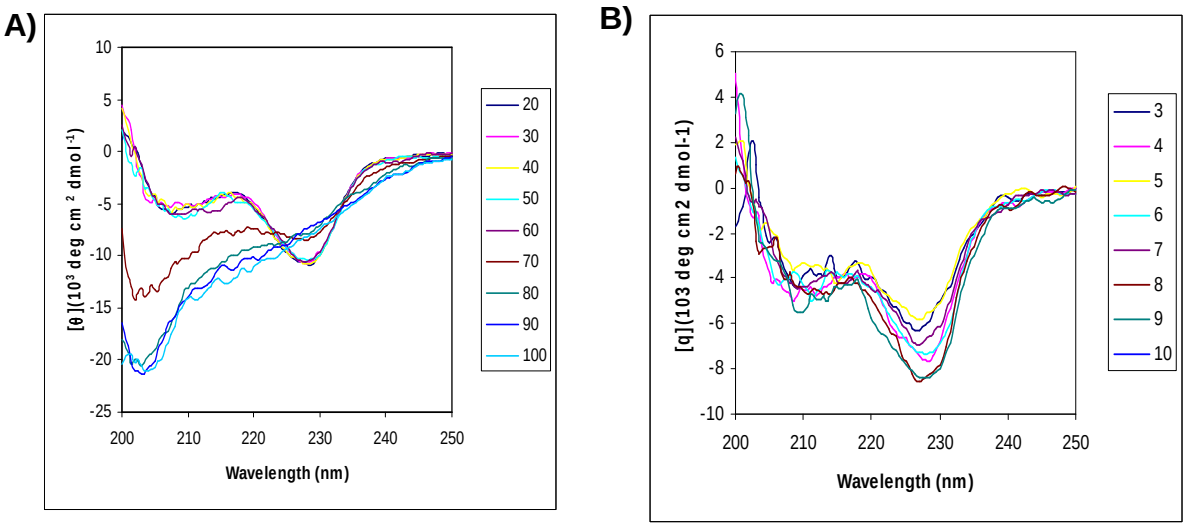


Fig 13: Effect of temperature and pH on secondary structure of ANTL

A) At different temperatures

The ANTL lectin (5 mg/ml) was gradually heated in 10 ºC from 20 -100 ºC using a circulating water peltier. At each temperature the lectin was incubated for 5 min and the spectrum was recorded. Secondary structure of ANTL is stable up to 60 ºC. On increasing the temperature beyond 60 ºC there is a gradual decrease in the ellipticity at 208nm

B) At different pH

The ANTL lectin (5 mg/ml) was dissolved in and incubated in the buffers of different pHs 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 (20mM sodium acetate/phosphate, Tris-HCl) and the spectra was obtained after 40 min.

As the pH from 7 increased or decreased there are slight conformational changes observed at 208 nm.

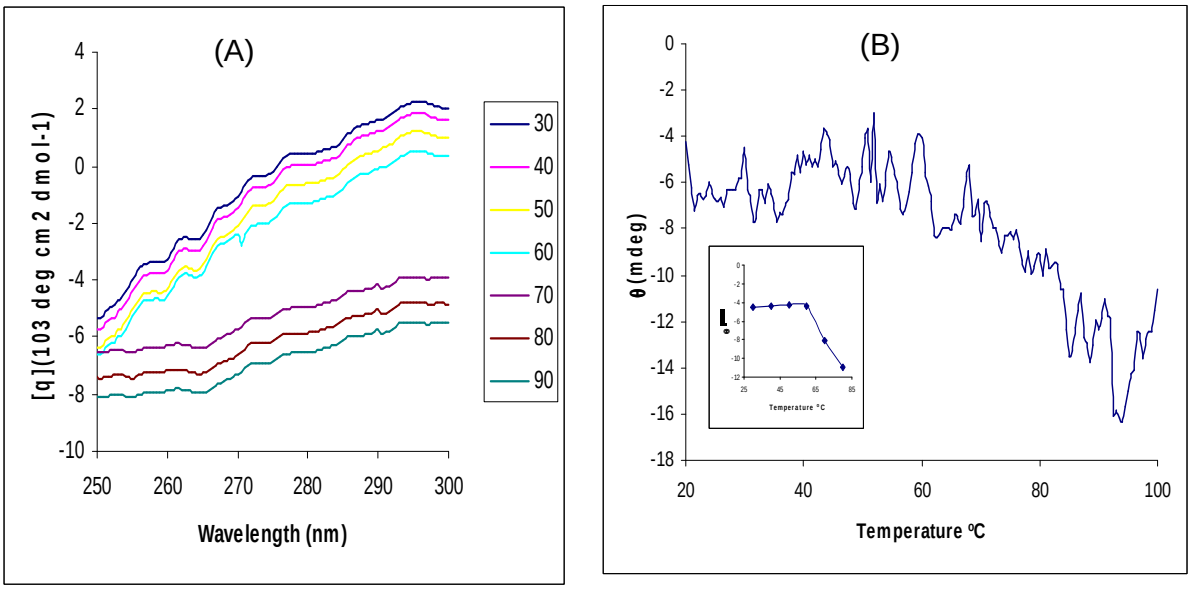
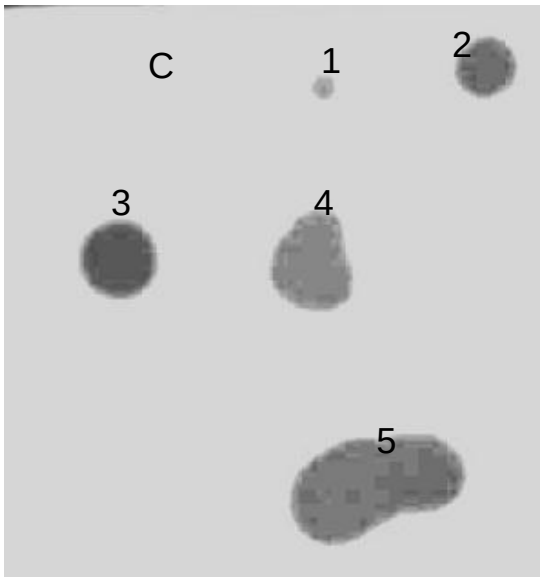


