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Purification, Characterization and Studies of Biological Activity of a Novel Lectin from Nymphaea nouchali Tuber

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Purification, Characterization and Studies of Biological Activity of a Novel Lectin from *Nymphaea nouchali* Tuber

A

Dissertation

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Dedicated

To

My Beloved

PARENTS

CERTIFICATE

This is to certify that the thesis entitled **"Purification, Characterization and Studies of Biological Activity of a Novel Lectin from *Nymphaea nouchali* Tuber"** has been prepared by Md. Abu Zubir under my supervision for submission to the Department of Biochemistry & Molecular Biology, University of Rajshahi, for the Degree of Doctor of Philosophy in Biochemistry and Molecular Biology. It is also certified that the materials included in this thesis are original research works and have not been previously submitted for the award of any other degree.

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DECLARATION

I do hereby declare that the whole work submitted as a thesis entitled **"Purification, Characterization and Studies of Biological Activity of a Novel Lectin from *Nymphaea nouchali* Tuber"** to the Department of Biochemistry & Molecular Biology, University of Rajshahi, Bangladesh for the Degree of Doctor of Philosophy in Biochemistry and Molecular Biology, are the original research works and have not been previously submitted elsewhere for the award of any other degree.

August, 2013

Md. Abu Zubair
Ph.D. Research Fellow

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ABSTRACT

A novel lectin (named as NNTL) have been isolated from the crude extracts of *Nymphaea nouchali* (White water-lily) tubers and purified by using anion-exchange chromatography on DEAE cellulose column followed by hydrophobic chromatography on HiTrap Phenyl HP column and then re-applying to anion-exchange chromatography on a HiTrap Q FF column. The purified NNTL moved as a single band and monomer in nature as judged by SDS-PAGE both in presence and absence of 2-mercaptoethanol and its molecular weight was estimated to be 27±1 kDa. The NNTL was glycoprotein in nature with neutral sugar content of 8%, which agglutinated rat, chicken and different groups of human blood cells. In the sugar inhibition study, O-nitrophenyl-ß-D-galactopyranoside was found to be most potent inhibitor and the minimum inhibitory concentration needed for visible agglutination was 25 mM. The lectin showed toxicity against brine shrimp nauplii with the LC_{50} value of 120±29 µg/ml and strong agglutination activity against four pathogenic bacteria (*Bacillus subtilis*, *Sarcina lutea*, *Shigella shiga* and *Shigella sonnei*). The NNTL was more thermostable and showed full activity at the temperature range 30 to 60^0C, and did not lose its activity between pH 5.0 to 9.0. The lectin was a divalent ion-dependent glycoprotein, which lost its activity markedly by treating with denaturants such as DTT and EDTA, but the activity was restored sequentially by the addition of Ca^{2+}, Ba^{2+} and Mg^{2+}.

The N-terminal sequences of the lectin upto 10-residues were identified except the first position and the sequence homology showed that NNTL was not identical with the sequence of any other reported lectins so far available. The analysis of amino acid composition revealed that NNTL was rich in leucine, methionine, and glycine residues as well as significant amounts of acidic amino acids.

The study of the conformational changes by fluorescence spectroscopy indicates that the tryptophan environment of NNTL was greatly affected upon binding to different concentrations of metal ions, sugars and denaturants and the stability of NNTL was markedly dependent on Ca^{2+} requirement.

The antifungal activity of both the lectin and non-lectin protein of *Nymphaea nouchali* tuber were assayed against five pathogenic fungi and it was found that the NNTL was devoid of any antifungal activity, whereas the purified protein (named NNTP-I) had notable effect only against *Candida albicans.* The study of antitumor activity of NNTL showed that the lectin inhibits 56% and 76% of proliferation of EAC (Ehrlich's Ascites Carcinoma) cells in vivo in mice at the dose of 1.5 and 3.0 mg/Kg/day respectively.

Furthermore, we also studied the antitumor effect of NNTL to assess the apoptotic nuclear morphology in fluorescence microscopy by staining with Hoechst 33342, but there was no nuclear damage markedly found in the EAC cells treated with NNTL. Subsequently, from the assessment of the expression of apoptosis regulating gene *bcl-1*, *bcl-X*, and *bax* by RT-PCR, it was observed that the apoptosis causing gene *bax* was not expressed in the experimental cell, suggesting that the lectin does not induce apoptosis.

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Chapter Three: Structure Analysis of NNTL

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1.1 General

Nymphaea nouchali commonly known as water lily is a popular aquatic plants, which has roundish, peltate, or heart-shaped leaves floating on the surface of the water. They are all aquatic perennial herbs not only beautiful to look at, but they also serve an important purpose in the pond, mainly in aiding its ecosystem. Water lilies spread across the waters surface, filling it with color and vibrancy all the while keeping the pond and the creatures in it safe and healthy. Both leaves and flowers grow from rootstalks imbedded in the mud of ponds or sluggish streams, the petioles and flower stalks sometimes attaining a length of several feet. Water lilies can remain in the pond year round. The lily will die off in the winter time and produce new leaves and flowers in the spring. There are reported to be about 50 species growing in different tropical countries. The common North American white water lily, or pond lily, is *Nymphaea odorata.* The European white water lily is *N. alba*. Both species have reddish leaves when young and large fragrant flowers. Other species of *Nymphaea* have pink, yellow, red, or blue flowers; many kinds are of hybrid origin. The lotus of ancient Egyptian art was usually the blue lotus (*N. caerulea*). The largest water lilies are those of the tropical South American species (*Victoria amazonica*) is occasionally grown in some gardens for its enormous orbicular floating leaves.

Bangladesh is a tropical riverine country, which is rich in water resources and is very much potential for production of aquatic plants. Water lily is available in various kinds of lakes, canals, ponds and most of the marshy land area in the country. In Bangladesh it is locally known as “Shapla”, the national flower of the country and two species mainly *N. nouchali* (white variety) and *N. pubescens* (blue variety) are available. Both species grow abundantly as a mixed population in almost all shallow natural water bodies, but the white variety is more frequent and popular in Bangladesh. Another species, *N. rubra* is often cultivated in ponds and tanks as an ornamental plant. Seasonal water lilies grow and bloom mainly in the rainy season and the mature tuber is found in the autumn and late

autumn. The peduncle is a popular vegetable to villagers and the tuberous rhizomes are also eaten. Seeds on frying are eaten as puffed-grains in some places.

Figure 1.1 White Water Lily **(***Nymphaea nouchali*) flower

1.2 Taxonomy of *Nymphaea nouchali* (Nymphaeaceae)

The scientific classification:

Kingdom: Plantae - Plants

Subkingdom: Tracheobionta - Vascular plants

Superdivision: Spermatophyta - Seed plants

Division; Magnoliophyta - Flowering plants

Class: Magnoliopsida – Dicotyledons

Subclass: Magnoliidae

Order: Nymphaeales

Family: Nymphaeaceae – Water-lily family

Genus: *Nymphaea* L. – waterlily

Species: ***N. nouchali*** – white water lily

(http://plants.usda.gov)

1.3 *Nymphaea nouchali* Tuber

The *Nymphaea nouchali*, i.e., waterlily produce a tuber that the plant uses to sustain itself in times of drought allowing the plant a chance at survival. In times of drought in the water lilies natural habitat, the pads will die off and the tuber will remain below the soil and becomes quite nut-like, protecting the tuber through the dry season. Once the rains return and the tuber becomes moist again, it will send up new growth from the terminal crown and a new plant will be born.

(a) **(b)**

Figure 1.2 (a) *N.noucahli* tuber, (b) After dried and peeling the tuber.

1.4 Uses of *Nymphaea* spp. As Food

The peduncle is a popular vegetable to villagers; the tuberous rhizomes are also eaten. The tubers are sweet and bitter at the same time and eaten as a starchy food staple. These tubers are especially important as a food reserve during times of famine or poor harvest, especially in the Northern regions of Bangladesh. Whole cooked tubers are commonly sold in markets as snack foods. *Nymphaea* tubers can be peeled, cut in small pieces, dried and ground into powder. The powder is then used for cooking into a thick porridge, which can be served with different types of stews and/or vegetables.

1.5 Medicinal Uses of *Nymphaea* spp.

Various species of *Nymphaea* have been also used as a medicinal plant by people of different cultures of the world. It is useful as a tonic in diarrhoea, dysentery, dyspepsia and general debility. The flowers are astringent and cardiotonic. The seeds are sweet, cooling, constipating, aphrodisiac, stomachic and restorative. It has found uses as a culinary delight food staple as well as being used internally as a treatment for gastrointestinal disorders and jaundice. Leaf is used in cutaneous and subcutaneous parasitic infection, eye treatments, and pregnancy. Seeds are used in sauces, condiments, spices and flavorings.

1.6 Plant as a Rich Source of Proteins

Proteins are a group of complex organic macromolecules that contain carbon, hydrogen, oxygen, nitrogen, and usually sulfur and composed of one or more chains of amino acids in a specific order; the order is determined by the base sequence of nucleotides in the gene that codes for the protein. Proteins are fundamental components of all living cells and known as the structural constituent of all living organisms. They include many substances, such as enzymes, hormones, and antibodies that are necessary for the proper functioning of an organism. Protein*s* are the true workhorses of the body, carrying out most of the chemical processes and making up the majority of cellular structures.

Proteins are available in all of living organisms, where animal kingdom is the main sources of protein but plant kingdom also possesses a lot of protein sources. Green leaves, barks, roots, tuber etc. contain small amount of protein; while seeds are the main sources of protein in plants. Pulses contained about 20 to 25% and oil seeds contain approximately 30 to 35% of proteins (Rutkowaski, 1970). In addition, wheat, barley, rice bran, maize etc. also contain significant amount of protein. Some plant seed proteins are toxic to other organisms.

1.7 Most of the Plant proteins are Glycoproteins

The glycoproteins that contain carbohydrate group attached covalently to the polypeptide chains represent a large group of wide distribution with considerable biological significance. Plant seed proteins contain in general, 1-3% carbohydrate but there are exceptional cases in which the carbohydrate content may be about 10-12% (Lis and Sharon, 1981). The percentage by weight of carbohydrate group in different glycoproteins may vary from less than 1% in ovalbumin to 80% in the mucoprotein (Lehninger, 1981).

Many different types of monosaccharide derivatives have been found in glycoproteins. The linear and branched side chain of glycoproteins may contain from two to dozens of monosaccharide residues, usually of two or more kinds. Some glycoproteins also contain oligosaccharide units. The sugar residues are generally mannose, galactose, lactose, xylose, glucose, raffinose, arabinose, glucosamine etc (Lis and Sharon,1981).

1.8 Lectins

Lectins are a group of proteins found in all types of living organisms, either in soluble or in membrane-bound form that recognize specific carbohydrate structures and thereby agglutinate cells by binding to cell-surface glycoproteins and glycoconjugates (Lis and Sharon, 1998). They are generally structurally complex molecules with one or more carbohydrate-recognition domains (Dodd and Drickamer, 2001). Lectins with specific carbohydrate specificity have been isolated and characterized from various plant tissues and other organisms. They are widely distributed in the plant kingdom, particularly among the legumes and to a lesser extent among the cereals grains. It is also found in animals, insects, and microorganisms (Linear *et al*., 1986; Sharone and Lis, 1989).

1.9 Background of the Lectin

The history of lectins dates back to 1888 when Stillmark discovered a toxic proteinaceous hemagglutinating factor in castor beans (*Ricinus communis*) which was named as "ricin" (Stillmark, 1888). In 1907 Landsteiner and Raubitschek reported for the first time the presence of nontoxic lectins in the legumes *Phaseolus vulgaris* (bean), *Pisum sativum* (pea), *Lens culinaris* (lentil), and *Vicia sativa* (vetch). Many more non-toxic plant hemagglutinins were discovered subsequently. Specificity of certain hemagglutinins toward erythrocytes of a particular human blood group within the ABO system was also established in the due course (Renkonen, 1948; Boyd and Reguera, 1949). This discovery of blood group specificity was the direct motive to the introduction of the novel term 'lectin' (from the Latin verb 'legere', which means 'to select') (Boyd and Shapleigh, 1954).

Lectins are currently attracting much of interest, primarily because they serve as invaluable tools in diverse areas of biomedical research. Because of their unique carbohydrate binding properties, lectins are useful for the separation and characterization of glycoproteins, glycopeptides, and glycolipids; following changes that occur on cell surfaces during physiological and pathological processes like, from cell differentiation to cancer; histochemical studies of cells and tissues; for tracing neuronal pathways; typing blood cells and bacteria, and fractionation of lymphocytes and of bone marrow cells for bone transplantation. In addition, lectins are excellent models to examine the molecular basis of specific reactions that occur between proteins and other types of molecules, both of low or high molecular weight, such as the binding of antigens to antibodies, of substrates to enzymes, of drugs to proteins and of hormones and growth factors to cells. The first Lectin to be purified on a large scale and available on a commercial basis was concanavalin A, which is now the most used Lectin for characterization and purification of sugar-containing molecules and cellular structures.

1.10 Lectin Mediated cell Agglutination

Agglutination is the most easily detectable manifestation of the interaction of a lectin with cells. The ability to agglutinate cells distinguishes lectins from other sugar-binding macromolecules, such as glycosidases and glycosyltransferases, and is therefore included in the definition of lectins (Goldstain *et al.*, 1980).

Although the interaction between lectins and cells is a well established phenomenon, however the way by which this interaction occurs is still unclear and published observations are sometimes contradictory. The traditional idea favours the carbohydrate-directed interaction as the main reason of binding of lectins to the cell surface. Furthermore, the agglutination mechanism itself is too complex to assume that lectins are only functional "bridges" that hold two cells together. The effect noticed when agglutination is studied with different cells and different lectins at various conditions, suggest that other factors are to be taken into account (Ochoa, 1979). In addition, agglutination is affected by some external conditions such as temperature, concentration of the cells, proper mix-up and so on. Agglutination can also be inhibited by various sugars on that the lectins are dependent.

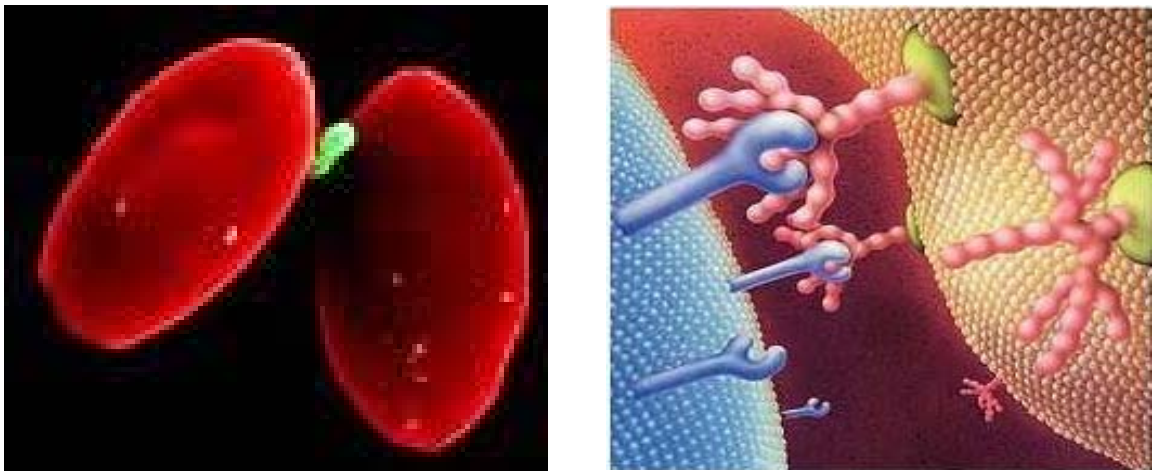


Figure 1.3 The mechanism of lectin action (Nathan Sharon and Halina Lis, 1993)

1.11 Classification and properties of Lectins

Lectins were first discovered more than 100 years ago in plants, but they are now known to be present throughout nature. Lectins are also prevalent in the microbial world, wherein they tend to be called by other names, such as hemagglutinins and adhesions (Varki *et al*., 2009).

Regardless of source, all of the effects of lectins are believed to be a manifestation of the ability of the lectins to specific kinds of sugar, thereby agglutinate red blood cells of various animals and possess many other characteristic properties such as exert wide range of biological effects on the cell.

Some of the major classes of lectins based on their sources and their biological properties are mentioned below:

I) Plant Lectin:

Plant lectins are the first and still the largest and best characterized group of lectin that have been studied extensively. It became a part that many of the lectins could be grouped into families with sequence homologies and common structural features. The largest family and best categorized grouped is *Leguminoseae* lectins. There are two small families of plant lectins, are those from *Gramineae* (cereals) and *Solanaceae* (potato and tomato) lectin (Sharon and Lis, 1990).

On the basis of structural and evolutionary development, most of these plant lectins have been classified as legume lectins, chitin-binding proteins, type-2 ribosome-inactivating proteins, monocot mannose-binding lectins, amaranthins, cucurbitaceae phloem lectins and jacalin-related lectins (Van Damme *et al.,* 1998). The rich sources of lectins in plants are particularly their organs such as seeds, tubers, bulbs, rhizomes, bark etc. and they have attracted great interest on according of their various biological activities, such as

cell agglutination (http.inbar.int/wiki/index.php/ Medicinal plants), antifungal (Sitohy *et al.,* 2007), antiviral (Tian, *et al.,* 2008) and antiproliferative activities (Liu *et al.,* 2010; Kaur *et al.,* 2005).

II) Animal Lectin:

Some proteins regarded as animal lectins were discovered before plant lectins, though many were not recognized as carbohydrate-binding proteins for many years after first being reported. As recently as 1988, most animal lectins were thought to belong to one of two primary structural families, the C-type and S-type (presently known as galectins) lectins. At least 12 structural families are known to exist, while many other lectins have structures apparently unique amongst carbohydrate-binding proteins, although some of those "orphans" belong to recognized protein families that are otherwise not associated with sugar recognition. While animal lectins undoubtedly fulfill a variety of functions, many could be considered in general terms to be recognition molecules within the immune system. More specifically, lectins have been implicated in direct first-line defence against pathogens, cell trafficking, immune regulation and prevention of autoimmunity (Animal lectins: a historical introduction and overview, 2002).

The carbohydrate recognition domains in vertebrate lectins fall into a number of structurally distinct families. Many of these lectin families have been known, such as the galectins (previously known as S-type) (Barondes *et al*., 1994), C-type (Drickamer *et al*., 1999), I-type (Angata *et al*., 2002), P-type lectins (Dahms *et al*., 2002), etc.

III) Invertebrate Lectins:

Lectins found in invertebrate animals are mainly present in the hemolymph and sexual organs (e.g., albumin glands and eggs). They are also present in the membranes of hemocytes, cells that function as primitive and rather unspecific immunological protectors (Cheng *et al.,* 1984)

Cellular slime molds have favorable properties for biological investigations. Slime mold is a broad term describing protists that use spores to reproduce. Slime molds were formerly classified as fungi, but are no longer considered part of this kingdom (http: //www.ucmp.berkeley.edu/protista/slimemolds.html.).

IV) Bacterial Lectins (Adhesins and Toxins):

Many bacterial lectins have been described, and they fall into two classes: (1) lectins (adhesins) that reside on the bacterial surface and facilitate bacterial adhesion and colonization, and (2) secreted bacterial toxins. Many bacterial lectins bind to membrane glycolipids, whereas only a few bind to glycoproteins. In some cases, binding specificity can explain the tissue tropism of the organism; for example, urinary tract infection by specific serotypes of *Escherichia coli* depends on binding mannose or blood group structures. Not all of these interactions are pathogenic, and some glycan–protein interactions between bacteria and host tissues play important roles in symbiosis. Many bacterial species, especially enterobacteria and *Salmonella* species are capable of producing surface lectins, in the form of submicroscopic hair-like appendages known as fimbriae (pili), present on the surface of the cells (Sharon, 1987). Type-1 fimbriae from *E. coli* are mannose specific and preferentially bind oligomannose and hybrid oligosaccharides of animal cell surface glycoproteins (Sharon and Lis, 1989).

Many heat-sensitive secreted bacterial toxins bind to glycans. The best-studied example is cholera toxin from *Vibrio cholerae*, which consists of A and B subunits, in the ratio AB_5. The B subunit binds to multiple GM_1 ganglioside receptors through glycan-recognition domains located on the base of the subunits, facilitating delivery of the toxin A subunit into the cytoplasm. The structures of related toxins from *Shigella dysenteria*, *Bordetella pertussis*, and *E. coli* have also been solved (Varki *et al*., 2009).

V) Fungal Lectins:

In fungi, lectins are known to occur in mycelium (Candy *et al.*, 2003), conidia (Tronchin *et al.*, 2002), sporomes (Raszeja, 1958), basidiomes (Konska, 1985) and fruiting bodies (Wang *et al.*, 2003). Fungal lectin are reported to participate in the formation of primordia, creation of mycelium structures to facilitate, penetration of parasitic fungi into the host organism as well as mycorrhization (Konska, 2006, Guillot and Konska, 1997).