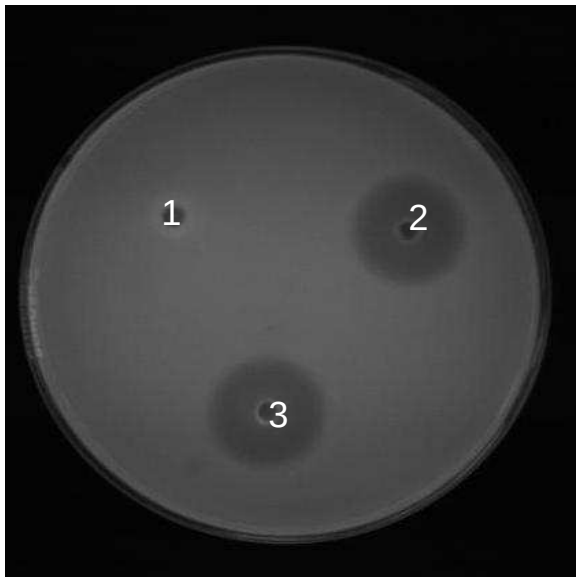
Fig 14: Effect of temperature on tertiary structure of ANTL

***A*) Near UV CD spectra of ANTL at different temperatures**
Spectra were recorded at 30, 40, 50, 60, 70, 80 and 90 °C, respectively.

B) Thermal unfolding of ANTL monitored by CD spectroscopy
The ellipticity (Θ) in the near UV region of the native protein was monitored at 280 nm as a function of temperature. The sharp decrease in the ellipticity around 70 °C indicates the transition from folded structure to the unfolded state. Inset shows the ellipticity change in the far UV region (208 nm) versus temperature, which also indicates a transition centered around 70 °C.

The isolated lectin possessed the chitinase activity, which was confirmed by the plate gel diffusion assay method (Fig 15). Ranipal is checked as a staining reagent which is less expensive than calcoflour white which is already established by the lab. Ranipal as Calcofluor white M2R binds to the glucan chains and linear b- (1, 4)-glucosidically linked units of *N*-acetylglucosamine. On binding to polysaccharide such as cellulose and chitin, this flourochrome highlights and emits a light blue light when exposed to UV. On degradation of this polymer to its individual subunits, this fluorescence is lost as indicated by a dark band against a fluorescent background (Fig 16).





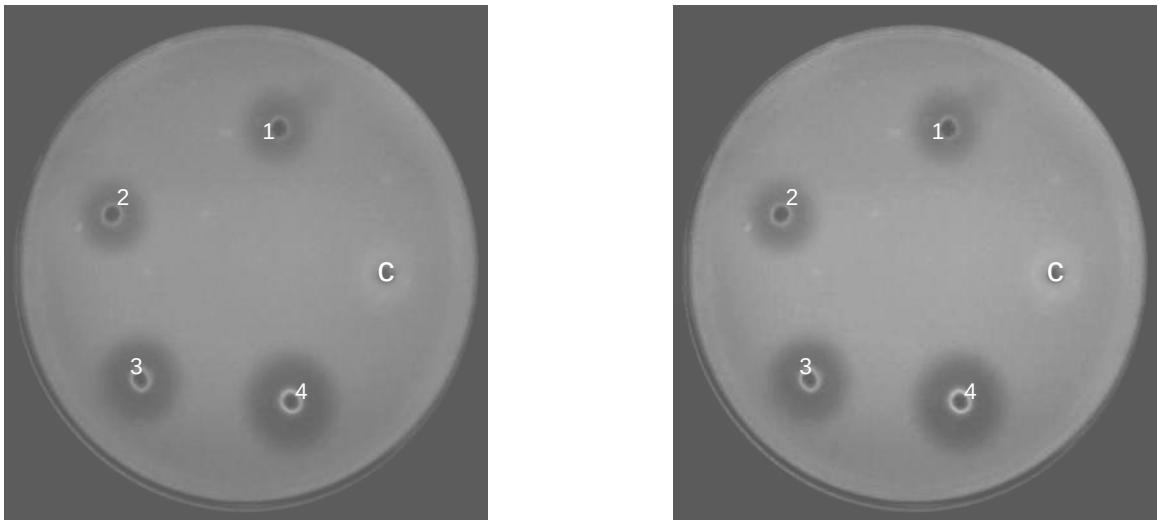
C-Control (10mM PBS); 1-1µg; 2-5µg; 3-10µg; 4-20µg; 5-40µg

1-Control (10mM PBS); 2 & 3- 20µg

Fig 15: Plate gel diffusion assay: Agarose gel is polymerized with 0.01% of glycol chitin incorporated into it. Wells are bored into the gel with the help of a cork borer and various concentrations of ANTL is loaded in to the well and incubated overnight. Gel is stained with 0.1% ranipal for 15 minutes followed by gently washing with distilled water. Zone of clearance appeared due to the solubilization of glycol chitin could be observed under uv-transilluminator.