VI) Viral Lectins (Hemagglutinins) :

The influenza virus hemagglutinin was the first GBP (glycan binding protein) isolated from a microorganism (~1950), and it is now one of the most thoroughly studied of all lectins. Wiley and associates crystallized the viral hemagglutinin, determined its structure in 1981, and later solved the structure of cocrystals prepared with sialyllactose (Varki *et al*., 2009).

Human influenza virus binds to erythrocytes and other cells by recognizing *N*-acetyl neuraminic acid present on the cell surface and this binding is a prerequisite for initiation of infection. Influenza A and B viruses and paramyxoviruses bind gangliosides containing *N*-acetyl neuraminic acid, whereas influenza C and bovine corona viruses bind receptors containing *N*-acetyl-9-*O*-acetyl neuraminic acid, the 9-*O*-acetyl group of which is critical for mediating cellular attachment (Schultze *et al*., 1993).

The detailed knowledge of the sialic acid-hemagglutinin interaction provides a possible basis for the design of antiviral drugs that would block viral attachment to cells.

1.12 Classification of Lectins based on the Sequence and Structural homology

The classification was proposed by Kurt Drickamer using some highly conserved amino acid sequence motifs in the CRDs (carbohydrate recognition domains) of lectins. Some of the major families are shown:

I) Galectin:

Previously known as S-type, are the major family of the lectins that bind ß-galactosides by means of a carbohydrate recognition domain (CRD) that has many conserved sequence elements (Barondes *et al*., 1994). Galectins have been found in a wide range of animal phyla, from mammals to nematode and even in protists (sponge and fungus) (Cooper *et al*., 2002).

II) C-typpe Lectin:

The most diverse animal lectins family. These are generally multi-domain proteins, in which C-type CRDs provide Ca^{2+}-dependent carbohydrate recognition activity and then initiate a broad range of biological process, such as adhesion, endocytosis, and pathogen neutralization (Drickamer and Taylor, 1993).

III) I-Type lectin:

I- type is a collective term introduced by Powell and Varki to describe carbohydrate-recognizing proteins that belong to the immunoglobulin (Ig) superfamily (Powell and Varki , 1995).

This classification is somewhat broader compared with C-type lectins or galectins, which are defined by conserved amino acid residues in their carbohydrate-recognition domains (CRDs).

IV) P-type lectins:

These lectins have two members, the cation-dependent mannose 6-phosphate receptor (CD-MPR) and the insulin-like growth factor II/mannsoe 6-phosphate receptor (IGF-II/MPR). These lectins play an essential role in the generation of functional lysosomes within the cell of higher eukaryotes by directing newly synthesized lysosomal enzymes bearing the mannose 6-phosphate (M6P) signal to lysosomes (Dahms *et al*., 2002).

1.13 Application of Lectins in different Fields

Lectins are currently attracting much interest, primarily because they serve as invaluable tools in diverse areas of biomedical research. They are being widely used for preparative and analytical purposes in biochemistry, cell biology, immunology, and related areas, particularly with glycoconjugates. According to Rüdiger and Gabius (2001), common applications of lectins in various disciplines include:

A. Biochemistry:

(i) Detection of defined carbohydrate epitopes of glycoconjugates in blots or on thin-layer chromatography plates.

(ii) Purification of lectin-reactive glycoconjugates by affinity chromatography.

(iii) Glycan characterization by serial lectin affinity chromatography.

(iv) Glycome analysis (glycomics).

(v) Quantification of lectin-reactive glycoconjugates in enzyme-linked lectin-binding assays (ELLA).

(vi) Quantification of activities of glycosyltransferases/glycosidases by lectin-based detection of products of enzymatic reaction

B. Cell biology:

(i) Characterization of cell surface presentation of glycoconjugates and their preceding intracellular assembly and routing in normal and genetically engineered cells.

(ii) Analysis of mechanisms involved in correct glycosylation by lectin-resistant cell variants. Fractionation of cell populations.

(iii) Modulation of proliferation and activation status of cells.

(iv) Model substratum for study of cell aggregation and adhesion.

C. Medicine:

(i) Detection of disease-related alterations of glycan synthesis.

(ii) Blood group typing and definition of secretor status.

(iii) Quantification of aberrations of cell surface glycan presentation, for example in malignancy.

(iv) Cell marker for diagnostic purposes including infectious agents (viruses, bacteria, fungi, parasites).

1.14 Biological Applications of Lectin

In the recent years, lectins have been proved to be attractive for research works due to their role in cell agglutination, toxicity, antifungal, antibacterial, antiviral, anti-proliferative and antitumor effects. The list of application of lectins is getting bigger and still the potentiality of these proteins is far from being completely explored.

(A) Anti-insect activity of lectins:

Lectins have been suggested as one of the promising agents against insect pests and have been engineered successfully into a variety of crops including wheat, rice, tobacco, and potatoes. This approach could be used as a part of integrated pest management strategies and caveat pest attack. In general, it seems that large-scale implementation of transgenic insecticidal and herbicide-tolerant plants does not display considerable negative effects on the environment.

Moreover, at least some transgenic plants can improve the corresponding environments and human health because their production considerably reduces the load of chemical insecticides and herbicides (Velkov *et al*., 2005). Lectins demonstrate anti-insect activity. They increase the mortality or delay the development of insect. When incorporated in an artificial diet, *Arisaema jacquemontii* lectin adversely affected the

development of *Bactrocera cucurbitae* larvae (Kaur *et al*., 2006_a). *Arisaema helleborifolium* lectin exhibited anti-insect activity towards the second instar larvae of *B. cucurbitae* (Kaur *et al*., 2006_b). The insecticidal property of lectins may be due to orchestration of enzymatic activity of larvae. After treatment with different lectins, the activity of esterases in larvae was increased whereas the activity of acid phosphatase and alkaline phosphatase decreased. Galectin-1 treatment of *Plutella xylostella* larvae brought about disruption of the microvilli and induced abnormalities in these epithelial cells (Chen *et al*., 2009). *Dioscorea batatas* lectin inhibited the emergence of *Helicoverpa armigera* larvae into adults by avidly binding to larval brush border and peritrophic membrane (Ohizumi *et al*., 2009).

(B) Antifungal activity of lectins:

The expression of *Gastrodia elata* lectins in the vascular cells of roots and stems was strongly induced by the fungus *Trichoderma viride,* indicating that lectin is an important defense protein in plants (Sá *et al*., 2009). Following insertion of the precursor gene of stinging nettle isolectin I into tobacco, the germination of spores of *Botrytis cinerea, Colletotrichum lindemuthianum,* and *T. viride* were significantly reduced (Does *et al*., 1999). Thus, lectins may be introduced into plants to protect them from fungal attack. Plant lectins can neither bind to glycoconjugates on the fungal membranes nor penetrate the cytoplasm owing to the cell wall barrier. It is not likely lectins directly inhibit fungal growth by modifying fungal membrane structure and/or permeability. However, there may be indirect effects produced by the binding of lectins to carbohydrates on the fungal cell wall surface. Chitinase-free chitin-binding stinging nettle (*Urtica dioica* lectin) impeded fungal growth. Cell wall synthesis was interrupted because of attenuated chitin synthesis and/or deposition (Van Parijs *et al*., 1991). The effects of nettle lectin on fungal cell wall and hyphal morphology suggest that the nettle lectin regulates endomycorrhizal colonization of the rhizomes. Severa1 other plant

lectins inhibit fungal growth. The first group includes small chitin-binding merolectins with one chitin-binding domain, e.g., hevein from rubber tree latex (Van Parijs *et al*., 1991) and chitin-binding polypeptide from *Amaranthus caudatus* seeds (Broekaert *et al*., 1992). The only plant lectins that can be considered as fungicidal proteins are the chimerolectins belonging to the class I chitinases. However, the antifungal activity of these proteins is ascribed to their catalytic domain.

(C) Antitumor Activity of lectin:

It is well documented that lectins have an antitumor effect. It has been found that treatment with anti- lectin antibodies can suppress growth of tumor cells in agarose, and inhibit lung colonization in vivo. Lectins have the potential use in cancer treatment strategies due to the fact that lectins present on the surface of tumor cells are capable of binding exogenous carbohydrate-containing molecules and internalize them by endocytosis. (http://www.ansci.cornell.edu/plants/index.html). Flammulina velutipes hemagglutinin-inhibited proliferation of leukemia L1210 cells (Ng *et al*. 2006). *Haliclona cratera* lectin displayed a cytotoxic effect on HeLa and FemX cells (Pajic *et al*., 2002); Dark red kidney bean hemagglutinin exerted an antiprolifirative activity toward leukemia L1210 cells (Xia and Ng, 2006); Small glossy black soyabean lectin impeded proliferation of breast cancer MCF7 cells and hepatoma (HepG2) cells (Lin *et al*., 2008); Del Monte banana lectin retarded proliferation of L1210 cells and hepatoma HepG2 cells (Cheung *et al*., 2009); Extralong autumn purple bean lectin inhibited the proliferation of hepatoma HepG2 cells by inducing the production of apoptotic bodies (Fang *et al*., 2010); Mistletoe lectin can be used in cancer patients to improve the quality of life (Semiglazov *et al*., 2006). *Polygonatum cyrtonema* lectin-treated human melanoma A375 cells (Liu *et al*., 2009a), *Pseudomonas aeruginosa* hemagglutinin-treated breast cancer cells (MDA-MB-468, and MDA-MB-231HM cells) (Liu *et al*., 2009c).

Lectins elicit apoptosis many different cancer cell lines, for examples, Korean mistletoe lectin-treated B16-BL6 melanoma cells (Park *et al*., 2001), Korean mistletoe lectin-treated human A253 cancer cells (Choi *et al*., 2004), *Agrocybe aegerita* lectin-treated HeLa cells (Zhao *et al*., 2009), Abrus agglutinin-treated Dalton's lymphoma cells (Bhutia *et al*., 2008_a) and HeLa cells (Bhutia *et al*., 2008_b), French bean hemagglutinin-treated breast cancer MCF-7 cells (Lam and Ng 2010_a), and recombinant protease-resistant galectin-9-treated myeloma cells (Kobayashi *et al*., 2010).

(D) Antiviral Activity of lectin:

The D-mannose-specific lectin from Gerardia savaglia was firstly reported to prevent infection of H9 cells with human immunodeficiency virus (HIV)-1. Furthermore, the lectin inhibited syncytium formation in the HTLV-IIIB/ H9-Jurkatcell system and HIV-1/human lymphocyte system by reacting with the oligosaccharide side chains of the HIV-1 gp120 envelop molecule (high-mannose oligosaccharides; Müller *et al*., 1988). A year later, the lectins concanavalin A, wheatgerm agglutinin, *Lens culinaris agglutinin, Vicia faba gglutinin, Pisum sativum* agglutinin and phytohaem (erythro) agglutinin were found to bind to gp120. They were able to inhibit fusion of HIV-infected cells with CD4 cells by a carbohydrate-specific interaction with the HIV-infected cells (Hansen *et al*., 1989). Plant lectins displayed anti-coronaviral activity, especially mannose-binding lectins, in severe acute respiratory syndrome coronavirus. They interfered viral attachment in early stage of replication cycle and suppressed the growth by interacting at the end of the infectious virus cycle (Keyaerts *et al*., 2007).

(E) Antibacterial Activity of lectin:

Lectins play a role in plant defense, not only against phytopathogenic invertebrates, herbivores or fungi, but also against bacteria. It must be through an indirect mechanism that is based on interactions with cell wall carbohydrates or extracellular glycans. It has

been suggested, for instance, that the potato lectin (which is considered as a cell wall protein) immobilized avirulent strains of *Pseudomonas solanacearum* in the cell wall (Sequeira and Graham, 1977).

Lectins localized at the root hairs are the entry sites for rhizobia. The lectins then aggregate the rhizobia in the root nodules and make them immobile (Hamblin and Kent, 1973; Bohlool and Schmidt, 1974; Diaz et al., 1989; Brewin and Kardailsky, 1997; and Hirsch *et al*., 1995). Type specificity of host-parasite interactions between leguminous plants and particular strains of rhizobia infecting them is determined by lectins. The expression of the pea lectin gene in white clover roots enabled them to be nodulated by a rhizobium strain specific for the peaplant (Van Eijsden *et al*., 1995).

In 1936, a using lectin in clinical microbiology began when Summer and Howell (Summer and Howell, 1936) had a report that Concanavalin A can agglutinated various *Mycobacterium spp.* Concanavalin-A could be precipitated various bacterial polysaccharides, with interacts specifically with bacterial cell walls containing glycosidic residues associated with teichoic acid. Concanavalin A aggregated a variety of Gram negative bacteria specifically *Helicobacter pylori* coccids and *Salmonella typhimurium* (Khin *et al*., 2000; Naughton *et al.*, 2000; Minor *et al*., 1973). It was also reported that fucose-specific *Tetragonolobus purpureas* (Lotus A) and N-acetyl glucosamine-specific *Triticum vulgaris* (WGA) lectins agglutinated *Helicobacter pylori* coccids (Khin *et al*., 2000) and EuniS lectin agglutinate *Staphylococcus aureus*, *Streptococcus sp., Klebsiella sp*. and *Pseudomonas aeruginosa* (Oliveira *et al*., 2008).

1.15 Lectins in Food

The ability to agglutinate human erythrocytes or representatives of human indigenous microflora was detected in 29 of 88 food items. Many foods contained substantial amounts of agglutinating activity, and lectins extracts could be diluted several folds and

still produce agglutination. Great variation was observed in agglutination activity in the same food item purchased from different stores or from the same store on different days. Sometimes a food that possessed substantial activity on one day was found to have little or even no activity on other day.

A survey of the fresh and processed foods found lectins in about 30% of the food stuffs tested, including such common foods as salad, fruits, spices, dry cereals and roasted nuts. However, dry heat may not completely destroy lectin activity. Hemagglutinating activity is found in the processed wheatgerm, peanuts, and dry cereals. Several lectins are resistant to proteolytic digestion e.g., wheatgerm agglutinin, tomato lectin and navy bean lectin. (http://www.ansci.cornell.edu/plants/index.html).

1.16 Toxicity of Lectins

Different lectins have different levels of toxicity, though not all are toxins. The high toxicity of castor beans was recognized during the last century when its extract was found to agglutinate a suspension of erythrocytes of different animal species. Since then, lectins were studied and extracted from plants, including fungus and lichens, as well as in animals.

Pathological lesions occur in animals injected with kidney beans extracts. Various tissues suffer from parenchymatous, fatty degeneration, and edema. Hemorrhages are observed in the stomach, the intestinal wall, and other organs. Morphological changes in rats fed raw kidney beans develop multiple histological lesions; observed a reduced intestinal absorption of glucose indicating hypoglycemia in rat.

Diet rich in raw soybean has a goitrogenic effect. This is indicated by the fact that fecal loss of thyroxin from the gut is higher in animals fed raw soybeans than in the controls. Raw soybean meals reduce fat and fatty acids absorption (not soybean trypsin inhibitor)

in young chicks. Such meals also depress the utilization of vitamin D in turkey. These effects are not found when the meals include heated soybean.

Ricin, abrin, crotin, and related toxins, produce similar macroscopic and microscopic pathological lesions. The intensive inflammation with destruction of epethelial cells, edema, hyperemia, and hemorrhages in the lymphatic tissues are very common. Several signs of toxicity may include: fatty degeneration and necrosis in the liver, degenerative lesions of the myocardium, and extension and presence of blood clots of capillaries of all organs. (http://www.ansci.cornell.edu/plants/index.html).

1.17 Tuber Lectins Isolated from Different Plant Sources

In general, plant tubers are rich in starch and indeed they are often considered solely as a source of carbohydrate for diets and industrial uses. However, they do contain protein which varies in amount from about 1-10% (D.Wt). Till now most of the lectins purified from plant sources are mature seeds, fruits, rhizome, and tuber. Plant tubers are known to possess defense-related proteins such as chitinase and lectins (Shewry, P. R., 2003) that are responsible for various insecticidal, anti-fungal anti-microbial functions. Different sugar specific lectins were purified from the tubers of different plants, such as, a mannose binding homotetrameric lectin of approximately 50 kDa was purified from edible arum maculatum tuber, another novel mannose-binding tuber lectin with in vitro antiproliferative activity towards human cancer cell bearing molecular weights of 48 kDa. A tuber lectin from *Arisaema jacquemontii* belonging to family *Araceae* by employing a single step affinity chromatography using column of asialofetuin-linked amino activated silica. An anti-insect and anti-cancer lectin has been isolated from *Arisaema helleborifolium* Schott by affinity chromatography using asialofetuin-linked amino activated silica beads.

1.18 AIM OF THE STUDY

Lectins are a class of proteins of non-immune origin, which show a very specific interaction with carbohydrates, and carbohydrate-containing compounds (e.g. polysaccharides, glycoproteins, and glycolipids), which can be free or bound in cell membranes.

Lectins have attracted much interest of the researchers in the world over last decades because of their unique biological properties and potential applications in various fields of biomedical research. Because of their carbohydrate binding specificity, they have wide range of applications in the purification and characterization of glycoconjugates and therefore it has immense value in analytical and preparative biochemistry. In the recent years, lectins have been proved to be attractive for research works due to their role in cell agglutination, preferential agglutination of tumour cells and mitogenicity, leucocytes transformation, tracing neuronal pathways, fractionation of lymphocytes and bone marrow cells for bone marrow transplantation etc. Through painstaking research, Lectin Labs identified the precise lectins that bind with those carbohydrate receptors that occur on many types of pathogens. By using the 'right' lectin, the company has developed lectin formulations, which are able to interfere with or destroy the development of the disease-causing process, even in cases where antibiotics are ineffective.

Thousands of lectins were purified from plant, animal, fungus and bacteria. However, plants are generally good sources of lectins and many lectins with different specificity were purified and characterized from wide varieties of plant sources. Plant lectins have many functions including growth regulation, carbohydrate transport, and plant defence through interaction with microorganisms as well as insect and mammalian predators.

Bangladesh is a tropical riverside country, and varieties of land and aquatic plants are available here. Hence, we have to plan to isolate novel lectins from variety of locally

cultivated species of plant sources that could have different biological activities but their natures are not fully elucidated.

Although different sugar specific lectins were purified from tubers of different sources, but till now purification of lectin was not reported from tubers of *Nymphaea nouchali.*

The objective of the present research works are summarized below:

- Purification of a lectin and some other proteins from *Nymphaea nouchali tuber, cultivated in Bangladesh.*
- Characterizations and physico-chemical studies of the lectin.
- Structural analysis (determination of N-terminal sequence and amino acid composition) of the lectin.
- Analysis of the Ca^{+2} dependent structural changes of the lectin by Fluorescence spectroscopy.
- Antimicrobial and cytotoxicity assay of the lectin.
- Antitumor study of the purified lectin against Ehrlich ascites carcinoma (EAC) cells *in vivo* in mice.
- Fluorometric study of the lectin treated EAC cells and apoptotic related genes (*bax, bcl-2* & *bcl-X*) expression by RT-PCR.

The present thesis illustrated the purification, characterization and studies of biological activity of a novel lectin from *Nymphaea nouchali* tuber, which comprises in five chapters. Different chapters are presented systematically in briefly as mentioned below:

- **Chaptre I** described the introduction and aim of the present research.

- **Chapter II** described the purification and characterization of a novel lectin (named NNTL) from *Nymphaea nouchali* tuber. This chapter also demonstrated the methods for purification of four proteins isolated from the *Nymphaea nouchali* tuber, and characterizations of the purified lectin are described with respect as well as test of erythrocytes agglutination and glycoprotein nature, and the study of lectin stability by several physical and chemical agents.

- **Chapter III** described the structural analysis of NNTL by sequence and compositional determination, and confirms the structural stability by fluorescence spectroscopy.

- **Chapter IV** described the study of biological activity of the novel lectin, which comprises very significant property as antimicrobial and cytotoxic effect of NNTL.

- **Chapter V** mentioned the anti-tumor activity of the purified lectin, where growth inhibition of the tumor cells and expression of apoptosis regulating genes have been studied.

2.1 INTRODUCTION

Animal kingdom is the rich sources of proteins but plants kingdom also possesses a lot of proteins and most of them are glycoproteins in nature. Lectins isolated from wide varieties of plants sources have recently attracted much interest for the researchers, because of their remarkable biological effects are mentioned in the previous **(chapter-1).** Lectins are being used increasingly to probe the structure of carbohydrate on the surface of the normal and cancer cells. Many authors have described the isolation, purification, chemical and biological activities of lectins from different plant sources. Water lily is available in Bangladesh and used as a medicinal plant by different cultures of the world. The water lily tuber is a good source of proteins but still now no detailed research work has been done on it.

This study describes the isolation, purification, characterization and structure-function analysis of *Nymphaea nouchali* tuber protein.

2.2 MATERIALS AND METHODS

2.2.1 Collection of *Nymphaea nouchali* tuber

During the late rainy season, water lily floating at ease in almost every water bodies in Bangladesh. The *Nymphaea nouchali* tuber were collected from the Sirajgong and Barishal region of the country. After collection the tubers were peeled off, sliced into small pieces and air dried at atmospheric temperature. The dried pieces of tuber were grinded into fine powder and was stored at 4°C in a airtight container.