CHITINASE ASSAY STAINED WITH CALCOFLOURWHITE

CHITINASE ASSAY STAINED WITH RANIPAL



C-Control (10mM PBS); 1-1µg;
2-5µg; 3-10µg; 4-20µg; 5-40µg

Fig 16: Different staining methods
A simple, rapid and inexpensive method to detect the chitinase activity within the gels is developed in the laboratory as an alternative for the earlier calcoflour white stain. The use of ranipal as an alternative staining agent for the detection of chitinases does not comprises with the sensitivity. There is no difference in the limits of detection between the two staining agent's calcoflour white and ranipal for the detection of ANTL.

Trudel and Asselin (1989) have developed an activity staining method by incorporating a soluble glycol chitin in electrophoresis gel. Lytic zone was observed by UV illumination with a transilluminator after staining with calcofluor white M2R. In this method, when substrate was directly incorporated into gel the bands showed a smear instead of well-defined band. We observed retardation of mobility of enzymes in the gel during the electrophoresis, which may be because of the presence of polysaccharide in the gel, which can be overcome by solid plate method. Native and Semi-native (β-mercaptoethanol is not present in the sample) gel overlays showed good activity and confirmed the chitinase activity in the PAGE gels as well (Fig 17). Kinetics studies of ANTL have revealed that the optimum concentration of the enzyme is 100 µg/ml and that of the substrate is 1 mg/ml the enzyme has shown an exponential activity till 48 hr and gradually became stable by 96 hour (Fig 18 & 19). The optimum temperature for the chitinase activity is found to be 40 ºC but gradually decreased with rise in the temperature and completely lost its activity after 70 ºC and the optimum p^H for the chitinase activity is found to be at 6 (Fig 20). These results are in excellent agreement with the hemagglutination and CD spectral studies.

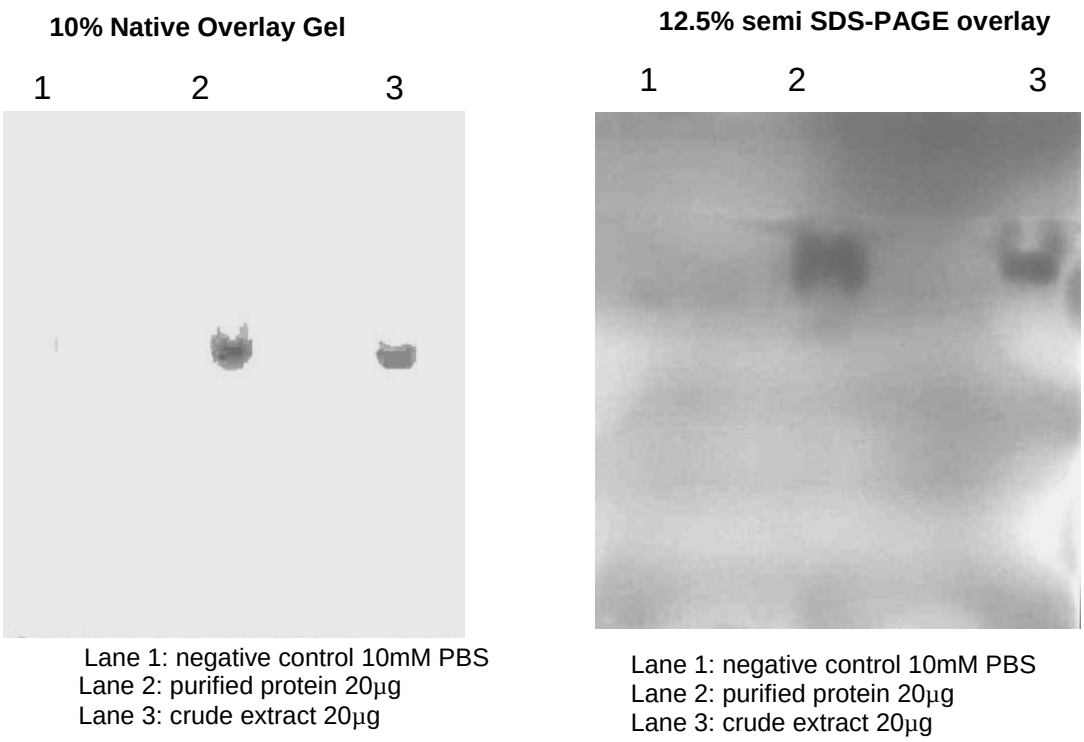
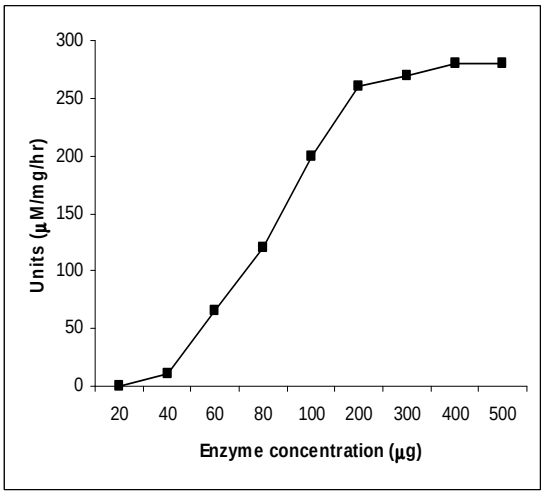


Fig 17: Overlay gels for chitinase activity

Native PAGE and semi SDS-PAGE (without β-mercaptoethanol) gels were carried out at 4ºC. After semi SDS-PAGE the gels were incubated at 37 ºC for 2 hour in sodium phosphate buffer pH 6 containing 1 % (v/v) Triton X-100 to remove SDS. The gels were then washed with distilled water and incubated at 37 ºC in sodium phosphate buffer pH 6 for 1 hour. Other gel containing 0.1% (v/v) glycol chitin as a substrate for the enzyme is polymerized. After polymerization, the gel with the substrate incorporated is overlayed on to the gel with the enzyme and incubated overnight. After incubation the gel with the substrate is stained with 0.1 %(w/v) ranipal and observed under uv-transilluminator.

The bands observed in the chitinase activity native and semi SDS-PAGE gels are consistent with the bands observed in the native and SDS-PAGE (without β-mercaptoethanol) gels.

(A) Enzyme concentration curve



(B) Enzyme activity time course

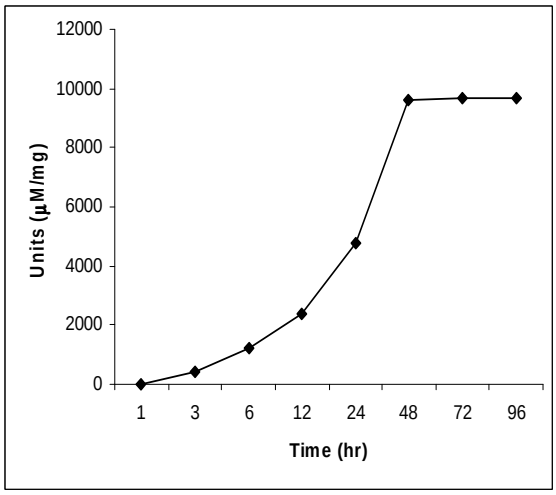


Fig 18: Chitinase activity enzyme concentration and time course curves

A) Substrate concentration is kept at 1 mg/ml and the assay is carried out with varied concentrations of enzyme

B) Substrate concentration is kept at 1 mg/ml and the enzyme concentration is kept at 100 µg and the assay is carried out with respect to time course

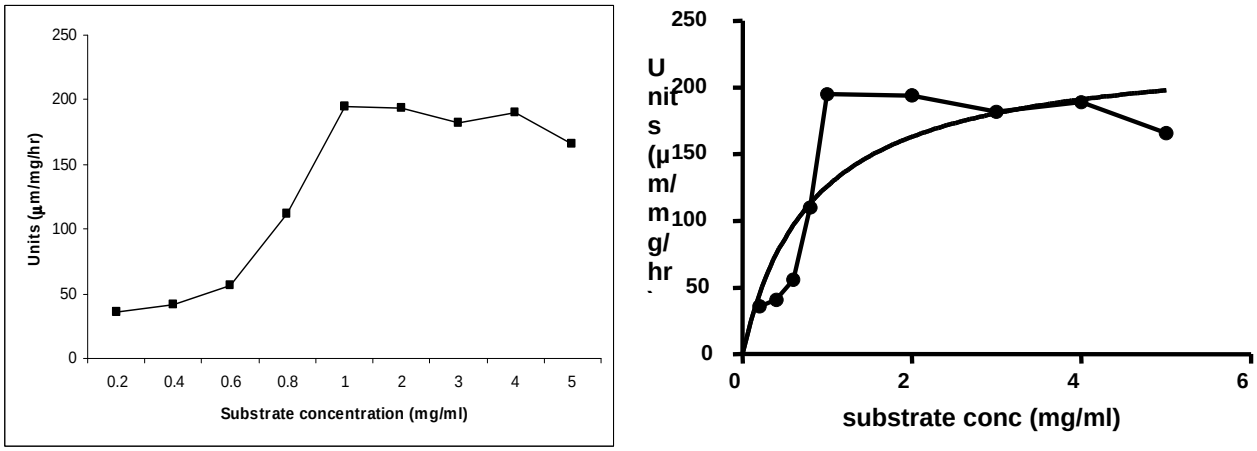


Fig 19: Substrate concentration curve

A) Substrate concentration curve, Enzyme concentration is kept at constant (500µg) and the assay is carried out with different concentrations of substrate.
B) Michaelis-menton curve as analyzed by prism 5 software