2.2.2 Preparation of fat-free powder

In order to isolate protein from *Nymphaea nouchali* tuber powder in biologically active form, all the operations were performed at 4°C. First the powder was suspended in a

beaker with n-Hexane and then homogenized with a homogenizer. The oily extract was then kept in a beaker at 4°C for an hour with occasional stirring. The homogenate was then filtered through a muslin cloth. The process was repeated to make the homogenate quite fat-free. The powder was then air dried at room temperature, and used for the extraction of protein.

2.2.3 Preparation of crude protein extract

The fat-free dry powder (100gm) was mixed with pre-cooled extracting solvent (10mM Tris-HCl buffer, pH 8.2 containing 0.15 M NaCl in the ratio gm/5ml solvent w/v) in a beaker and kept overnight at 4°C with occasional stirring . After a while, the suspension was filtered through a clean muslin cloth and finally the filtrate was collected by centrifugation at 12,000 rpm at 4°C for 20 minutes. Then the clear supernatant obtained was dialyzed against distilled water for 24 hours and then against 10 mM Tris- HCl buffer, pH 8.2 for 12 hours at 4°C. After dialysis crude sample was centrifuged again at 12,000 rpm at 4°C for 15 min and the supernatant was collected as crude protein extract. The crude protein extract was stored in a deep freezer and centrifuged every time before use.

2.2.4 Purification of proteins from crude extract

The following techniques were followed for the purification of crude protein extract:

2.2.4.1 Ion-exchange chromatography on DEAE Cellulose column

The crude protein samples after dialysis against distilled water and against 10 mM Tris-HCl buffer, pH 8.2 were subjected to anion-exchange chromatography on a DEAE - cellulose column (1.5 ×12 cm) at 4°C which was previously equilibrated with the same buffer. The column was first washed by 30-50 ml of 10 mM Tris-HCl buffer, pH 8.2 to collect the unbound fraction and finally the proteins were eluted by the sodium chloride salt with increasing of concentration from 0-0.5 M in 10 mM Tris-HCl buffer, pH 8.2. The eluted fraction was collected in an automated fraction collector adjusting 2.5 ml/tube at a flow rate of 1ml/min. Absorbance of each fraction was measured at 280 nm and the protein concentration was determined by the Lowry method (Lowry *et al*., 1951).

2.2.4.2 Hydrophobic chromatography on HiTrap phenyl HP column

The eluted fraction of DEAE cellulose column was dialyzed against distilled water for 12 hours and was mixed with an equal volume of 20 mM Tris-HCl buffer, pH 8.2 containing 1.0 M $(NH_4)_2SO_4$ and subjected to hydrophobic chromatography on a HiTrap phenyl HP column (5ml), which was previously equilibrated with the same buffer. First we collected the unbound fraction and then the bound fraction was eluted by a decrease of ammonium sulfate concentration in the same buffer from 0.5-0.0 M. The eluted fractions were applied to the next step for further purification.

2.2.4.3 Ion-exchange chromatography on HiTrap Q FF column

The unbound fraction from hydrophobic column, which showed haemagglutination activity was dialyzed against distilled water for 12 hours and then against10 mM Tris-HCl buffer pH 8.2 for 12 hours and finally subjected to ion-exchange chromatography on a

HiTrap Q FF column (5 ml) was previously equilibrated with the same buffer. The elution was performed by the increase of salt concentration from 0.0-0.5 M NaCl in 10 mM Tris-HCl buffer pH 8.2.

The bound fraction obtained from hydrophobic chromatography was also dialyzed against DW and 10 mM Tris-HCl buffer pH 8.2 and then applied for purification further to the anion-exchange chromatography on DEAE-cellulose column and the elution was performed by the increase of sodium chloride salt concentration from 0-0.5 M in 10 mM Tris-HC l buffer, pH 8.2.

2.2.5 Test of Purity

Sodium dodesyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

The purity of separated *Nymphaea nouchali* tuber proteins obtained from hydrophobic and ion-exchange chromatography was tested by using SDS-PAGE in 12.5 and 15% (w/v) polyacrylamide gel according to the method of Laemmli (1970).

2.2.6 Characterization of the Purified Proteins

2.2.6.1 Determination of Molecular mass by SDS/PAGE method

Polyacrylamide gel electrophoresis in presence of SDS seperates proteins almost exclusively on the basis of their mass. Protein-SDS complexes will therefore all move towards the anode (positive electrode) during electrophoresis and their movements are inversely proportional to their molecular weights. Molecular mass of the purified proteins from *N.nouchali* tuber was determined by SDS/PAGE using 15% (w/v) polyacrylamide gel according to the method of Laemmli (1970). In this experiment Bovin serum albumin (67 kDa), Ovalbumine (45 kDa), Carbonic anhydrase(29kDa), Trypsin inhibitor (20 kDa), and Lysozyme(14.6 kDa) were used as marker proteins.

2.2.6.2 Haemaglutination Assay

Materials:

(a) Collection and preparation of 2% blood

Different human blood types (A, AB, B, and O) were collected from healthy donor (about 1 ml of every group) in saline. Animal blood samples were also collected from mouse and hens from departmental animal house. All of these blood samples were centrifuged at 3000 rpm for 5 minutes. This procedure was repeated three times to prepare 2% blood cells.

(b) Preparation of Haemagglutination buffer

20 mM Tris-HCl buffer was prepared containing 0.95% NaCl & 10 mM $CaCl_2$, and the pH was maintained to 7.8.

(c) Purified protein solution

Procedure:

The haemagglutination assay was performed in a 96-well microtitre plates in a final volume of 100 µL, containing 50 µL of 2% suspension of albino rat erythrocytes previously washed with 0.95% NaCl and 50 µL of two-fold serially diluted lectin solutions. The plate mixture was placed in a shaker incubator adjusting 37^0C for 5 minutes. After that the plate was kept at room temperature for 30 minutes, and haemagglutination was examined under microscope. The agglutination titer of the maximum dilution giving positive agglutination was recorded (Atkinson, *et al.*1980).

2.2.6.3 Haemagglutination-inhibition Studies

Materials:

(a) Haemagglutination buffer: 20 mM Tris-HCl buffer, pH 7.8 containing 1% NaCl & 10 mM $CaCl_2$.

(b) 2% RBC in 0.95% NaCl solution.

(c) Sugars and protein used for inhibition Study:

D-Glucose,	L-Fucose	*O*-Nitrophenyl-ß-D-glucopyranoside
D-Galactose	Inositol(meso) inactive	*O*-Nitrophenyl-ß-D-galactopyranoside
D-Maltose	N-Acetyl-D-glucosamine	4-Nitrophenyl-α-D-glucopyranoside
D-Mannose	N-Acetyl-D- galactosamine	4-Nitrophenyl-α-D-galactopyranoside
D- Melibiose	Methyl-α-D-glucopyranoside	4-Nitrophenyl-α-D-mannopyranoside
D-Xylose	Methyl-α-D-galactopyranoside	4-Nitrophenyl-ß-D-glucopyranoside
D-Raffinose	Methyl-α-D-mannopyranoside	4-Nitrophenyl-ß-D-mannopyranoside
L-Arabinose	Methyl-ß-D-glucopyranoside	Fetulin (glycoprotein)
L-Rhamnose	Methyl-ß-D-galactopyranoside	

Procedure:

The haemagglutination inhibition test was performed in the presence of different sugars as follows-

25µl of haemagglutination buffer were added to the selected every well of the 96 well titre plate. Then 25 µl of sugar solutions were added to the first well of the titre plate and serially diluted upto 8 well & then 25 µl of purified protein solutions were added to the wells of the titre plate. Finally 50 µl of 2% blood cells (RBC) in saline were added to the wells of titre plate, and then this plate were shacked by microshaker and incubated at 37^0C for 60 minutes (Atkinson *et al.,* 1980).

2.2.6.4 Determination of the Protein Concentration

Concentration of the purified protein of *Nymphaea nouchali* tuber was determined according to the method of Lowry *et al*., (1951).

2.2.6.5 Test for Glycoprotein and Determination of Sugar content

2.2.6.5.1 Phenol-sulfuric acid method

Phenol in the presence of sulfuric acid can be used for quantitative colorimetric micro determination of sugars and their methyl derivatives, oligosaccharide and polysaccharides. The sugar content of NNTL was determined following the phenol-sulfuric acid method (Dubois *et al.*, 1956) using D-glucose as standard.

2.2.6.5.2 Periodic Acid-Schiff's (PAS) staining method

The glycoprotein nature of the purified protein was detected by the PAS staining method (Fairbanks *et al*., 1971) as follows:

Reagents and Solutions:

(A) Preparation of Staining (PAS) Reagent:

1. Dissolved 5g of basic Fuchsin in 900 ml of boiling distilled water,
2. Cooled to approximately 50ºC and slowly add 100 ml of 1N HCl.
3. Cooled to approximately 25ºC and dissolved 10 g of $K_2S_2O_5$,
4. Shaked for 3 min and incubated in the dark at room temperature for 24 hours.
5. Added 5 gm of fine activated charcoal and shake for 3 min.
6. Filtered the solution (should be clear).
7. Stored at 4ºC in a foil-covered bottle.

(B) Test for the Schiff's Reagent:

Poured 10 ml of 37% formalin into a watch glass. Added a few drops of the schiff reagent, and observed the following changes-

- Rapidly develops Red-purple color –Good
- Delayed development of deep blue-purple color – Deteriorated.

Procedure:

(a) Applying sample in PAGE:

The 15% polyacrylamide gel was prepared and the protein pattern of the selected fractions was determined by SDS-PAGE according to the method of Laemmli (1970).

(b) Staining of the gel:

After recovery, the gel was stained with Periodic Acid-Schiff's base staining solution in dark for 10 min. at 4^0C.

(c) Distaining of the gel:

The gel was removed from the staining solution and washing the gel with 0.58% potassium metabisulfite in 3% acetic acid (in water). The washing was continued with this solution at 30 minute intervals for at least 1 hour or until background (if any) is eliminated.

2.2.6.6 Determination of pH stability

The organized native structure of a protein is greatly affected by the changes with pH in different experimental conditions. The stability of NNTL was examined at different pH solution by observing the haemagglutination activity of rat erythrocytes.

Buffer solutions: The following buffers were used for pH stability assay:

(1) 0.1 M sodium acetate (pH 3.0 to 6.0),

(2) 0.1 M phosphate (pH 7.0),

(3) 0.1 M Tris–HCl (pH 8.2),

(4) 0.1 M glycin-NaOH (pH 9.0 to 10.0).

The NNTL samples (0.25 mg/ml) was incubated with the buffer solutions within the pH ranges from 3.0 to 10.0, containing 0.15 M NaCl at room temperature for 8 h. The pH of the protein solutions were adjusted to pH 7.8 by dialyzing against 20 mM Tris–HCl buffer (pH 7.8) containing 0.15 M NaCl for 12 h. Then the retained relative haemagglutination activity was determined for each lectin separately.

2.2.6.7 Determination of Heat stability

The thermal stability of NNTL was examined in various temperature and their effects on the haemagglutination activities have been observed. The purified lectin samples (0.5 mg/ml) mixed with 10mM Tris-HCl buffer saline, pH 7.8 and incubated in a controlled water bath at distinct temperatures in the range of 30-90ºC for 30 minutes. After cooling the heated sample at room temperature, the relative haemagglutination activity was determined by agglutination with rat erythrocytes in a microtitre plate. 50 µl of NNTL was serially diluted with an equal amount of haemagglutination buffer, pH 7.8 and the haemagglutination activity was observed. The non-heated lectin sample was used as a control, which denoted 100% activity.

2.2.6.8 Effect of Denaturants and Metal ions

NNTL in 0.1 M Tris-HCl buffer saline was incubated with 50 mM DTT (dithiothreitol) and 4 M of Urea at room temperature for 12 h. NNTL in the same buffer without denaturants was used as a control and its activity was considered as 100%. Haemagglutination activity of NNTL was also checked in the presence and absence of some metal ions to determine the dependency on divalent cations.

The effect of different metallic salts on the stability of NNTL was also observed. The purified lectin solutions were incubated with 0.1 M EDTA for 2 hr at room temperature. Then the solutions were dialysed against 20 mM Tris-Hcl buffer, pH 7.8 for 12 hr at 4ºC and finally the haemagglutination activity was determined in the presence and absence of each Ba^{2+}, Ca^{2+} and Mg^{2+} in the haemagglutination buffer.

2.3 RESULTS

2.3.1 Purification of *Nymphaea nouchali* Tuber Proteins

Proteins was purified from crude protein extracts of *Nymphaea nouchali* tuber through ion-exchange chromatography on DEAE cellulose column, followed by hydrophobic chromatography on HiTrap Phenyl HP column and finally re-applying to anion-exchange chromatography on a HiTrap Q FF column.

2.3.1.1 Ion-exchange chromatography on DEAE Cellulose column

At first proteins was purified from crude protein extracts of *Nymphaea nouchali* tuber through ion-exchange chromatography on DEAE cellulose column (1. 5× 12 cm). As shown in Fig. 2.1, the components of the crude extract were eluted as two distinctive peaks as F-1 and F-2. The F-2 fraction although showed as major peak in the figure, but it cannot used for further study, as it contained mostly intense colour material. On the other hand, F-1 fraction contain negligible amount of colour but cantain mostly protein absorption, which was confirmed by Lowry method. Unbound fraction (results not shown) as well as bound fraction, F-2 showed no such detectable haemagglutination activity, whereas fraction F-1 showed strong haemagglutination activity was collected, pooled and used for further purification by hydrophobic chromatography.

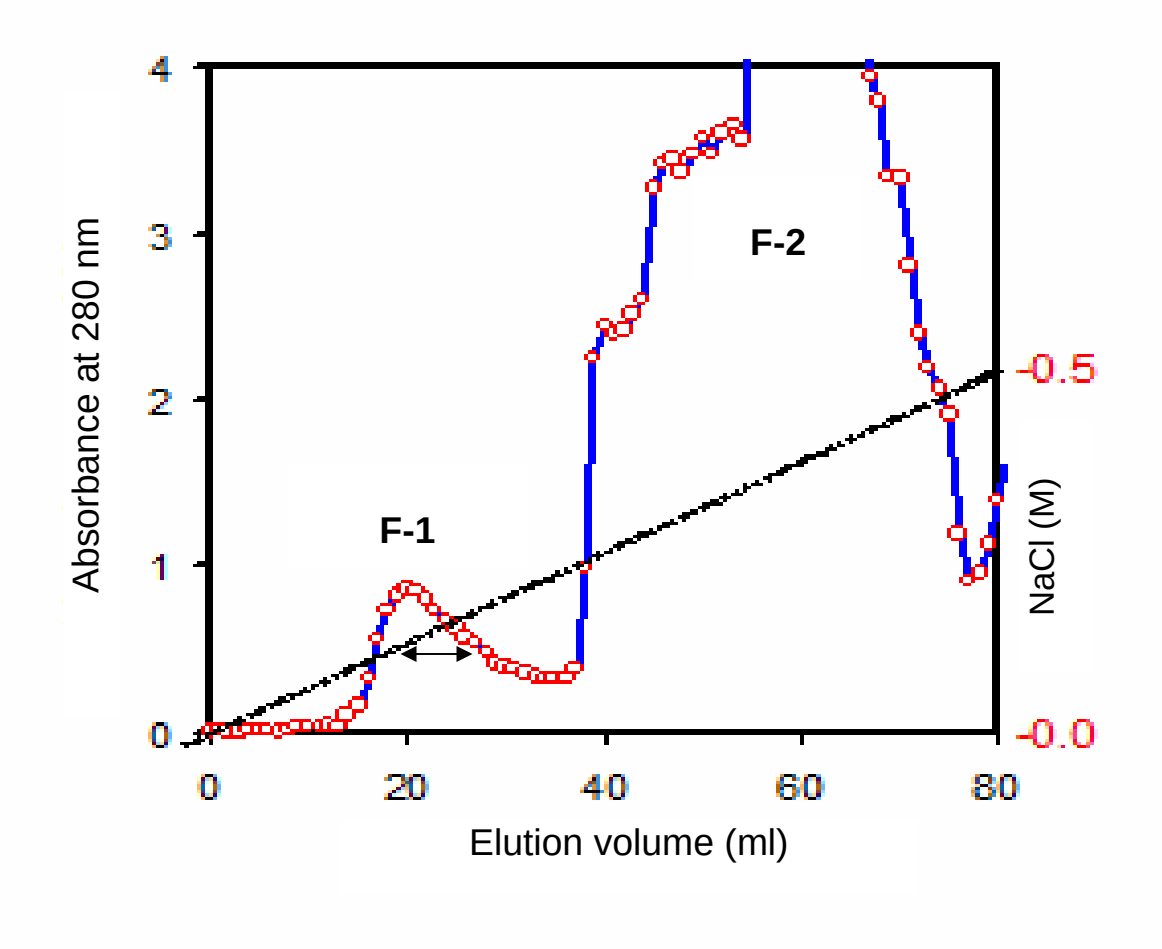


Figure 2.1 Ion exchange chromatography of NNTL on DEAE Cellulose column. Crude protein was applied to a DEAE cellulose column (2.5 x 12 cm) previously equilibrated with 10 mM Tris-HCl buffer pH 8.2. Proteins were eluted with the same buffer with the gradually increase of NaCl gradient from 0-0.5 M. The elution profiles were monitored at 280 nm. Fractions (2.5 ml/tube) were collected at a 1 ml/min flow rate.

2.3.1.2 Hydrophobic chromatography on HiTrap phenyl HP column

The F-1 fraction obtained from ion-exchange chromatography was dialyzed against distilled water and 10 mM Tris-HCl buffer, pH 8.2 and subjected to hydrophobic chromatography on a HiTrap Phenyl HP column (5ml), which was previously equilibrated with the same buffer containing 0.5 M $(NH_4)_2SO_4$. Elution was performed by the same buffer and collected the unbound (UB) fraction, whereas the bound (B) fraction was occurred by a linear decrease of ammonium sulfate concentration in the same buffer as shown in Figure-2.2. The UB fraction showed haemagglutination activity, whereas the bound fraction (B) didn't show any activity. The UB fractions contained some impurities as checked by SDS-PAGE, hence subjected to an anion-exchange chromatography for further purification.

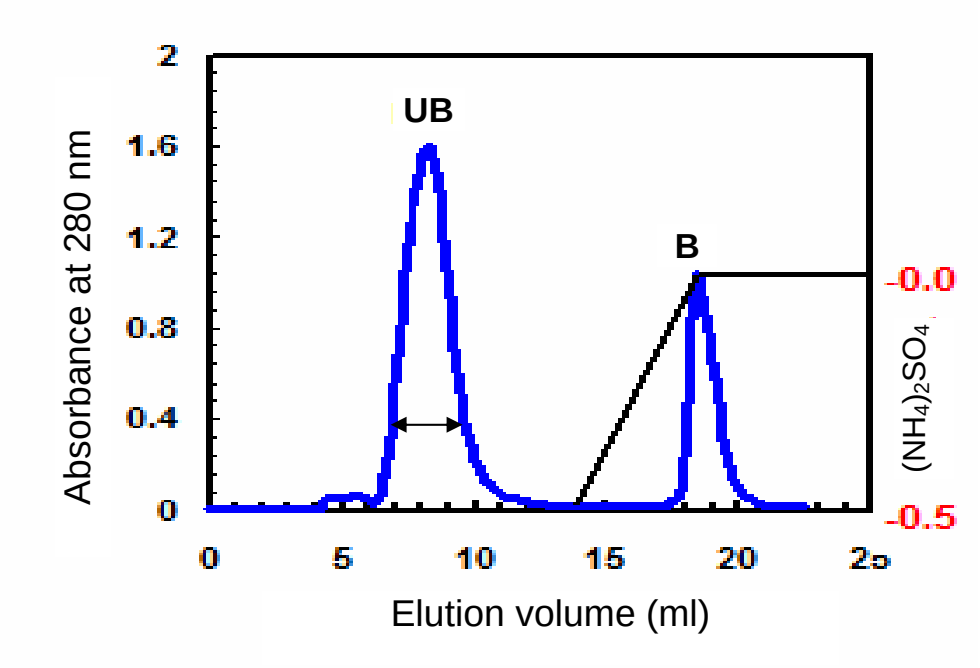


Figure 2.2 Hydrophobic chromatography of F-1fraction obtained by ion-exchange chromatography was applied to a HiTrap Phenyl HP column (5ml) previously equilibrated with the 10 mM Tris-HCl buffer containing 0.5 M $(NH_4)_2SO_4$. Bound fraction was eluted by the same buffer without $(NH_4)_2SO_4$ at flow rate 1 ml/min.

2.3.1.3 Ion-exchange chromatography of unbound fraction on HiTrap Q FF column

The UB fraction obtained from hydrophobic chromatography were re-applied to anion-exchange chromatography on a HiTrap Q FF column, which was previously equilibrated with 10 mM Tris-HCl buffer, pH 8.2. The elution was performed by the increase of salt concentration from 0.0-0.5 M NaCl in the same buffer. Mainly one minor peak Q(A) and another major peak Q(B) were obtained as shown in Figure-2.3. Only the QB fraction showed hemmaglutinating activity and the purity was confirmed by SDS-PAGE.