V_{max} - 231.3 k_m - 0.83

(A)Temperature optima **(B) pH optima**

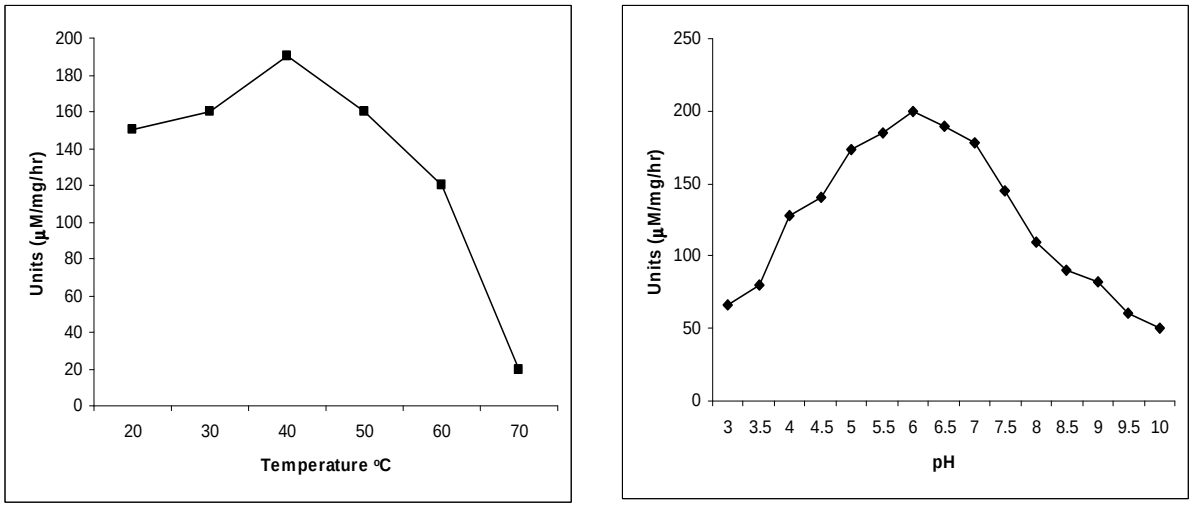


Fig 20: Temperature and pH optima studies for chitinase activity

A) Substrate and purified lectin (ANTL) were incubated at different temperatures viz 20ºC, 30ºC, 40ºC, 50ºC, 60ºC, 70ºC for 6 hr and the assay is carried out by above said method.Chitinase activity is maximum at 40 ºC and gradually decreased upon increasing the temperature and lost its activity completely after 80 ºC.

(B) Substrate and purified lectin (ANTL) were incubated in the buffers of different pHs 3, 3.5, 4.0,4.5, 5.0, 5.5, 6.0, 6.5, 7.0,7.5, 8.0, 8.5, 9.0, 9.5,10.0 (20mM sodium acetate(3.0-5.5)/sodium phosphate(6.0-8.0), Tris-HCl(8.5-10.0) for a period of 6 hr at 40ºC and then the assay is carried out. Chitinase activity is maximum at pH 6. upon increasing or decreasing of pH the activity is gradually lost.

The effect of ANTL on insect larval development of two pests *Corcyra cephalonica* and *Achaea janata* was investigated by incorporating the lectin in artificial diets at concentrations ranging from 0.1% to 0.4%. These values were chosen because they are within the range of lectin concentrations found in seeds and were similar to concentrations used in other studies with purified plant lectins. The effect of ANTL on larval development was monitored by feeding the larvae with ANTL in an artificial diet(semi crushed sorghum seeds are mixed with various concentrations of ANTL 0.1%-0.4% (w/w) and ten neonatal larvae are reared on this artificial diet for 5 days) and then determining the number and mass of the surviving fourth instar larvae. The concentration–response curve for the effect of ANTL on the survival and mass of *Corcyra cephalonica* larvae has shown ~50% mortality at the concentration of 0.3% (w/w) and ~50% mass loss is observed at 0.15% (w/w) concentration (Fig 21).

A) SURVIVAL **B) LARVAL MASS**

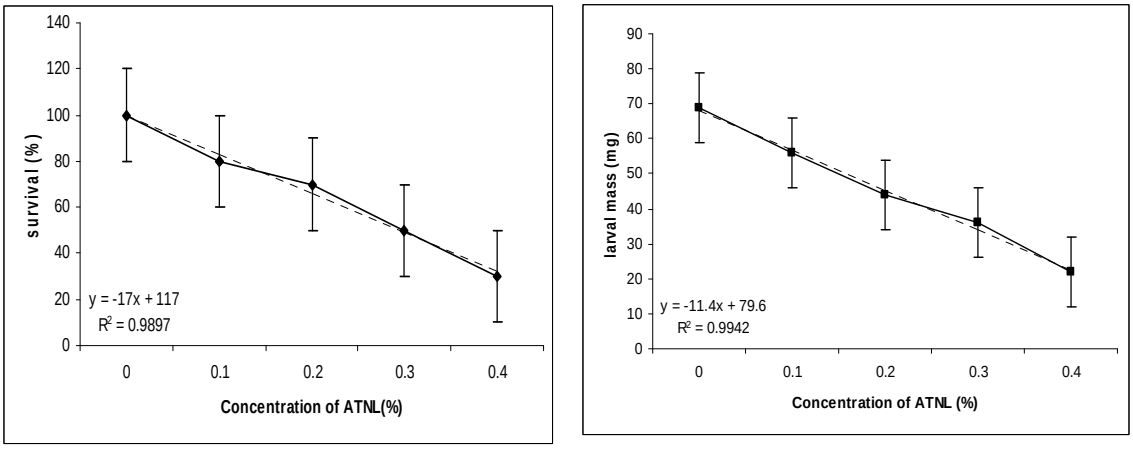


Fig 21: Effect of various concentrations of ANTL on *Corcyra cephalonica* larvae Semi crushed sorghum seeds are mixed with various concentrations of ANTL 0.1%-0.4% (w/w) and ten neonatal larvae are reared on this artificial diet For 5 days.
~50% mortality is observed at the concentration of 0.3% (w/w)
~50% mass loss is observed at 0.15% (w/w) concentration

Regression analysis showed that a 0.1% increase in the concentration of ANTL resulted in a 3.0% increase in mortality (r2 =0.98) and a 0.2 mg decrease in mass (r2 =0.99). The food consumption by *C. cephalonica* larvae reared on an artificial diet containing 0.4% ANTL decreased by 45% and the faecal production has got a decrease of 63% with respect to the controls with no ANTL. No significant change is observed when the larvae of *A.janata* have been fed artificial diet with 1% ANTL. When food consumption was expressed relative to body mass there was a 25% decrease in consumption by *C. cephalonica* larvae fed ANTL. The mean total protein content of faecal and midgut extracts of C. *cephalonica* larvae fed with 0.4 % ANTL showed a significant decrease of ~16% and ~23% respectively compared to the controls (Fig 22). The higher faecal protein content in lectin-fed insects resulted in a significant decrease in the mean total protein content of crude gut extracts. The trypsin-like activity of gut and faecal extracts in control and lectin-treated larvae on the consumption of an artificial diet containing 0.4% ANTL significantly decreased by ~21% the faecal protease activity by ~14% (Fig 23). The efficiency with which *C. cephalonica* larvae that were fed ANTL converted food into new body material relative to the amount of food eaten (ECI), or relative to the amount of food actually absorbed from the gut (ECD) decreased by ~42% and ~49%, respectively, whereas the efficiency with which ingested food was assimilated (AD) the metabolic cost (MC) increased by ~15% and ~38% respectively (Table 16) compared to larvae that received the control diet.

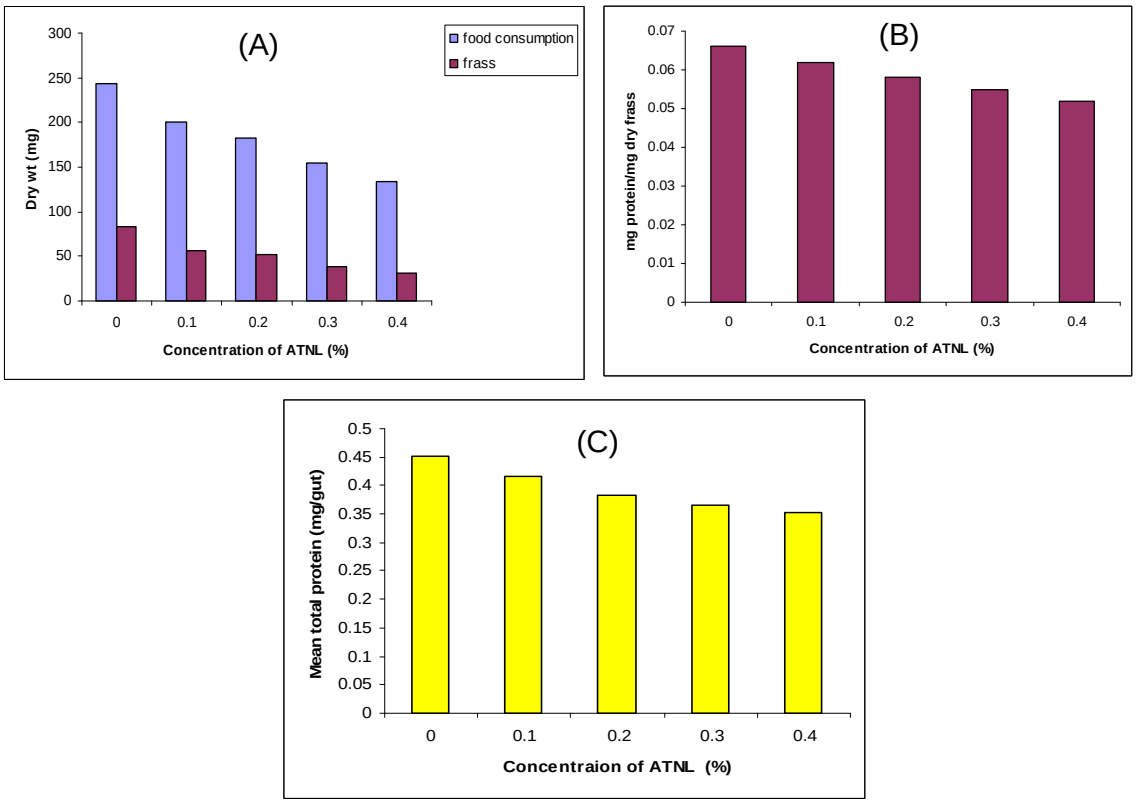


Fig 22: Physiological parameters measured in *Corcyra cephalonica* larvae

Larvae are fed with an artificial diet with 0.1%-0.4% (w/w) ANTL. Controls are without ANTL A) Diet consumption and faecal production B)Faecal protein content C)Total protein content of midgut extract

The food consumption by C. cephalonica larvae reared on an artificial diet containing 0.4 %(w/w) ANTL decreased by ~45%and faecal production was lower than in control larvae, with a decrease of ~63%. The faecal protein and the mean total protein content of gut extracts showed a significant decrease of ~16% and ~23% respectively.