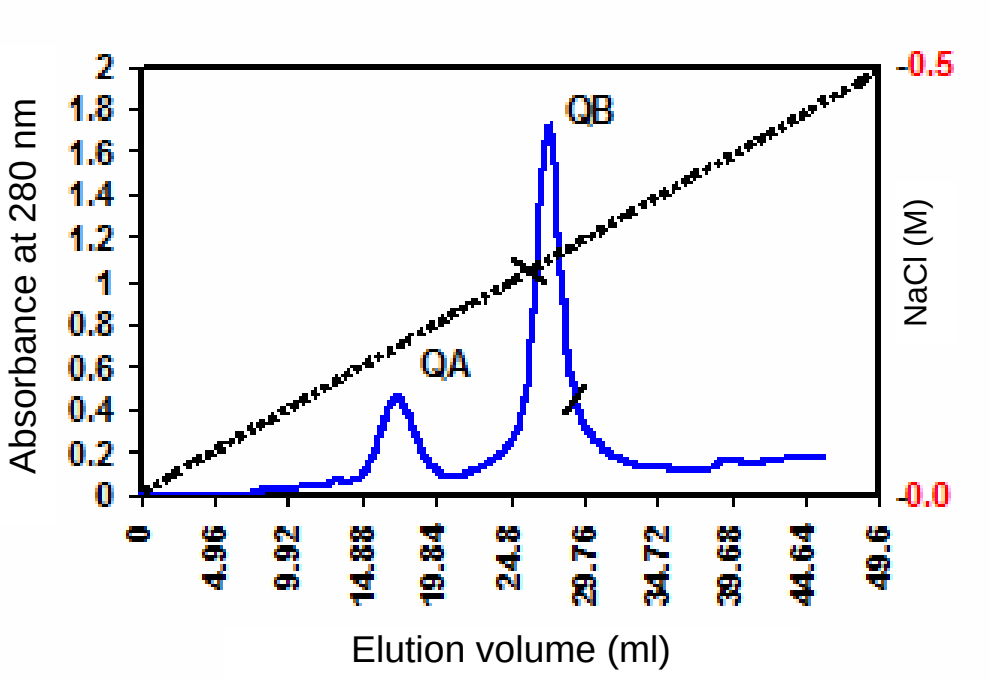


Figure 2.3 Ion-exchange chromatography of the UB fraction obtained from hydrophobic chromatography, was dialyzed against 10 mM Tris-HCl (pH 8.2) and then applied to a HiTrap Q FF column (5 ml) previously equilibrated with the same buffer. Proteins were eluted with 10 mM Tris-HCl (pH 8.2) with the gradually increase of NaCl gradient from 0.0-0.5 M. Fractions were collected at 1 ml/min flow rate.

2.3.1.4 Ion-exchange chromatography of the bound fraction on DEAE column

The bound fraction (B) obtained from hydrophobic chromatography on HiTrap Phenyl HP column did not show any visible haemagglutination activity against rat RBC. This fraction was also subjected to ion-exchange chromatography on DEAE cellulose column for purification of other non-lectin proteins. Here we obtained three major peaks F-1, F-2 and F-3 as shown in the Figure follows. The purity of these fractions were checked by SDS-PAGE.

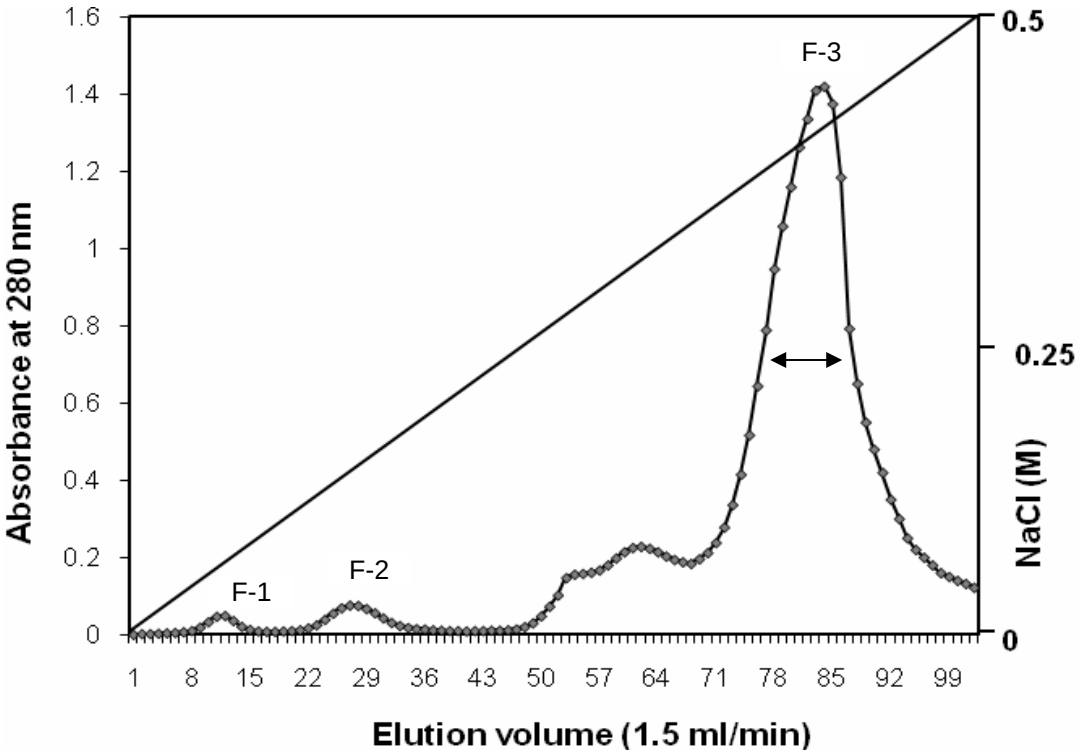
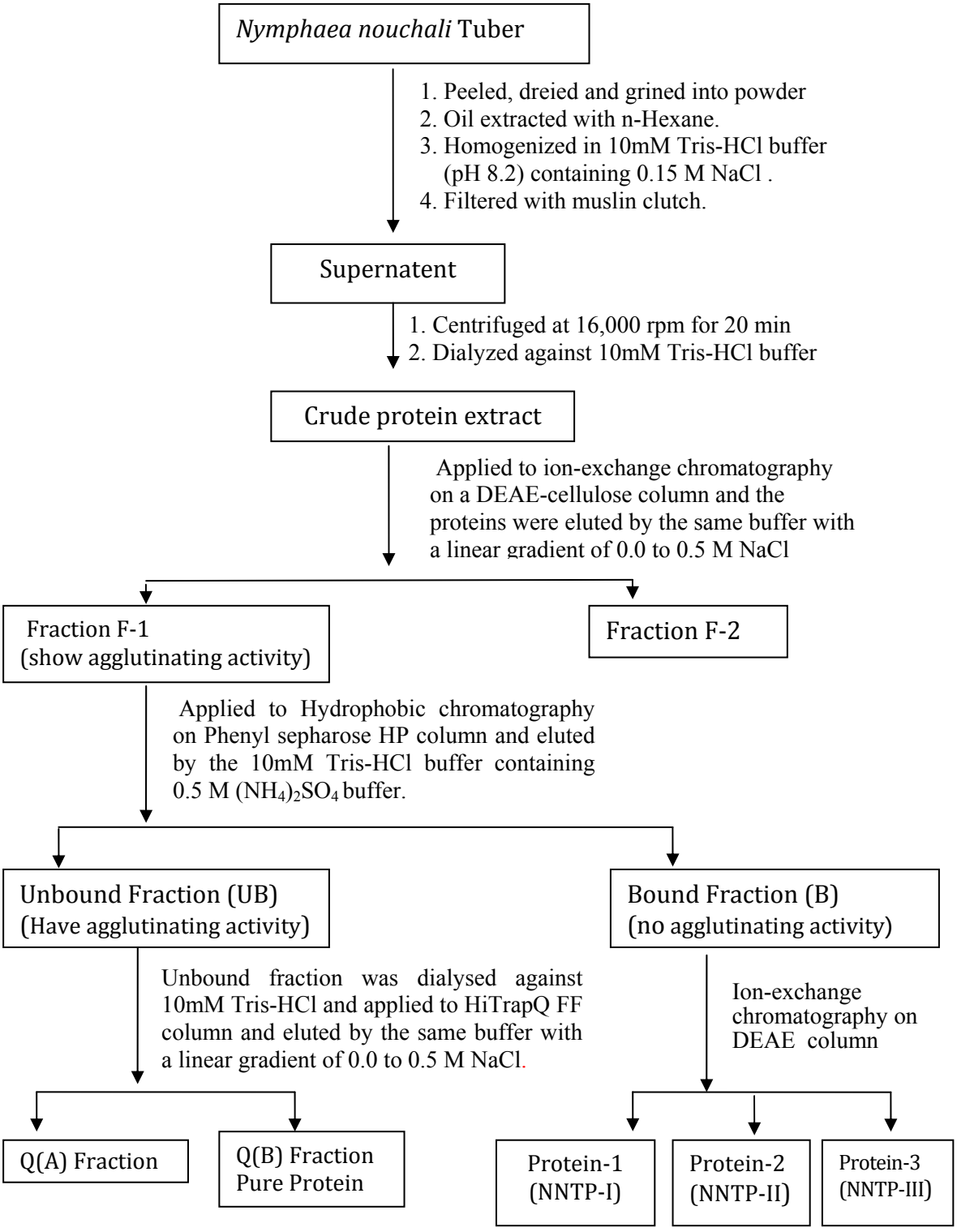


Figure 2.4 Ion-exchange chromatography of the bound (B) fraction obtained from hydrophobic chromatography, was applied to ion-exchange chromatography on DEAE cellulose column, equilibrated with 10 mM Tris-HCl buffer (pH 8.2). Proteins were eluted by the same buffer with the gradual increase of NaCl gradient from 0.0 - 0.5 M.

2.3.2 Diagram of the proteins purification from *Nymphaea nouchali* Tuber



2.3.3 Test of Purity and Determination of the Molecular mass

The homogeneity of the protein fractions obtained from different chromatography stages were checked by SDS-PAGE on 15% and 12.5% polyacrylamide gel. The molecular weight of the purified protein was then determined by SDS-polyacrylamide gel electrophoresis using Bovine serum albumin (MW. 67 kDa), Ovalbumin (MW. 45 kDa), Carbonic anhydrase (MW. 29 kDa), Trypsin inhibitor (MW. 20 kDa) and Lysozyme (MW. 14.6 kDa) as reference proteins. The molecular weight of the purified lectin as determined by SDS-PAGE was estimated to 27.0±1.0 kDa in the presence and absence of 2-mercaptoethanol (Fig. 2.8).

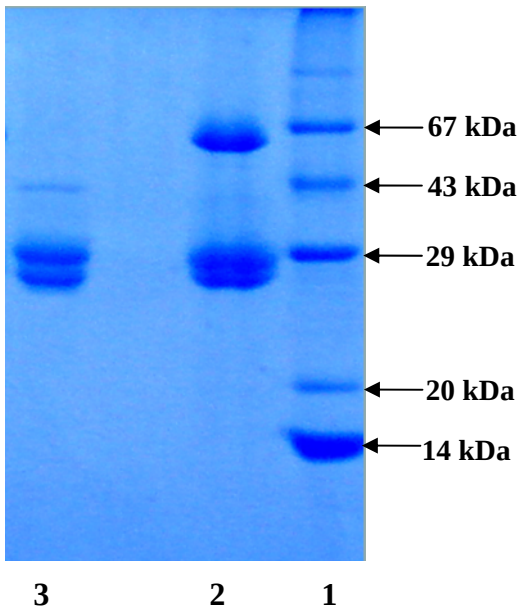


Figure 2.5 SDS-PAGE of *Nymphaea nouchali* tuber proteins obtained from Hydrophobic Chromatography on 15% polyacrylamide gel.

Lane 1: marker proteins; **Lane 2**: Bound fraction (B); **Lane 3**: Unbound fraction (UB).

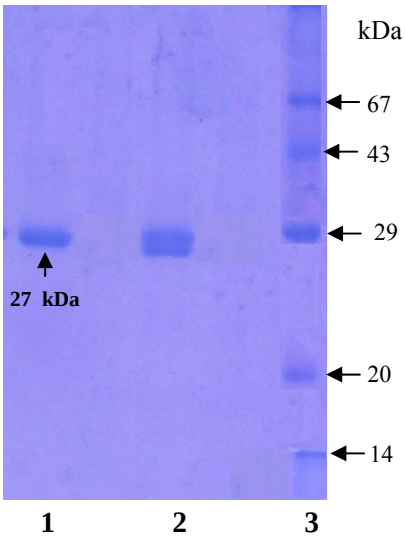


Figure 2.6 SDS-PAGE of Q (B) fraction, obtained from Ion-exchange Chromatography on HiTrapQ FF column of the Unbound fraction (UB) on 12.5% polyacrylamide gel. **Lane-1**: Pure Lectin (NNTL) with 27 kDa. **Lane-2**: Unbound fraction of Hydrophobic chromatography, **Lane-3**: Marker protein.

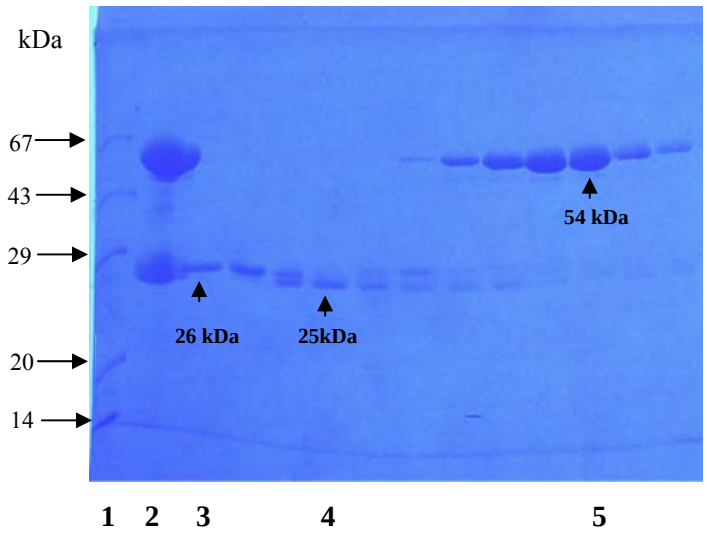


Figure 2.7 SDS-PAGE of non-lectin proteins, obtained form Ion-exchange Chromatography on DEAE column of the Bound fraction (B) on 15% polyacrylamide gel. **Lane-1**: Marker proteins, **Lane-2**: Bound fraction (B), **Lane-3:** Pure protein (NNTP-I) with 26 kDa, **Lane-4**: NNTP-II with 25 kDa; **Lane-**5: NNTP-III with 54kDa.

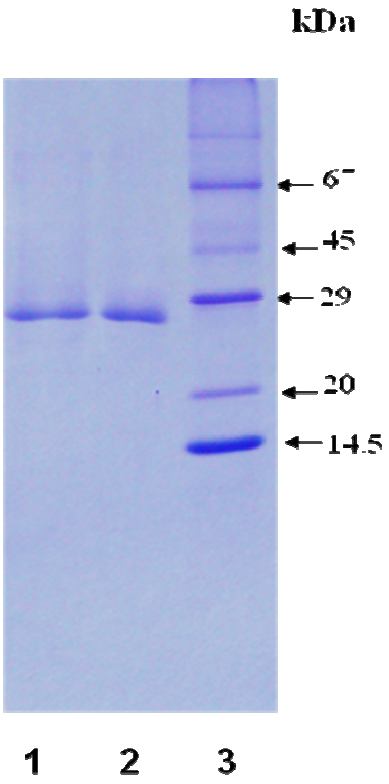


Figure 2.8 SDS-PAGE of pure NNTL in presence and absence of 2-mercaptoethanol on 15% polyacrylamide gel.

Lane 1: NNTL in the presence of 2-mercaptoethanol

Lane 2: NNTL in the absence of 2-mercaptoethanol

Lane 3: Marker proteins.

Table 2.1 Summary of the purification of *Nymphaea nouchali* tuber Proteins

Purification steps	Fractions	Total Protein (mg)	Total activity (titer/mg×10^3)	Recovery of activity (%)	Yields (%)
Crude		13700	1080	100	100
Anion-exchange Chromatography on DEAE cellulose column	F-1	210	537	49.7	1.5
Hydrophobic chromatography on HiTrap Phenyl HP	Unbound fraction	105	269	23.7	0.76
Anion exchange Chromatography on HiTrap Q FF	QB fraction (NNTL)	30	153.6	14.2	0.2

Haemagglutination activity (titre) = Reciprocal of highest dilution showing visible haemagglutination.

$$\text{Recovery of activity} = \frac{\text{Observed hemagglutination activity}}{\text{Initial hemagglutination activity}} \times 100$$

$$\text{Fold} = \frac{\text{Observed specific activity}}{\text{Initial specific activity}}$$

$$\text{Yield} = \frac{\text{Amount of pure protein}}{\text{Amountof crude protein}} \times 100$$

2.3.4 Characterization of the Purified Proteins

2.3.4.1 Haemagglutination Assay

The result showed that the purified protein QB agglutinates all human blood types and other animal erythrocytes as tested. As the QB fraction only contained the haemagglutination activity and SDS-PAGE also demonstrated the purity of protein present in this fraction, so protein must be categorized as lectin and indicated in the present thesis as Nymphaea Nouchali Tuber Lectin (NNTL).

The minimum agglutination concentration of the NNTL was found to be 8 µg/ml for rat red blood cells, 16 µg/ml for different groups of human erythrocytes (A, B, O and AB) and chicken blood cells.

Table 2.2 Agglutination of different blood types by the Pure Protein (i.e., QB).

Blood	Group	Protein (µg/ml) *
Human blood	A+	16
	B+	16
	O+	16
	O-	16
	AB-	16
	AB+	16
Chicken blood	-	16
Albino rat	-	8

*****Minimum NNTL concentration required for a visible agglutination.

2.3.4.2 Haemagglutination inhibition study

The sugar specificity of NNTL was evaluated against different mono- and oligo-saccharides in various concentrations by inhibiting the agglutination of rat red blood cells. The results of the haemagglutination inhibition of the NNTL are shown in Table-2.3. The result suggested that *O*-nitrophenyl-ß-D-galactopyranoside was the best inhibitor for inhibiting the agglutinatination of NNTL. Among the tested saccharides two others namely, *O*-nitrophenyl-ß-D-glucopyranoside and N-acetyl-D-galactosamine were also found to be specifically inhibiting the agglutinatination of the purified lectin, NNTL.

Table 2.3 Haemagglutination inhibition assay of the NNTL by different sugars and their derivatives at different concentration.

Sugar	Inhibition	Conc.(mM)
O -Nitrophenyl-ß-D-galactopyranoside	I	25
O -Nitrophenyl-ß-D-glucopyranoside	I	200
N-Acetyl-D-galactosamine	I	200
N-Acetyl-D-glucosamine	NI	200
D-Xylose	NI	200
L- Fucose	NI	200
D-Mannose	NI	200
D-Maltose	NI	100
L-Arabinose	NI	200
D-Raffinose	NI	200
D-Glucose	NI	200
L-Rhamnose	NI	200
D-Melibiose	NI	200
Inositol (meso) inactive	NI	100
D-Galactose	NI	200
Methyl-α-D-glucopyranoside	NI	200
Methyl-α-D-galactopyranoside	NI	200
Methyl-α-D-mannopyranoside	NI	200
Methyl-ß-D-galactopyranoside	NI	100
4-Nitrophenyl-α-D-galactopyranoside	NI	50
4-Nitrophenyl-α-D-mannopyranoside	NI	50
4-Nitrophenyl-α-D-glucopyranoside	NI	100
4-Nitrophenyl-β-D-mannopyranoside	NI	100
4-Nitrophenyl-ß-D-glucopyranoside	NI	100
Methylyl-β-D-glucopyranoside	NI	200
Fetulin (glycoprotein) (4mg/ml)	NI	200

Symbol: 'I'means Inhibition**; 'NI'** means no Inhibition**.**

2.3.4.3 Determination of the Protein Concentration

The absorbance of 1.0 at 280 nm for NNTL corresponded to 0.25 mg of protein as determined by the Lowry method using BSA as standard.

2.3.4.4. Test for Glycoprotein and Estimation of Sugar

The glycoprotein nature of the NNTL was detected by the positive phenol-sulfuric acid test as well as by PAS staining. The NNTL gave an orange-yellow colour with phenol-sulfuric acid solution and its neutral sugar content was estimated to be 8% using glucose as a standard. The purified lectin also gave red-purple color visible band on the gel by staining with the PAS, as shown below in Fig. 2.9 (Lane P).

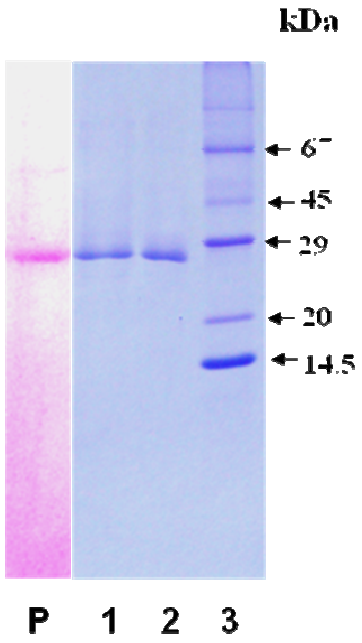


Figure 2.9 Glycoprotein Test of NNTL on 15% polyacrylamide gel. Lane 1: NNTL in presence of 2-mercaptoethanol; **Lane 2**: NNTL in absence of 2-mercaptoethanol; **Lane 3**: Marker proteins; and **Lane P**: Periodic acid-Schiff (PAS) staining of the purified lectin.

2.3.4.5 Effect of pH

To examine the stability of NNTL, the haemagglutinating activity against rat red blood cells was performed at different pH values and the results presented in the Fig. 2.10. The activity of the lectin decreased sharply below pH 5.0, i.e., at the more acidic region its activity was found to be decreased about 50% and 90% at pH 4.0 and 3.0 respectively. NNTL showed maximum activity between the pH 5.0 and 9.0, but at pH 10.0 the activity was decreased sharply from 100% to 50%.

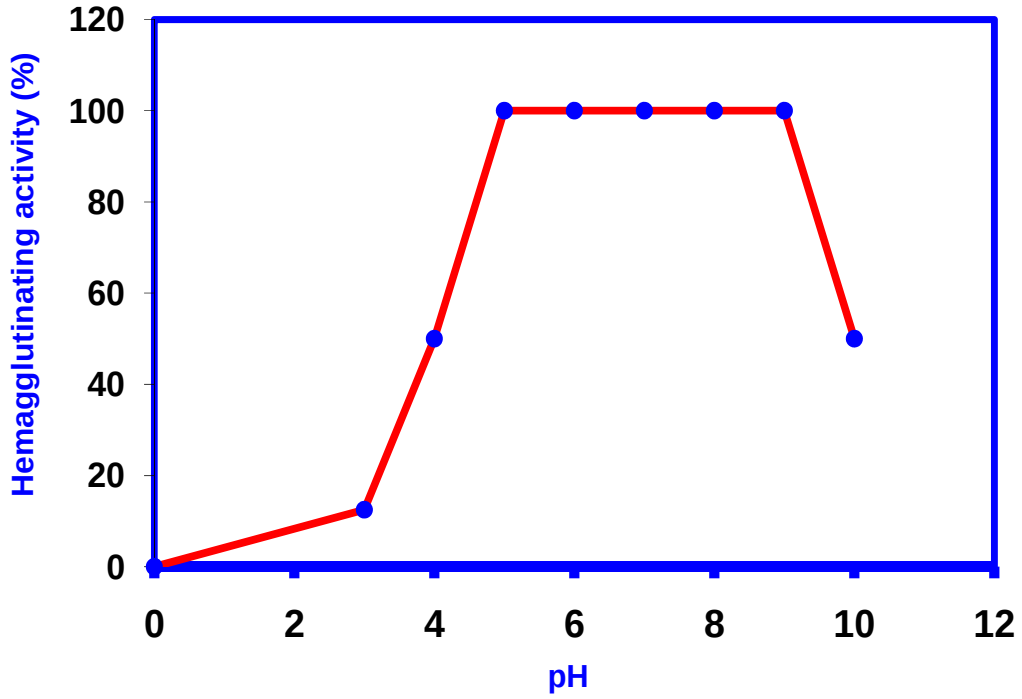


Figure 2.l0 Haemagglutination activities of NNTL at different pH values.

2.3.4.6 Effect of Temperature

The activity of NNTL was affected with the changes of temperature. The haemagglutinating activity was assayed by incubating the lectin at different temperature (ranging from 30-90ºC) for 30 min. The maximum activity was observed between the temperature 30 to 60ºC, and the activity of the lectin was decreased sharply above 60ºC and at 70ºC it becomes 50%. Thereafter upto 90ºC the activity was found to be almost unchanged.

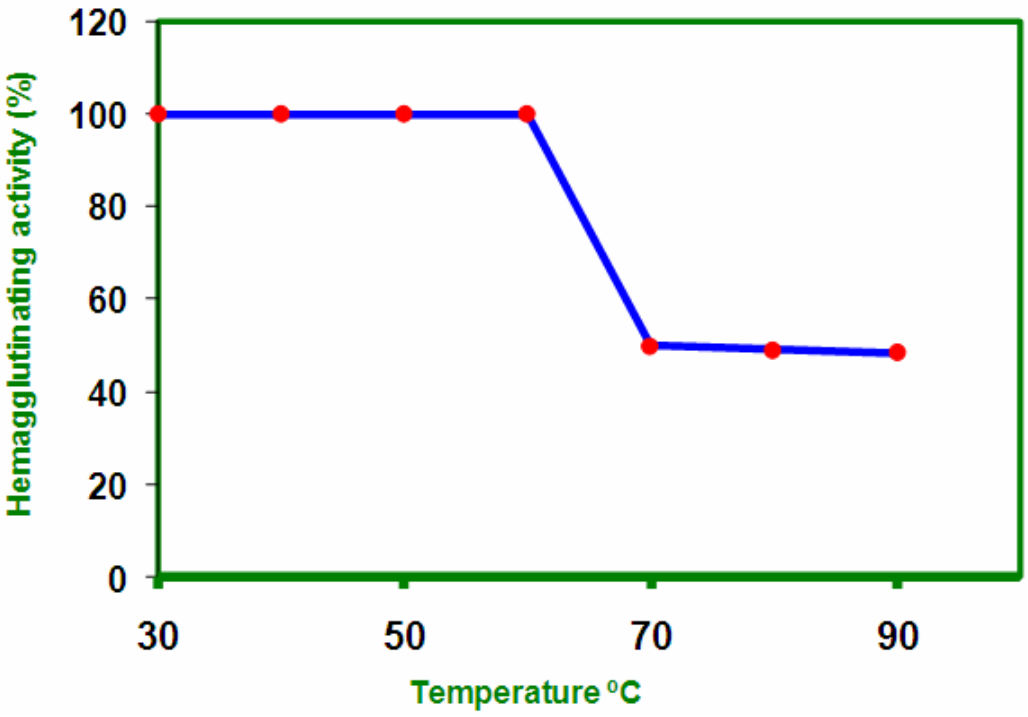


Figure 2.11 Haemagglutination activities of NNTL at different Temperatures.

2.3.4.7 Effect of Denaturants and Metal ions on NNTL activity.

As shown in Fig. 2.12, a remarkable effect was observed on the stability of NNTL, when lectin was treated with denaturants, such as DTT, Urea and EDTA. In presence of 50 mM DTT and 4 M urea the lectin lost its activity by 50% and 77.5% respectively as compared to that of the control. On the other hand, the NNTL did not show any agglutination activity when treated with EDTA in the absence of divalent ions. But after addition of 10 mM of each Ba^{2+}, Ca^{2+} and Mg^{2+} to the haemagglutination buffer NNTL showed strong agglutination activity.

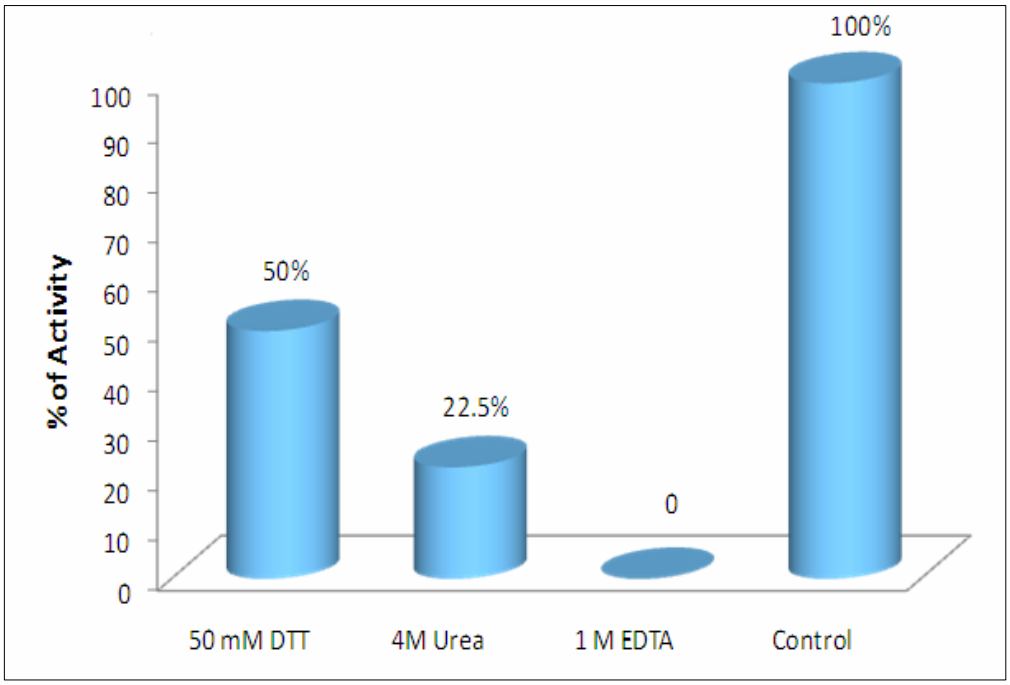


Figure 2.12 Effect of DTT and Urea on the haemagglutination activity of NNTL

2.4 DISCUSSION

A lectin was purified from *Nymphaea nouchali* tuber by using anion-exchange chromatography on DEAE cellulose column followed by hydrophobic chromatography on HiTrap Phenyl HP column and then re-applying to anion-exchange chromatography on a HiTrap Q FF column and the pure lectin was designated as NNTL. The purified NNTL moved as a single band on SDS-PAGE both in presence and absence of 2-marcaptoethanol and the molecular weight was estimated to 27±1 kDa, which is very close to the lectin from *Kaempferia rotunda Linn*. tuberous rhizome having 29±1 kDa (Kabir *et al*., 2011). Whereas lectin purified from many other tubers of *Arisaema erubescens* (Wall) Schott, *Arisaema jacquemontii* (Kaur *et al*., 2006_b), *Arisaema helleborifolium* (Kaur *et al*., 2006_b), *Arum maculatum* (Majumder *et al*., 2005), *Helianthus tuberosus* L. (Suseelan *et al*., 2002) and *Solanum tuberosum* L. tubers (Millar *et al*., 1992, Pramod *et al*., 2006, Van Damme *et al*., 2006) bearing the molecular weights ranging from 12 kDa to 20 kDa, and their characteristics are quite different from presently purified lectin. Again in presence of 2-mercaptoethanol lectin showed single band with the said molecular weight, which confirm that the lectin have no more subunit. It was similar with the lectin purified from *Kaempferia rotunda* Linn. having MW of 29.0 kDa under both reducing and non-reducing condition (Kabir *et al*., 2011).On the other hand, lectin from yam tuber (*Dioscorea batatas*) as DB1 with molecular mass of 20 kDa consisting of 10 kDa subunits, DB3 of 120 kDa composed of one 66 kDa subunit and two 31 kDa, whereas DB2 was isomer consisting of 31.0 kDa (Gaidamashvili *et al*., 2004).

The NNTL as well was a potent agglutinin for different types of animal erythrocytes (as observed against human blood types and rat and chicken RBC). The ability to agglutinate different animal erythrocytes is a common feature of the plant lectins, as studied by some researchers in Thiland (Sopit Wongkham *et al*., 1995). Lectin from the seeds of *Egyptian*

Pisum sativum and *Archidendron jiringa* Nielsen has haemagglutinating activity against human blood groups, rabbit, mouse, rat, chicken, guinea pig, geese and sheep erythrocytes (Sitohy *et al*., 2007 and Charungchitrak *et al.,* 2011). The minimum agglutination concentration of NNTL towards rat erythrocytes was 8 µg/ml. DB1 (*Dioscorea batatas* protein1) and DB2 (*Dioscorea batatas* protein 2) from *D.batatas* tuber (Gaidamashvili *et al*., 2004) agglutinate rabbit erythrocytes at 2.7 and 3.9 µg/ml respectively; whereas AJL (*Arisaema jacquemontii* lectin) from *A. jacquemontii* tuber (Van Damme *et al.*1998) required 11.5 µg/ml. The purified NNTL showed no human blood group specificity as it was agglutinated A^+, B^+, O^+, AB^+, AB^- and O^- blood group as tested. This hemaglutinating nature was similar to other lectins, for example, EspecL. (*Erythrina speciosa* lectin) and BBL (*Belamyia bengalensia* lectin) and a lectin from *Phaseolus coccineus* seeds (Chen *et al.,* 2009) can agglutinate all human blood groups (Konozy *et al.*, 2003 and Banerjee *et al.*, 2004). Different result was also observed for HTTL (*Helianthus tuberosus* lectin) and AJL (*Arisaema jacquemontii* lectin) , which could not agglutinate any human blood group (Suseelan *et al.*, 2002; Manpreet Kaur *et al*., 2006).

From the study of sugar specificity of NNTL, it was found that the lectin-induced haemagglutination was inhibited by *O*-nitrophenyl-ß-D-galactopyranoside followed by *O*-nitrophenyl-ß-D-glucopyranoside and N-acetyl-D-galactosamine. Among these sugars *O*-nitrophenyl-ß-D-galactopyranoside was the most potent inhibitor and the minimum inhibitory concentration was 25 mM. Only a few lectins found in literature have such complex glycan type specificity. *O*-nitrophenyl-ß-D-galactopyranoside specific lectins were purified from edible mushroom *Pleurotus citrinopileatus* and Samta tomato (Li *et al.*, 2008, Wang *et al.*, 2006), with the molecular mass 32.4 and 79.0 kDa.

In our investigation, NNTL was detected as a glycoprotein and the estimated content of neutral sugar was 8% , which correlates with the general range for carbohydrate

content in *Erythrina* Lectins (Konozy *et al*., 2003), TCSL (Kabir *et al*., 2012), HTTL (Suseelan *et al*., 2002) and AJL (Kaur *et al*., 2006_a) contained only 3-12%, 4.2%, 5.3% and 11.5% sugars, respectively. Whereas potato lectin contains 50% sugar on weight basis (Pramod *et al*., 2006).

The study of thermal denaturation showed that the activity of NNTL was stable at 30-60ºC. Afterwards the lectin activity starts declining and lost the activity about 50% when incubated along 70-90ºC. Heat stability of NNTL was found to be similar or higher as compared with some other tuber lectins from different sources. This result showed good agreement with that of KRL (Kabir *et al*., 2011), AJL (Kaur *et al*., 2006_a), AHL (Kaur *et al*., 2006_b) and HTTL (Suseelan *et al*., 2002) which are stable up to 60ºC, but higher than DB3 from yam tuber is stable up to 50ºC (Gaidamashvili *et al*., 2004). On the other hand TCSL showed wider temperature stability between 30-70ºC (Kabir *et al*., 2012).

NNTL showed the maximum haemagglutination activity (lectin activity) between the pH values ranging from pH 5.0-9.0, indicating that the lectin was active around the alkaline condition. Similar result was observed in lectin from *Pisum sativum* seeds, stable at pH 5.0 to 9.0 (Sitohy *et al*., 2007) and also to those of the lectin from *Annona muricata* seeds (Damico *et al*., 2003). On the other hand, above and below of this optimum pH value the purified lectin lost its activity significantly, indicating the changes in structure of lectin-binding sites or ionization of the groups associated with the sugar binding. Again the hemaglutination activity of NNTL was also found to be higher at pH 10.0 than that of at pH 3.0, which suggests that the activity of NNTL is favorable in basic pH. The pH stability of NNTL is found to be very close to EspecL (Konozy *et al*., 2003), AJL (Kaur, *et al*., 2006_a) and HTTL (Suseelan *et al*., 2002).

Several denaturants are destructive causing for denaturation of the native structure of lectin. By treatment with DTT and urea, the haemagglutination activity of NNTL lost by 50% and 77.5% respectively. Such property also similar to the lectin of *Arisaema helleborifolium* Schott, when treated with 3.0 M urea reduces 50% of the activity (Kaur *et al*., 2006_b). The denaturation by these agents indicates the globular nature of lectins that was stabilized mainly by hydrophobic interactions (Nelson *et al*., 2001). On the other hand NNTL lost its haemagglutination activity when incubated with chelating agent EDTA, but subsequent addition of 10 mM of Ca^{2+}, Ba^{2+} and Mg^{2+} the activity of the lectin was restored. The overall investigation suggest that NNTL is a metal ions dependent lectin, which was also found in EspecL (Konozy *et al.*, 2003) and DB3 (Gaidamashvili *et al*., 2004) when treated with chelating agent EDTA. Whereas EDTA treatment did not affect the agglutinatination activity of DB1 (Gaidamashvili *et al*. 2004), AJL (Kaur *et al*., 2006_a) and HTTL (Suseelan *et al.*, 2002). The dependency of lectin activity on Ca^{2+}, Mg^{2+} and Mn^{2+} also observed for the lectins from *A. jiringa* (Charungchitrak *et al*., 2011) and *Dioclea altissima*, and indeed the requirement for divalent metal ions is a general physico-chemical property of most plant lectins, suggesting that they are essential for the haemagglutination activity (Moreira *et al.*, 1996; Wongkham *et al.*, 1995).

3.1 INTRODUCTION

Protein function can be understood in terms of its structure. Indeed, the three-dimensional structure of a protein is closely related to its biological function. Proteins that perform similar functions tend to show a significant degree of structural homology (Chan and Dill., 1993; Voet and Voet., 1990).

In general, a protein consists of a linear chain of a particular sequence of the 20 naturally occurring amino acids. Sequences vary in length, containing anywhere from ten to thousands of amino acids. The generation of a protein sequence is much easier than the determination of a protein structure. But sequencing is first and firmest step to deduce the structure. The structure of a protein gives much more insight in the function of the protein than its sequence. The organized native structure and the stability of a protein is known to be affected from the effect of external factors, denaturing agents and number of other metal ions. Therefore, a number of methods for the computational prediction of protein structure from its conformational change at the active site have been developed.

The purified novel lectin from *Nymphaea nouchali* (NNTL) were subjected to determination of amino acid analysis, determining the N–terminal sequence, identification of homology, its similarity and dissimilarity with other lectin, measurement of fluorescence emission spectra, structure functional relationship etc.

3.2 MATERIALS AND METHODS

3.2.1 Amino acid analysis

Amino acid analysis can be used to quantify protein and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis.

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies.

A generalized method for N-terminal amino acid analysis follows:

(i) Treated the peptide with a reagent which will selectively label the terminal amino acid,

(ii) Hydrolyze the protein to its individual amino acid constituents,

(iii) Determine the amino acid by chromatography and comparison with standards.

The amino acid analysis of NNTL was carried out in acidic condition with a Biochrom 20 Plus Amino acid Analyzer after the sample was hydrolysis in a sealed, evacuated ample at 110^{o} C. with 6 M HCl for 24 h.

3.2.2 Determination of N-Terminal Amino Acid Sequence

The purified lectin was first subjected to SDS-PAGE and then the lectin was immobilized on a polyvinylidene difluoride (PVDF) membrane by electroblotting and after that the protein band was excised from the membrane. Finally, N-terminal sequence was determined by Edman degradation using an amino acid analyzer and the N-terminal sequence homology was analyzed using the BLAST database search.

3.2.2.1 Edman degradation:

The Edman degradation is a very important reaction for protein sequencing, as it allows the ordered amino acid composition of a protein to be discovered. A reaction scheme for sequencing a protein by the Edman's degradation have to be maintained. The peptide to be sequenced is adsorbed onto a solid surface - one common substrate is glass fiber coated with polybrene, a cationic polymer. The Edman's reagent phenylisothiocyanate (PITC) is added to the adsorbed peptide, together with a mildly basic buffer solution of 12% trimethylamine. This reacts with the amino group of the N-terminal amino acid. The terminal amino acid can then be selectively detached by the addition of anhydrous acid. The derivative then isomerizes to give a substituted phenyl thiohydantoin (PTH) which can be washed off and identified by chromatography, and the cycle can be repeated. The efficiency of each step is about 98%, which allows about 50 amino acid residues to be reliably determined.

3.2.2.2 Determination of homology:

BLAST

In bioinformatics, "Basic Local Alignment Search Tool", or BLAST, is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. The sequence homology of NNTL was analyzed by using the database search in the website: *www.ncbi.nlm.nih.gov/blast*.

3.2.3 Study of Fluorescence Emission Spectra of the NNTL

Fluorescence spectra of NNTL were recorded on a Shimadzu Spectrofluorometer RF-5301 PC at room temperature. The concentration of NNTL was 50 and 40 µg/ml, the native and treated samples (with Ca^{+2}, *O*-nitrophenyl-ß-D-galactopyrano side and urea), were placed in a (1 ×1 × 4.5) cm quartz cuvette for measurement. The samples were excited at 280 nm and the emission spectra were recorded in the range of 300–400 nm and widths for the excitation and emission monochromators were maintained at 5 nm.

3.3 RESULTS

3.3.1 Amino acid composition of NNTL

From the standard sample 18 peaks of the different specific amino acids were obtained (Figure 3.1), which has eluted at different retention time including the area as mentioned in the Table 3.1. After obtaining the data (retention time and area) on the peaks of different amino acids from NNTL (Figure 3.2 and Table 3.2) and the total amino acid compositions (% of area) of NNTL were calculated by comparing with area (%) of standard sample (Table 3.3).