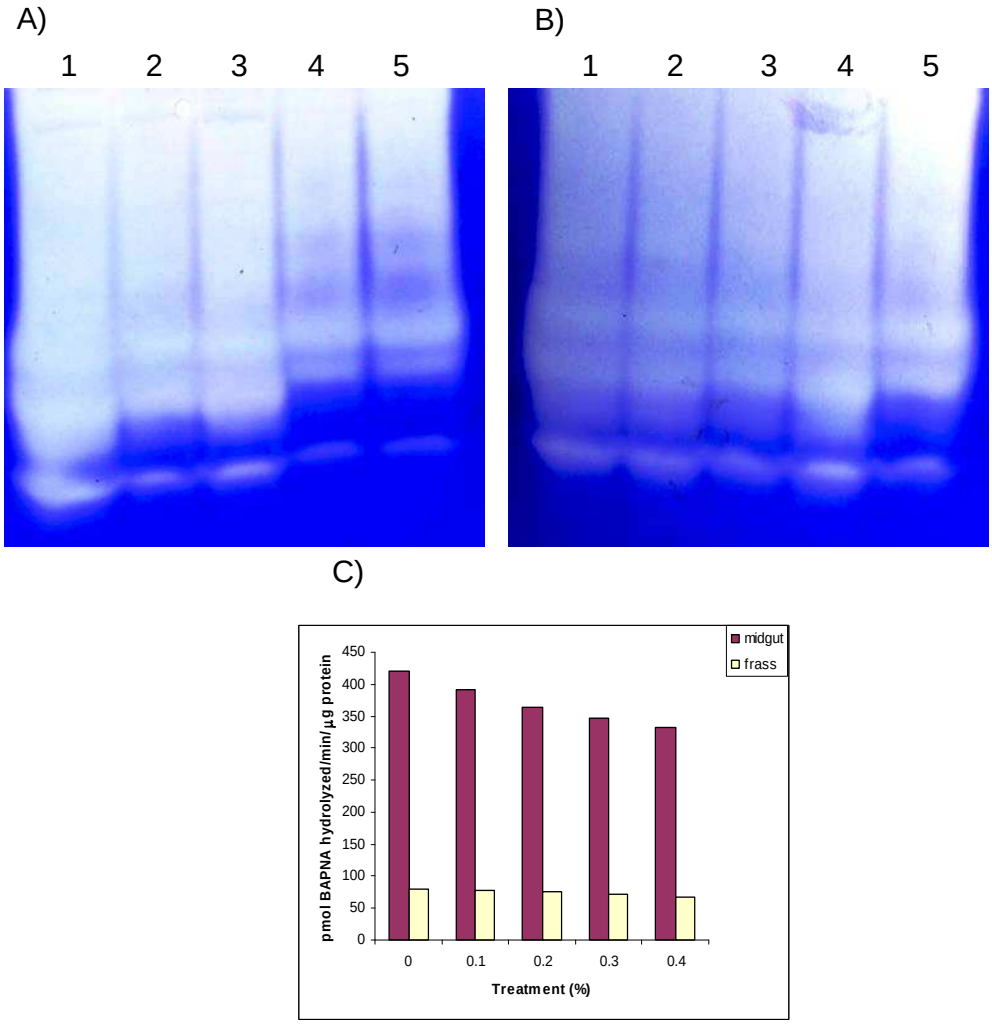


Fig 23: Proteolytic activity of C. cephalonica larvae fed with ANTL.

(A) & (B) Protease assays after SDS-PAGE in midgut and faeces respectively. 1-Control (no ANTL), 2- 0.1%, 3- 0.2%, 4-0.4%, 5- 0.5%. Proteolytic activity appeared as a clear zone against a dark blue background. (c) Trypsin like activity in the midgut and faeces of C. cephalonica larvae fed a diet containing 0.1%-0.4% ANTL.

Table 16: Nutritional parameters measured in *Corcyra cephalonica*

Treatment (%)	ECI (%)	ECD (%)	AD (%)	MC (%)
0.0	28.3	43.1	65.8	56.9
0.1	27.8	38.6	72.1	61.4
0.2	24.1	33.8	71.4	66.2
0.3	23.3	31.3	74.6	68.7
0.4	16.4	21.3	76.8	78.7

Nutritional indices of *C. cephalonica* fourth-instar larvae on various concentrations of (0.1%- 0.4%) ANTL-treated and control diets. Ingestion and digestion of larvae fed on ANTL treated diets was significantly decreased whereas the approximate digestibility and metabolic cost are significantly increased compared to the control diets.

A lectin from *Koelreuteria paniculata* seeds added to an artificial diet at a concentration of 1.0% (w/w) reduced the larval mass of *A. kuehniella* by 84% (Macedo *et al*., 2003); *Galanthus nivalis* lectin (GNA) was toxic to *Lacanobia oleracea* at a concentration of 2.0% (Fitches *et al*., 1997), and pea (*Pisum sativus*) lectin was detrimental to *Chilo partellus* at a concentration of 0.5% (Law and Kfir, 1997). As shown here, ANTL was effective only to *C.cephalonica* larvae. Reduced growth and decrease in faecal production resulted by the decrease in the food consumption suggests the changes from behavioral and physiological (post ingestive) effects (Koul and Isman, 1991; Venzon *et al*., 2004), possibly caused by the longer retention of food in the gut to maximize AD. The increase in the AD is to meet the increased demand for nutrients (Mirela *et al*., 2007) and compensate for the decrease in the food consumption, the deficiency in foodstuff conversion (reduction in ECI and ECD), perhaps by diverting energy from biomass production into detoxification (Nathan and Kalaivani, 2005). Some lepidopterans can maintain their growth rate and body mass independently of food quality

by altering their food consumption and use in response to changes in food quality by such compensatory effects. Serine proteases are the predominant digestive enzymes in the lepidopteran gut and this activity was found in the gut and faecal extracts analyzed here. Lectins can indirectly affect enzyme regulatory mechanisms by perturbing the organization of the peritrophic membrane (Fitches and Gatehouse, 1998). Such an action could account for the increased tryptic activity of faecal extracts, as well as the decrease in the protein concentration of gut contents and the enhanced faecal protein concentration. Even though the exact mechanism of the lectins interfering with the hydrolytic gut enzymes of the insects is unclear, they may involve binding to sites other than the active site (Kim *et al*., 1976). On the other hand, lectins may increase the number of enzyme active sites by altering their accessibility to the substrate (Erickson *et al*., 1985). Lectins may also bind to substrates, thereby increasing the enzymatic activity, or may bind to both the enzymes and their substrates to increase the affinity between them. The antinutrient activity of plant lectins could be explained primarily by the predominant binding of lectins to glycan receptors at the intestinal surface and blocking them from enzyme. A prerequisite for toxicity is that the lectin should be able to survive the hostile proteolytic environment of the insect midgut. Depending on their resistance to gut proteolysis and on their specificity for carbohydrate receptors, lectins may bind to different parts of the small intestine to cause functional and morphological alterations (Pusztai *et al*., 1990). The incubation of ANTL with gut proteases from *C. cephalonica* larvae did not resulted in degradation of the lectin that started within first minutes but required several hours for completion Various insecticidal lectins, such as those of *Talisia esculenta, Bauhinia monandra* leaf lectin, *Griffonia simplicifolia* seed lectin II and some storage proteins, such as vicilins and zeatoxin (maize globulin) (Mirela *et al*., 2007), are

resistant to degradation by insect digestive enzymes. Resistance to degradation by pest metabolic systems is clearly beneficial for plant defensive mechanisms.