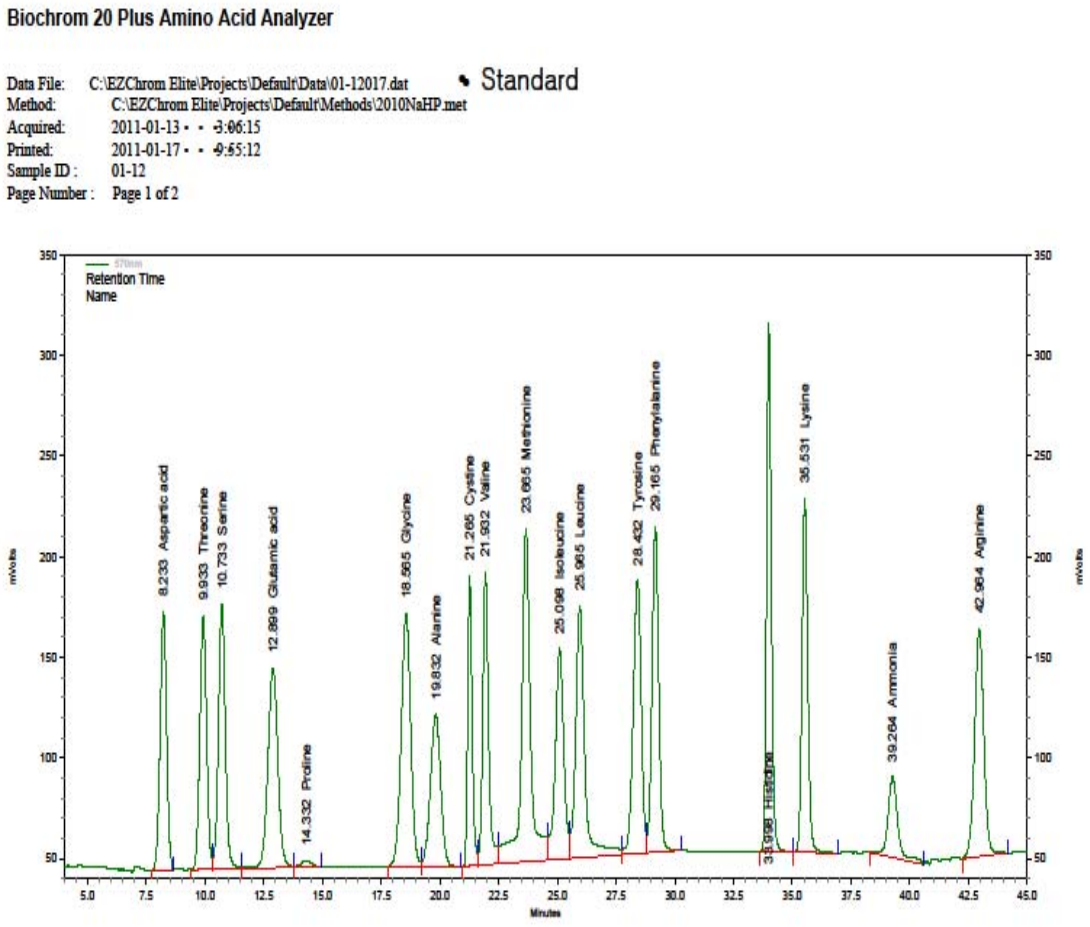


Figure 3.1 Amino acid analysis of Standard

Table 3.1 Data obtained from the Standard graph

Peak no.	Amino acid	Retention Time	Area	Area (%)
1	Aspartic acid	8.233	2455137	5.029
2	Threonine .	9.933	2561610	5.247
3	Serine	10.733	2702083	5.534
4	Glutamic acid	12.899	2977087	6.098
5	Proline	14.332	103680	0.212
6	Glycine	18.565	3504274	7.177
7	Alanine	19.832	2286211	4.683
8	Cysteine	21.265	1875567	3.841
9	Valine	21.932	2492968	5.106
10	Methionine	23.665	4500359	9.217
11	Isoleucine	25.098	2618979	5.364
12	Leucine	25.965	3383267	6.929
13	Tyrosine	28.432	3237707	6.631
14	Phenylalanine	29.165	3206735	6.568
15	Histidine	33.998	3710314	7.599
16	Lysine	35.531	2999236	6.143
17	Ammonia	39.264	1154636	2.365
18	Arginine	42.964	3054404	6.256
Totals			**48824254**	**100.00**

***** Tryptophane has not included in the Table, as it was destroying during acid hydrolysis (6 M HCl at 110^{o} C for 24 h).

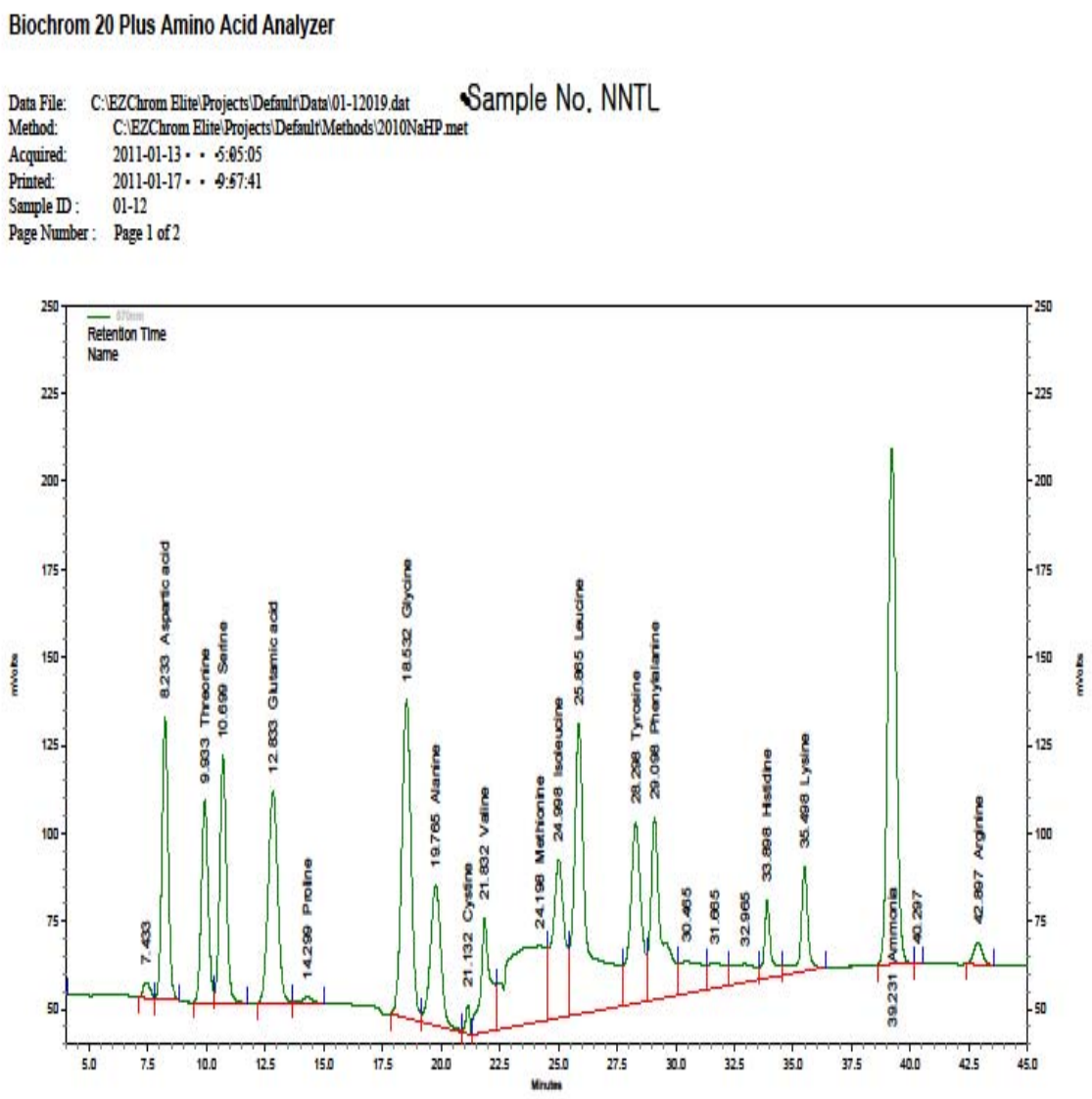


Figure 3.2 Amino acid analysis of applied sample (NNTL).

Table 3.2 Data obtained from the amino acid analysis curve of NNTL

Peak no.	Amino acid	Retention Time	Area	Area (%)
1		3.166	137463	0.480
2		7.433	90291	0.322
3	Aspartic acid	8.233	1472930	5.249
4	Threonine	9.933	1173909	4.184
5	Serine	10.699	1402624	4.999
6	Glutamic acid	12.833	1719718	6.129
7	Proline	14.299	56374	0.201
8	Glycine	18.532	2492207	8.882
9	Alanine	19.765	1253995	4.469
10	Cysteine	21.132	95104	0.339
11	Valine	21.83 2	936216	3.337
12	Methionine	24.198	2541086	9.056
13	Isoleucine	24.998	1600008	5.702
14	Leucine	25.865	3467113	12.356
15	Tyrosine	28.298	1710789	6.097
16	Phenylalanine	29.098	1709076	6.091
17		30.465	622319	2.218
18		31.665	382304	1.362
19		32.965	372091	1.326
20	Histidine	33.898	440958	1.572
21	Lysine	35.498	641342	2.286
22	Ammonia	39.231	3581959	12.766
23		40.297	1740	0.006
24	Arginine	42.897	157801	0.562
Totals			**28059417**	**100.000**

Table 3.3 Amino acid composition of NNTL

Amino acids	***Mol %***
Aspartic acid	**6.4**
Threonine	**5.1**
Serine	**6.1**
Glutamic acid	**7.5**
Proline	**0.3**
Glycine	**10.9**
Alanine	**5.5**
Cysteine	**0.4**
Valine	**4.1**
Methionine	**11.1**
Isoleucine	**7.0**
Leusine	**15.2**
Tyrosine	**7.5**
Phenylalanine	**7.5**
Histidine	**1.9**
Lysine	**2.8**
Arginine	**0.7**
Total	**100**

From the Amino acid analysis of NNTL, 17 amino acids were obtained. It was found that NNTL contained higher percentage of Leucine, Methionine and glycine with concentration of 15.2%, 11.1%, and 10.9% respectively. The present data also revealed that NNTL contained significant amounts of acidic amino acids (Glutamic acid and Aspartic acid), but lesser amount of Cystein (0.4%) as well as Proline (0.3%).

3.3.2 N-terminal Sequences of NNTL.

The N-terminal sequences upto 10th residues of the lectin was determine by Edman degradation method and the data are presented in the Table 3.4. Except the first residue, the N-terminal sequence of NNTL was determined to be: **XPEEADYLTE** (X-Proline- Glutamic acid - Glutamic acid - Alanine - Aspartic acid- Tyrosine- Leucine- Threonine- Glutamic acid)**.**

Table 3.4 Sequence homology of NNTL with other Proteins

Proteins	Sequences											Accession No
NNTL	**2**	**P**	**E**	**E**	**A**	**D**	**Y**	**L**	**T**	**E**	**10**	
Putative dehydrogenase	182	**P**	**E**	**E**	**A**	**D**	**Y**	**L**	**T**	–	189	ZP_01898845.1
Clostripain	213	**P**	**K**	**E**	**A**	**D**	**Y**	**L**	**T**	**E**	221	YP_504219.1
Protein rocB	11	**P**	**E**	**Q**	**A**	**E**	**Y**	**L**	**T**	**E**	19	ZP_03228294.1
NAD-dependent epimerase/ dehydratase	319	–	**E**	**E**	**A**	**D**	**Y**	**L**	**T**	**D**	326	YP_003798514.1
Reticulocalbin 2	253	**P**	**E**	**E**	**V**	**D**	**Y**	**M**	**T**	**E**	261	EDL95577.1
Taipoxin-associated calcium binding protein 49	184	**P**	**E**	**E**	**V**	**D**	**Y**	**M**	**T**	**E**	192	AAC05132.1
TonB family protein	65	**P**	**E**	**E**	**A**	**D**	**Y**	**L**	**A**	**D**	73	YP_001002515.1
Permease	200	**P**	**E**	**E**	**A**	**D**	**Y**	**S**	**T**	**E**	208	YP_003406033.1
Zinc-binding dehydrogenase family oxidoreductase Oxidoreductase	182	**P**	**E**	**E**	**A**	**D**	**Y**	**L**	–	–	188	YP_259548.1
3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, YrbI family	143	–	**E**	**E**	**A**	**D**	**Y**	**I**	**T**	**E**	150	Y p_003828733.1
Thioredoxin reductase	160	–	**E**	**E**	**A**	**D**	**Y**	**L**	**T**	–	166	CBL04572.1
Putative ATP-binding protein	870	**P**	**E**	**Q**	**A**	**D**	**Y**	**L**	**K**	**E**	878	ZP_06198416.1
Prolyl aminopeptidase	110	**P**	**E**	**E**	**Q**	**A**	**D**	**Y**	**L**	**T**	118	ZP_04700458.1

3.3.3 Study of Fluorescence Emission Spectra of NNTL

To study the structural stability of NNTL, the measurement of fluorescence spectra were done at different states as shown in Figure 3.3 and 3.4 .The effect of Ca^{2+} in the salt form and *O*-Nitrophenyl-ß-D-galactopyranoside on the structure of NNTL were measured using fluorescence emission spectra at 280 nm. As shown in Figure 3.3, the presence of $CaCl_2$ upto 1mM the fluorescence emission spectra were increased significantly in compare to control with maximum at around 324-326 nm. Further no changes were observed if the salt concentration was increased more than 1 mM.

Again, as shown in the Figure 3.4, the fluorescence emission spectra of the lectin in the presence of specific ligand, were quenched moderately in the presence of 15 µg/ml *O*-nitrophenyl-ß-D-galactopyranoside containing 1 mM $CaCl_2$. On the other hand, the quenching of the native lectin spectra were more pronounced, when the concentration of sugar increased from 15 to 30 µg.

The effect of Ca^{2+} on the stability of NNTL was also performed by incubating with 4 M urea in the presence and absence of 1 mM $CaCl_2$. The incubation was monitored for a shorter time period (90 min) and a longer time period (8 h) at room temperature. When NNTL was incubated with 4 M urea in the presence o f $CaCl_2$ for 90 min and 8 h, no significant change in intensity was found. On the other hand, a remarkable change was observed when NNTL was incubated with 4 M urea in absence of $CaCl_2$ after 90 min and it was more significant after 8 h, as shown in Figure 3.5.

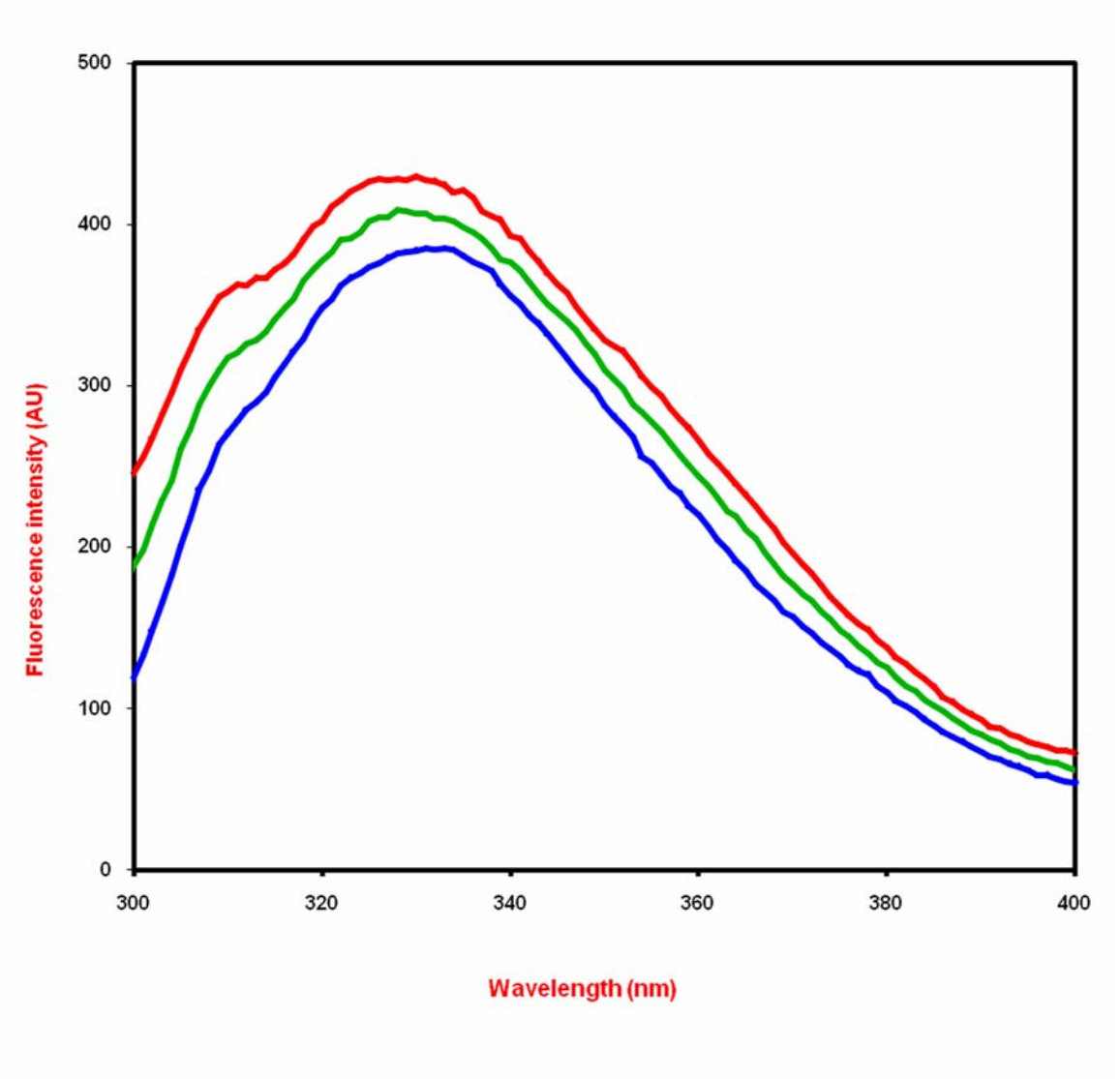


Figure 3.3 Fluorescence emission spectra of 50 µg/ml NNTL in absence *O*-nitrophenyl-ß-D-galactopyranoside.

(——) NNTL in TBS,

(——) TBS containing 0.5 mM $CaCl_2$,

(——) TBS containing 1.0 mM $CaCl_2$.

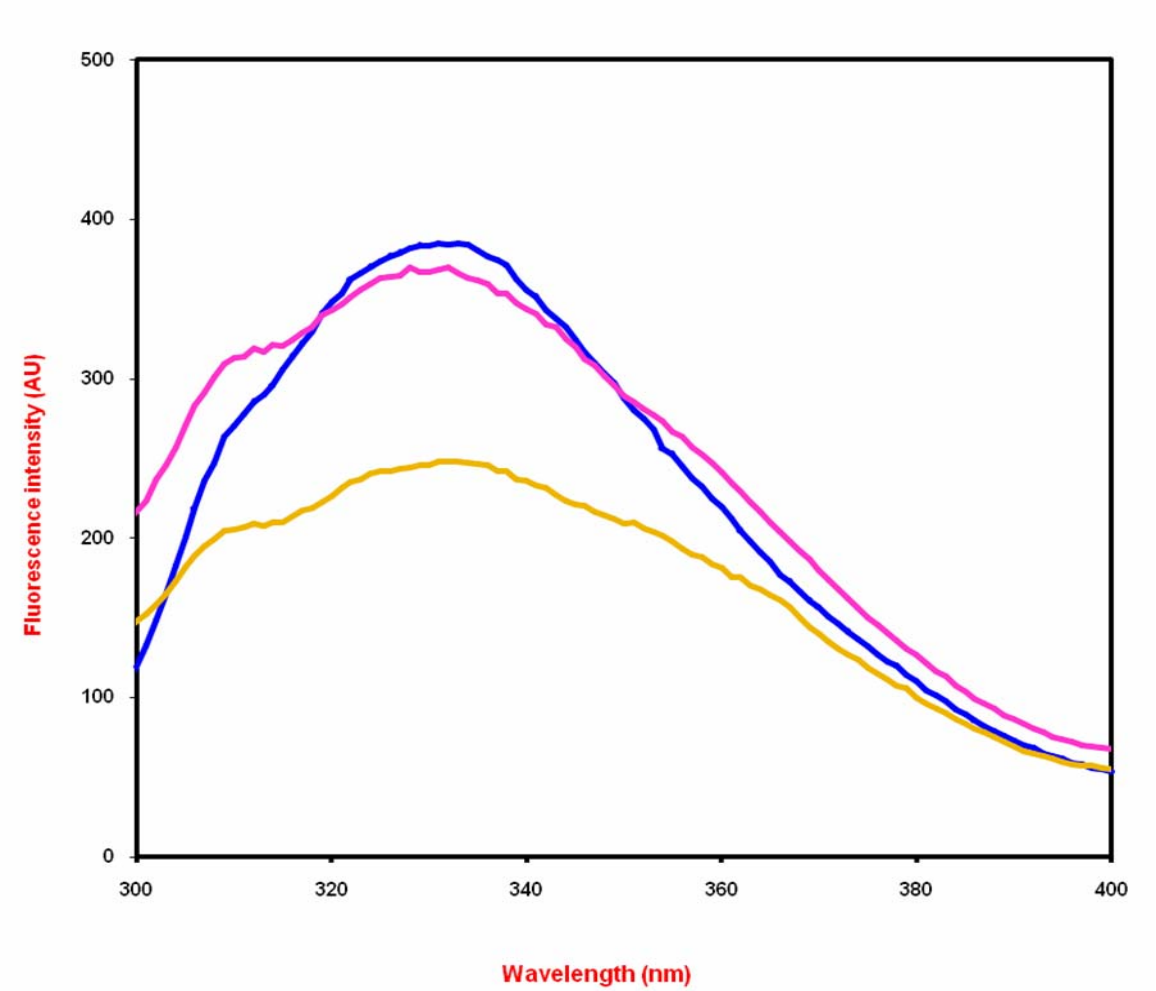


Figure 3.4 Fluorescence emission spectra of 50 µg/ml NNTL in presence of *O*-nitrophenyl-ß-D-galactopyranoside.

(——) NNTL in TBS as control,

(——) TBS containing 15 µg/ml *O*-nitrophenyl-ß-D-galactopyranoside and 1.0 mM $CaCl_2$,

(——) TBS containing 30 µg/ml *O*-nitrophenyl-ß-D-galactopyranoside and 1.0 mM CaCl2.

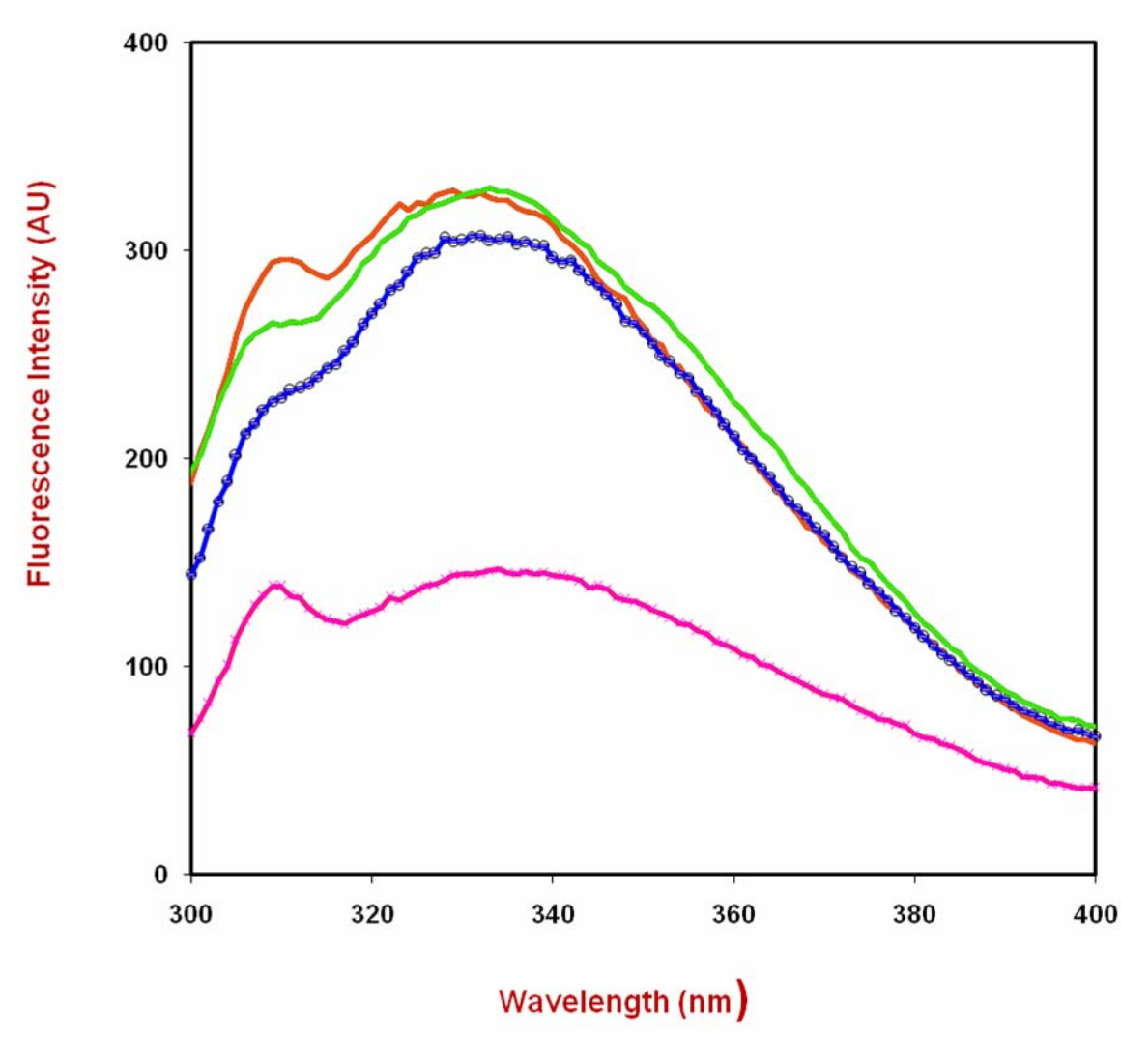


Figure 3.5 Effect of urea on NNTL (40 µg/ml) activity in presence and absence of $CaCl_2$.

(——) TBS containing 1 mM $CaCl_2$ and 4M urea incubated for 90 min,

(——) TBS containing 1mM $CaCl_2$ and 4 M urea incubated for 8 h,

(**ooo**) In absence of $CaCl_2$, TBS containing 4M urea incubated for 90 min

(——) In absence of $CaCl_2$, TBS containing 4M urea incubated for 8 h.

3.4 DISCUSSION

The amino acid composition analysis of NNTL revealed that the lectin was rich in leucine (15.2%), methionine (11.1%), and glycine (10.9%) residues as comparable to many other plant lectins. High contents of glycine were also reported for the lectin of *Helianthus tuberosus* L. tuber (Suseelan *et al*., 2002); HTA I, HTA II (Nakagawa *et al*., 1996), and chitin-binding protein from *Solanum tuberosum L*. tuber (Millar D.J. *et al*., 1992), however they contain lesser amounts of leucine and methionine. The amino acid analysis of NNTL also showed the large contents of acidic amino acids, but negligible amounts of cystein (0.4%); which is similar to the composition of lectin from *Erythrina speciosa* reported by Konozy *et al*. (2003). As this experiment was conducted in acidic condition, the acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids. Tryptophan is destroyed, and cysteine is typically recovered as cystine. Hence tryptophane was not detected by the study, although fluorescence data clearly indicated that NNTL contained tryptophane.

The N-terminal sequences of NNTL have determined from 2^{nd} position upto 10^{th} position sequentially using Edman's degradation method and the sequence was determined as XPEEADYLTE. The homology of the sequence was searched by using the standard protein BLAST database, but was not identical with the sequence of any other lectin, Only similarity was found with the regions of some proteins and enzymes other than N-terminal sequence as shown in Table 3.4. This result suggested that NNTL is a novel lectin.

In this investigation, dependency of divalent cations (especially Ca^{2+}) on lectin for structural stability was examined by the measurement of fluorescence spectra, which have already been confirmed by hemagglutination assay. In the presence of $CaCl_2$ up to 1mM concentration intensity was increased remarkably indicating that the binding of Ca^{+2} to

the lectin changes the environment of Ca^{+2} binding site of the lectin, which may possibly near to the tryptophan residues. From the result it can be concluded that NNTL is a Ca^{+2} dependent protein, and it showed the emission spectra at 330 nm when native lectin is excited at 200 nm, suggesting the presence of tryptophan residues in the hydrophobic region of lectin. But when the concentration of $CaCl_2$ was increased more than 1 mM, no change of the intensity was observed. It may assumed that Ca^{+2} affect the environment of tryptophan residue upto 1 mM concentration, and at this maximum concentration the binding site of the lectin might be completely saturated with Ca^{+2}.

Furthermore, on addition of *O*-nitrophenyl-ß-D-galactopyranoside to the lectin solution, the fluorescence intensity decreased remarkably as compared to that in presence of Ca^{2+} salt. From this finding it may be suggested that the changes in the fluorescence spectra induced by Ca^{2+} and *O*-nitrophenyl-ß-D-galactopyranoside might be attributed to the change in the environment of the tryptophan residue(s) at or near the carbohydrate binding site of NNTL (Sallay *et al*., 2001). Whereas *Erythrina speciosa* is a lactose-specific lectin, but there was no significant change in the spectra upon binding of lactose to lectin (Konozy *et al*., 2003).

On the other hand, Ca^{2+} stabilization of NNTL was also examined by treatment with urea. It was found that when NNTL was incubated with 4M urea in presence of $CaCl_2$ for 90 min and 8 h, there was no profound change in the tryptophan region. Whereas in absence of $CaCl_2$, the fluorescence intensity decreased remarkably when incubated with 4 M urea for 90 min and the intensity was fall largely when the incubation period was increased to 8 h, which suggested Ca^{2+} was responsible for the conformational change of NNTL. The study of the conformational changes by fluorescence spectroscopy indicates that the tryptophan environment of NNTL was greatly affected upon binding to different concentrations of metal ions, sugars and denaturants.

4.1 Bacterial Agglutination Study

4.1.1 Introduction

Agglutination is the most easily detectable manifestation of the interaction of a lectin with cells, and to this very day is used to reveal the presence of lectin in a biological source. The sensitivity of lectin to a bacterial cell can be investigated against several gram-positive and gram-negative strains. It is a dose dependent method, and in minimum concentration form precipitation at which lectin may specific to the sugar moiety is present on the surface of the bacterial cells. Agglutination may also inhibited by using an appropriate sugar specific for that lectin.

4.1.2 Materials

(a) 20mM Tris-HCl buffer, pH 7.8, 1% NaCl & 10mM $CaCl_2$

(b) Protein solution

(c) Bacterial species:

Bacillus cereus, Staphylococcus aureus, Bacillus subtilis, Bacillus megaterium, Sarcina lutea, Escherichia coli, Klebsiella sp, Shigella shigae, Shigella sonnei, Shigella dysenteriae, Salmonella typhi, in 1% NaCl.

4.1.3 Procedure

Bacteria were grown at 37^0C over night in nutrient broths (liquid nutrient media) and then the precipitate containing bacterial cell were collected by centrifugation at 4×10^3 r.p.m for 3 minutes, washed with 20 mM Tris-HCl buffer saline, pH 7.8 and re-suspended in the same buffer with a turbidity of 2.0 at 640 nm.

50 µl of each bacterial suspension was mixed with serial dilution of NNTL to a final volume of 100 µl in 96 well microtitre plate. The plate was agitated for 2 min and the mixture was kept at room temperature for 60 min. Finally bacterial agglutinating activity was monitored under a light microscope.

4.2 Antifungal Assay

4.2.1 Introduction

Antifungal activities of purified proteins were tested against five fungi by using disc diffusion technique, as it is essentially a quantitative or semi quantitative test indicating the sensitivity or resistance of microorganism to the test material. In vitro antifungal screening is a useful technique for the detection of new compounds for the development as potential antifungal agents.

4.2.2 Preparation of culture media

Potato Dextrose Agar (PDA) media was used to perform the antifungal activity test and for subculture of the test organism. The composition of the media was as follows.

Potato Dextrose Agar media (Origin: Hi Media, India).

Component	**Amount**
Peeled and sliced potato	20 gm
Dextrose	2 gm
Agar	2 gm
Distilled water	100 ml

4.2.3 Test organisms

The fungal strains used in the sensitivity test are given below. The pure cultures of the strains were taken from the Institute of Biological Science, University of Rajshahi.

(i) Candida albican, *(ii) Aspergillus nigar*

(iii) Aspergillus flavus, *(iv) Mucor sp.*

(v) Fusarium Vasinfactum,

4.2.4 Preparation of discs

(a) Sample discs: Filter paper discs (5mm diameter) were taken in sterile petri- dishes. The fungal mycelia were placed over the solid potato dextrose agar. The sterile filter paper discs were soaked with 20 µl of the lectin (100µg/ml) and other proteins solution (5 mg/ml) that is at a dose of and were distributed over the plates.

(b) Control discs: The control discs were soaked with 10 mM Tris-HCl buffer saline, pH 8.0 and applied on the same plates.

4.2.5 Procedure

Antifungal activities of the *Nymphaea nouchali* tuber lectins were performed using sterile petridishes (100 × 15 mm) containing 30 ml potato dextrose agar. Fungal mycelia were placed over the solid PDA and the sterile filter paper discs (5 mm in diameter), both the sample discs and control discs, were distributed over the plate. Incubation of the petridishes were carried out at 30^0C until the mycelial growth enveloped the control discs and formed crescents of inhibition around the sample discs soaked with protein samples.

4.3 Cytotoxicity Study of NNTL

4.3.1. Introduction

This bioassay indicates toxicity as well as a wide range of pharmacological activities (e.g. anticancer, antiviral, insecticidal, pesticidal, AIDS etc.) of the compounds. Here *in vivo* lethality bioassay is conducted by using the simple zoological organism, brine shrimp naupli (*Artemia salina*). This bioassay can be used as a convenient monitor for screening and fractionation in the discovery and monitoring of bioactive natural products (Melaughlin *et al.,* 1990; Perssone *et al.,* 1980; Meyer *et al.,* 1982).

4.3.2 Procedure

a) Preparation of simulated seawater

38 g of sea-salt (non ionized NaCl) was weighed accurately, dissolved in one liter of sterilized distilled water and then filtered off to get clear solution. The pH of the seawater was maintained to 7.0 by the addition of sodium tetraboraet.

b) Hatching of brine shrimp

Simulated sea water was taken in the small tank and the shrimp eggs (1.5 g/l) were added to one side of the tank and this side was covered. The shrimps were allowed for one day to hatch and matured as nauplii (larvae). Constant oxygen supply was carried out and constant temperature (around 37˚C) was maintained during the hatching time. The hatched shrimps were attracted to the lamp on the other side of the divided tank through dam. These nauplii were taken for the bioassay.

c) Preparation of sample

2 ml of the lectin solutions (conc. 4 mg/ml) in Tris-HCL buffer, pH 8.4 were dialyzed against distilled water for 3 hours at 4˚C.

d) Application of the test sample and brine shrimp nauplii to the vials

At room temperature 25, 50, 100 and 200 µl of NNTL were taken in different vials and 5 ml of artificial sea water was added to each vial containing 10 brine shrimp nauplii. Three replicate were used for each treatment and control. All the tests were performed at around 30˚C, under the continuous light regime. After 24-hour of incubation, the number of survivors in each vial were counted; and the percent (%) of mortality of the nauplii was calculated at each concentration. The concentration-mortality data were analyzed and the LC_{50} values were determined using Probit analysis (Finney *et al.,* 1971).

4.4 RESULTS

4.4.1 Bacterial agglutination

Agglutination of several bacterial strains confirms the interaction between the lectins and the strains. NNTL were subjected for bacterial agglutination, and these interactions with several pathogenic bacteria were observed. NNTL agglutinated *Bacillus cereus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Sarcina lutea*, *Klebsiella species*, *Escherichia coli*, *Shigella shiga* , *Shigella sonnei* and *Salmonella typhi*; but there was no aggregation found against *Bacillus megaterium and Shigella dysenteriae*. The minimum concentration of NNTL solution needed for the agglutination of each bacterium is summarized in Table 4.1. From the data obtained in the present study, the agglutination pattern of NNTL might be arranged in the following order:

Bacillus subtilis >Sarcina lutea > Shigella shiga > Shigella sonnei > Staphylococcus aureus > Bacillus cereus > Escherichia coli > Klebsiella species.

Table 4.1 Minimum concentrations of NNTL needed for agglutination of some pathogenic bacteria.

Bacterium	**NNTL (µg/ml)**
Bacillus cereus	9.7±2.5
Staphylococcus aureus	3.7±1.5
Bacillus subtilis	0.42±0.17
Bacillus megaterium	NA
Sarcina lutea	1.0±0.39
Klebsiella species	40±10.3
Escherichia coli	39.2±11.5
Shigella shiga	1.1±0.43
Shigella dysenteriae	NA
Shigella sonnei	2.2±0.86
Salmonella typhi	40±10.3

Further, these observations were also confirmed by taking photographs under microscope after interaction of NNTL with the experimental organisms as shown in the following figures.

Photographic image of the agglutination: Fig. 4.1 *Bacillus cereus,* **Fig. 4.2** *Sarcina lutea,* **Fig. 4.3** *Bacillus subtilis.*

In absence of NNTL (control) **In Presence of NNTL**

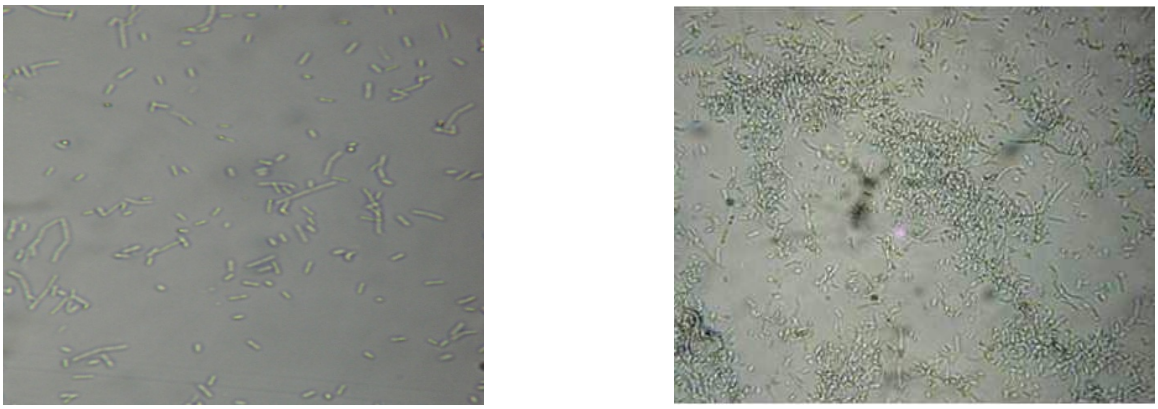


Fig-4.1: *Bacillus cereus*

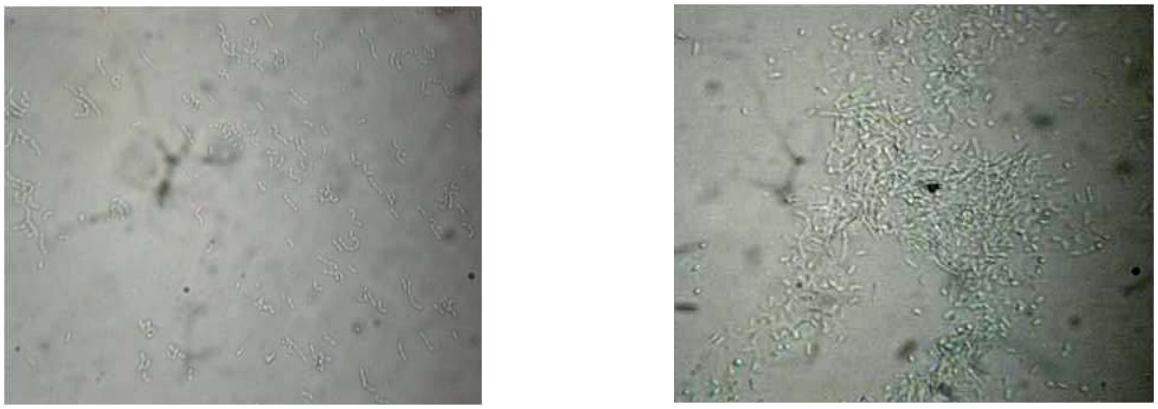


Fig 4.2: *Sarcina lutea*

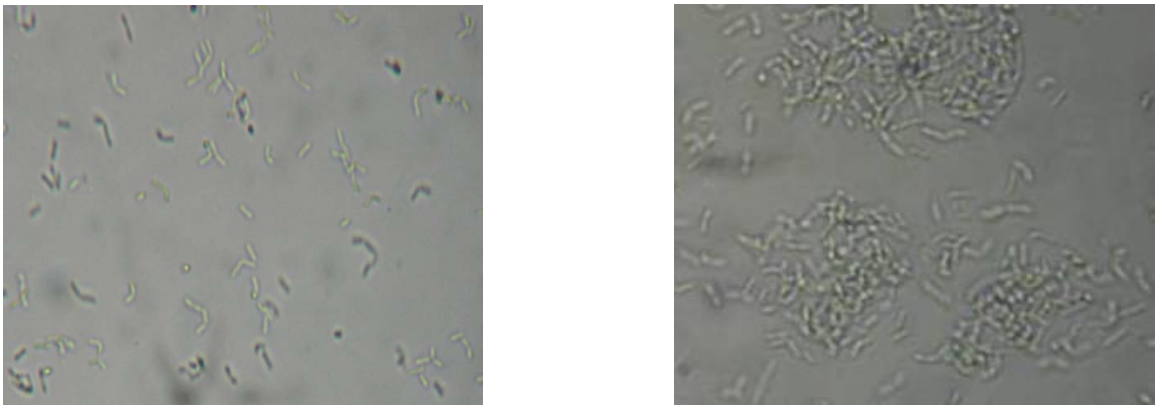


Fig. 4.3: *Bacillus subtilis*

Photographic image of the agglutination: Fig. 4.4 *Klebsiella sp.*, **Fig. 4.5** *Shigella siga*, **Fig. 4.6** *E.coli*,