In conclusion, the divergent effects of ANTL to *C. cephalonica* larvae indicate that insects can adapt to the presence of plant lectins in their diets by using strategies to bypass or destroy these molecules. The insecticidal activity of ANTL may involve: (1) binding to chitin components (or equivalent structures) in the insect gut, (2) interaction with glycoconjugates on the surface of epithelial cells along the digestive tract, (3) binding to the sugar moiety of any of the glycosylated digestive enzymes and/or assimilatory proteins present in midgut extracts, and (4) resistance to enzymatic digestion by midgut proteases. The physiological responses of pests to insecticidal proteins provide important information for the development of management strategies that can be applied to transgenic plants protected against insect attack (Christeller *et al*., 2005).

The nutritional indices suggested that ANTL has a multi mechanistic mode of action and an antifeedent for both insects. The toxicity in *Corcyra* apparently resulted from the change in the gut membrane environment and consequent disruption of digestive enzyme recycling mechanisms.

In contrast, the inclusion of 1% (w/w) ANTL did not significantly decrease the survival or weight of *Achaea janata* (data not shown).

Among the three tested fungi, *Rhizoctonia Solani*, *Aspergillus Niger*, *Fusarium moniliforme*, ANTL has inhibited the growth of only *R.solani* at a concentration of 250 µg/ml (Fig 24).

In conclusion, Chitin Affinity chromatography has been employed to purify chitin-binding lectin from *Aponogeton natans* tubers. The lectin was eluted from the column in a single step with 4-fold purification, as a single symmetrical peak with 70.3% yield which is a Glycoprotein with an apparent molecular mass of 66 kDa and is made of two

types of subunits (33kDa & 32kDa). The carbohydrate content is found to be 8.2% of the total protein. The hemagglutination activity of the lectin was found to be specific for different human blood groups viz. A (+vet), B (+vet), O (+vet), AB (+vet), rabbit and rat. The lectin activity was heat stable up to 60°C and showed optimum hemagglutination activity at pH 6. The lectin was affected by denaturing agents such as Lithium chloride (2M), Potassium ferricyanide (2M), Geranium chloride (2M), Urea (2M) and Per Iodic acid (2M) and EDTA has no effect up to 30 mM. The most effective quenching is brought about by acrylamide with 46.9 % quenching of the total fluorescence of the native protein at a quencher concentration of 0.5 M. I- and Cs+ could quench only 26.6 % and 2.5 % respectively, at the same concentration. Low quenching of the charged quenchers indicates that most of the fluorescent tryptophan residues in ANTL are buried in the hydrophobic core of the protein and are in a significantly non-polar environment. Denaturation with 6 M Gdn. HCl results in the increased quenching percentage to 84.7 %, 47.5 % and 7.1 % with acrylamide, iodide ion and cesium ion, respectively, consistent with the unfolding of the protein. CD spectral studies indicate that ANTL is a protein with a higher content of α-helix (53 %) than β- sheet (21 %) and unordered structures (16%). The protein is thermally quite stable up to 60°C and undergoes an unfolding transition at ~70°C, The isolated lectin possessed the chitinase activity, which was confirmed by the plate gel diffusion assay method and Ranipal is checked as a staining reagent which is less expensive and as effective as calcoflour white which is already established by the lab. Native and Semi-native (β-mercaptoethanol is not present in the sample) gel overlays has confirmed the chitinase activity in the PAGE gels as well. The optimum concentration of the enzyme is found to be 100 µg/ml and the optimum concentration of the substrate is found to be 1 mg/ml. The optimum temperature for the chitinase activity is found to be 40 ^{o}C but gradually decreased with rise in the temperature and completely lost its activity

after 70 ºC. The optimum pH for the chitinase activity is also found to be at 6. The enzyme has shown an exponential activity till 48 hr. The cytotoxic studies of the enzyme on insect cell lines (sf9 cells) and mammalian cell lines (SUP T1 and U266) has revealed that there is no effect of the lectin on these cell lines up to the concentration of 20µg till 72 hrs incubation in sf9 & SUP T1 cell lines but there is 50% decrease in the proliferation at 5µg in U266. Aponogeton natans lectin is found to be potent mitogen for it stimulated the Proliferative response in murine and human spleen lymphocytes at the concentration of 5µg/ml. FITC-conjugated lectin studies revealed that the lectin is bound to all the cells but the exact mechanism with which it is inducing responses in some cell lines is not clearly understood. Regarding the insecticidal activity tested against two pests *Corcyra cephalonica* and *Acheae janata*, ANTL has produces ~50% mortality and mass loss in *Corcyra* at the concentrations of 0.3%(w/w) and 0.15%(w/w) respectively when incorporated into the artificial diet. In contrast, the inclusion of 1% (w/w) ANTL did not significantly decrease the survival or weight of *Acheae janata.* The nutritional indices suggested that ANTL has a multi mechanistic mode of action and an antifeedent for both insects. The toxicity in *Corcyra* apparently resulted from the change in the gut membrane environment and consequent disruption of digestive enzyme recycling mechanisms.

ANTL is a chimeric lectin which has both chitin binding specificity and chitinase activity. It is the second chimeric lectin reported from monocots.

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