In absence of NNTL (control) **In Presence of NNTL**

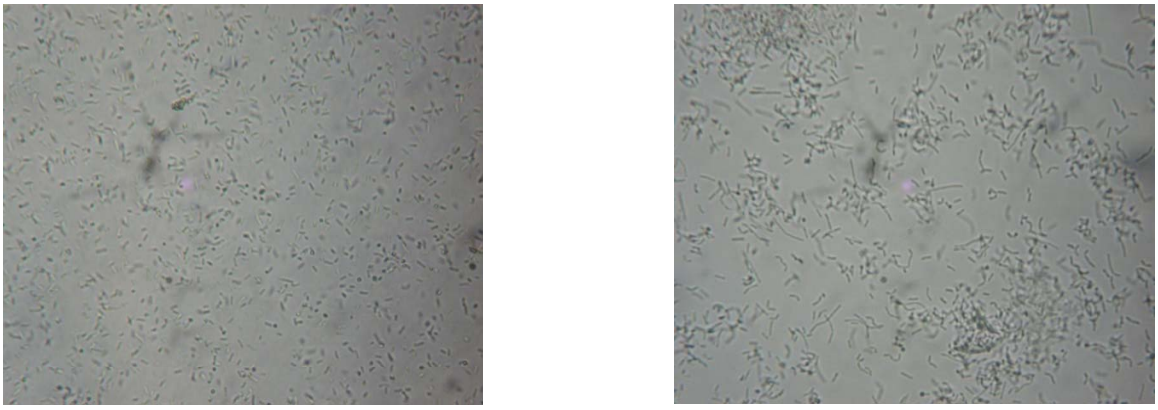


Fig. 4.4: *Klebsiella sp.*

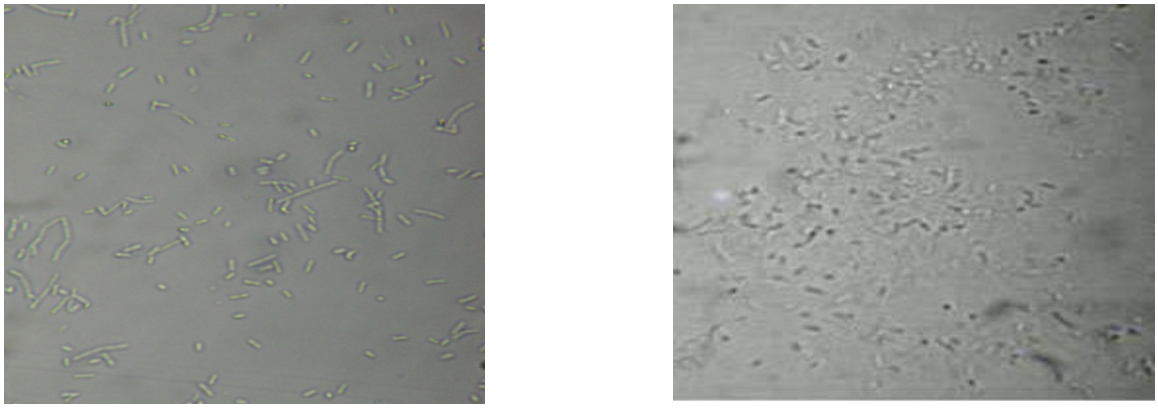


Fig-4.5: *Shigella siga*

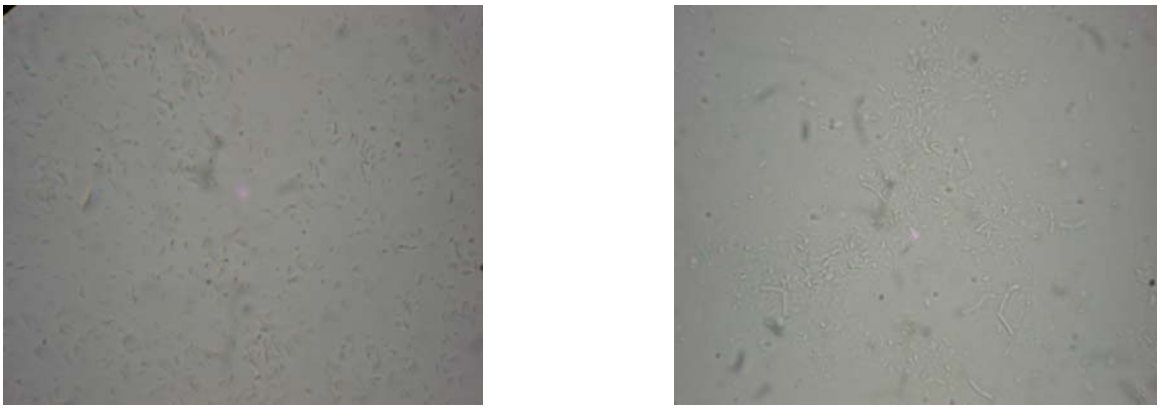


Fig-4.6: *E.coli*

Photographic image of the agglutination: Fig.4.7 *Shigella sonnie;* and **no agglutination :** Fig**. 4.8** *Bacillus megaterium*, **Fig. 4.9** *Shigella dysenteria.*

In absence of NNTL (control) **In Presence of NNTL**

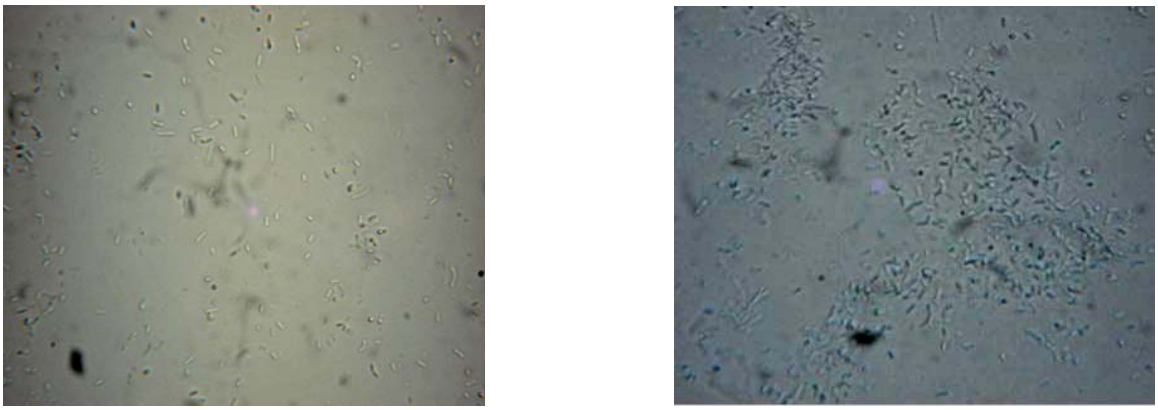


Fig. 4.7: *Shigella sonnie*

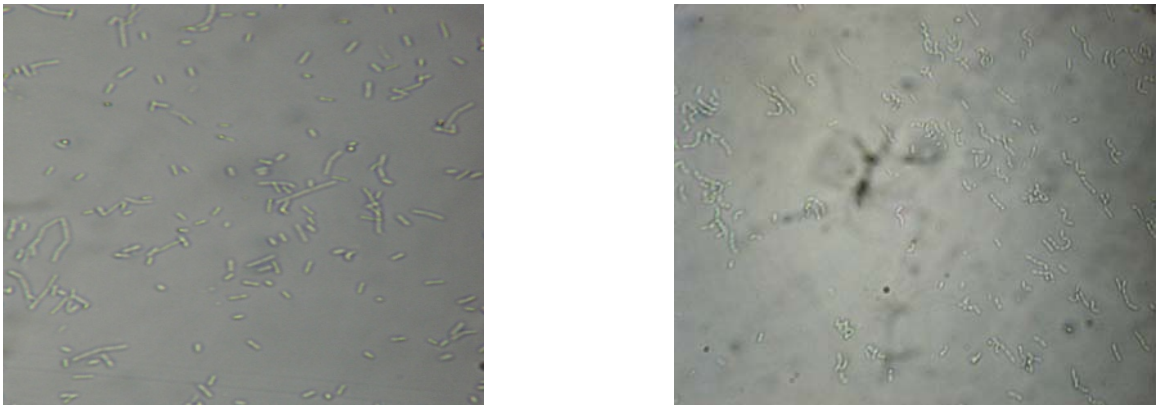


Fig. 4.8: *Bacillus megaterium*

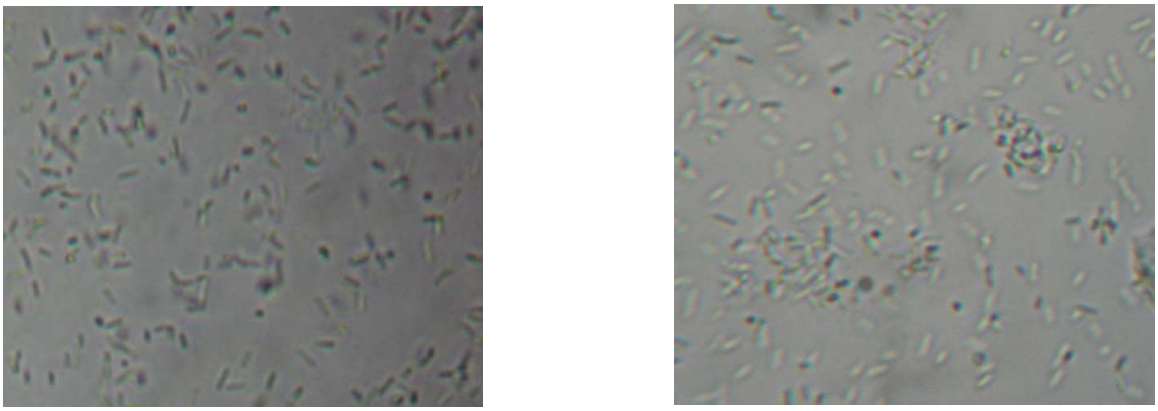


Fig. 4.9: *Shigella dysenteria*

4.4.2 Antifungal activity

The antifungal activities of NNTL and other purified three proteins (NNTP-I, NNTP-II, and NNTP-III) against five pathogenic fungi were investigated by using the doses of 20 µl/disc. The standard TBS saline disc was used for comparison. In the present study NNTL did not show antifungal activity against any of the following fungus *Candida albicans, Mucor sp, Fusarium Vasinfactum, Aspergillus nigar* and *Aspergillus flavus*. On the other hand, among the other three proteins studied, only one protein namely NNTP-I showed notable antifungal activity against *Candida albicans* (Table 4.2 and Figure 4.10).

Table 4.2 In vitro antifungal activities of NNTP-I

Test fungi	**NNTP-I (**20µl/disc**)**
Candida albicans	+
Mucor sp.	–
Fusarium vasianfactum	–
Aspergillus nigar	–
Aspergillus flavus	–

Symbol: ' **+** ' showed sensitivity; ' **–** ' no sensitivity.

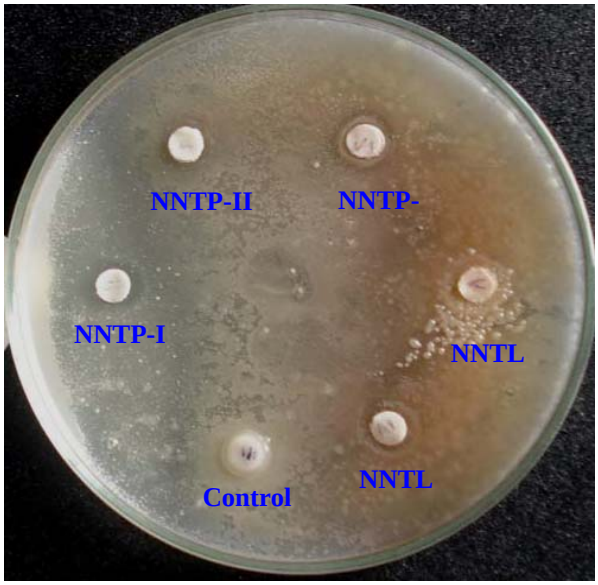


Figure- 4.10 Antifungal activities of NNTP-I against *Candida albicans*

4.4.3 Brine-Shrimp lethality bio-assay

In brine-shrimp lethality bioassay, the purified lectin showed positive results indicating that the lectin is cytotoxic in nature. The mortality rate of brine shrimp nauplii was found to be increased with the increase of concentration of the lectin as shown in Figure 4.11. The LC_{50} value for NNTL was 120±29 µg/ml, suggesting the cytotoxic nature of the purified lectin.

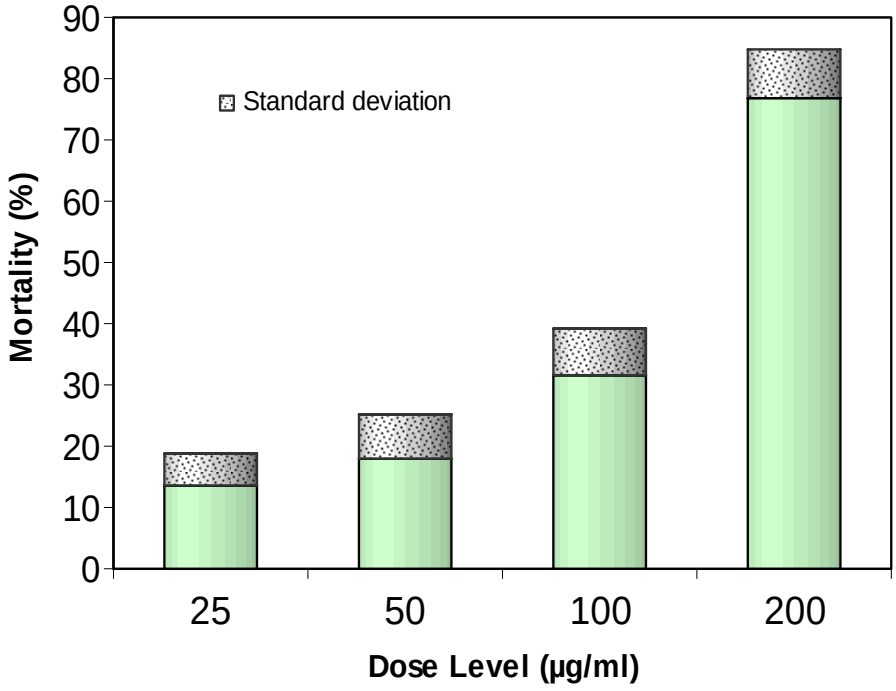


Figure 4.l1 Percentage of mortality rate of brine shrimp nauplii treated with NNTL solution after exposure for 24 h.

4.5 DISCUSSION

The interaction between the lectin and strains was investigated by the study of agglutination of NNTL against several gram-positive and gram-negative bacterial species. The bacterium *Bacillus subtilis* was the most sensitive to NNTL as it was agglutinated at 0.42±0.17 µg/ml of protein concentrations in contrast to control. Moreover, *Sarcina lutea, Shigella shiga,* and *Shigella sonnei* were strongly aggregated also by NNTL and the minimum agglutination concentration were 1.0±0.39, 1.1±0.43 and 2.2±0.86 µg/ml respectively. On the other hand, *Shigella dysenteriae* and *Bacillus megaterium* did not show any agglutination with NNTL. The bacterial agglutination might be due to the presence of cognate glycan antigen on the cell surface of induced bacteria. This significant property also observed in many of the plant lectins, such as *Trichosanthes cucumerina* lectin (TCSL) also agglutinate both gram- positive and gram-negative bacteria as *Shigella shiga, Shigella sonnei* and *Bacillus megaterium (*Kabir *et al., 2012). Concanavalin A* the firstly isolated lectin aggregated a variety of gram-negative bacteria specifically *Helicobacter pylori* coccids and *Salmonella typhimurium* (Khin *et al*., 2000; Naughton *et al.*, 2000).

The antifungal activity of the lectin and other purified proteins were also studied, but NNTL was devoid of antifungal action on five fungal species tested. Large number of lectin have been purified, only a few of them manifested antifungal activity, e.g., *Pisum sativum* lectin (PSL) from Egypsian seed inhibited the growth of *Aspergillus flavus*, *Trichoderma viride* and *Fusarium oxysporum* (Sitohy *et al*., 2007); whereas, lectin from Snake guard seeds (TCSL) and *Canavalia gladiata* seeds did not show any antifungal activity (Kabir *et al*., 2012; Wang *et al*., 2005). On the other hand, one of the non-lectin proteins purified from *Nymphaea nouchali* tuber, i.e., NNTP-I exhibited notable antifungal effect against *Candida albicans*. Many of the proteins purified from higher plants play a defensive role against plant pathogens as well as pathogenic fungi (Popovic *et al*., 2012).

Different lectins have different levels of toxicity, though not all lectins are toxic. The toxicity of NNTL was evaluated against brine shrimp nauplii. It was dose-dependent and the mortality rate of brine shrimp nauplii was found to be increased with the increase in concentration of the lectin. In the present study, 50% mortality (LC_{50}) of the nauplii occurred at 120±29 μg/ml concentration of NNTL, whereas LC_{50} of MSL (mulberry seed lectin) and *Kaempferia rotunda* lectin (KRL) were only 21.87 μg/ml and 18±6 μg/ml respectively (Absar *et al.*, 2005; Kabir *et al.*, 2011). These result suggested that NNTL is less toxic than that of MSL and KRL.

5.1 Antitumor Activity of NNTL

5.1.1 Introduction

The study of antitumor activity in vivo against Ehrlich Ascites Carcinoma (EAC) cells in swiss albino mice has been assigned as an essential part of biological investigation of plant lectin.

EAC cells is a spontaneous murine mammary adenocarcinoma (Ehrlich and Apolant 1905) adapted to ascites form (Loewenthal and Jahn 1932) and carried in outbred mice by serial intraperitoneal (i.p.) passage. The ascitic form is produced by infecting tumor cell suspension into the mouse peritoneal cavity. The ascitic tumor develops as a milky white fluid containing large rounded tumor cells. One million of tumor cells multiply to yield about 25-100 million tumor cells/ml. Host carrying such a tumor survives for about 14-30 days.

5.1.2 Materials and Methods

5.1.2.1 Experimental Animal

Swiss Albino male mice of 3-4 weeks of age and weighing 20-25 grams were collected from International Center for Diarrheal Diseases Research, Bangladesh (ICDDR'B) for the experimental studies.

5.1.2.2 Experimental tumor model

The transplantable tumor (Ehrlich's Ascites Carcinoma) was used in this experiment. The initial inoculum of EAC cells was kindly provided by the Indian Institute of Chemical Biology (IICB), located at Kolkata, India. The EAC cells were thereafter maintained in our laboratory in Swiss Albino mouse by intraperitoneal (i.p.) transplantation.

5.1.2.3 Transfusion of Ascite Tumour:

The EAC cells were propagated intraperitonealy in our Departmental research laboratory biweekly and the cells were collected from a donor swiss albino mouse bearing 6-7 days old ascites tumor. The collected cells were diluted with normal saline(0.98% NaCl solution) and adjusted the cell number to 3×10^6 cells/ml by counting with the help of a haemocytometer. The viability of tumor cells was checked by trypan blue dye (0.4%) exclusion assay. Tumor cells that showed 90% viability were used for transplantation.

Tumor suspension of 0.1 ml was injected intraperitoneally (i.p.) to each swiss albino mouse. Strict aseptic condition was maintained throughout the transplantation process.

5.1.2.4 Determination of cell growth inhibition of test sample

To determine the cell growth inhibition (Leonand *et al*., 1960) of the lectin, three groups of EAC cell bearing mice (5 in each group) weighing 20-25 gm were used. Treatments were started after 24 hours of tumor inoculation and continued for five days. Two groups of mice treated with the pure NNTL at a concentration of 3.0 mg/kg/day and 1.5 mg/kg/day, and the remaining group was used as control. Mice in each group were sacrificed on the sixth day and the total intraperitoneal tumor cells were harvested by normal saline and counted by a haemocytometer. The total number of viable cells in every mouse of the treated groups was compared with those of the control (EAC cells only). The percentage of inhibition was calculated by the following formula:

$$\%\,\text{of inhibition} = 100 - \left\{ \left(\frac{\text{Cells from NNTL treated mice}}{\text{Cells from control mice}} \right) \times 100 \right\}$$

5.2 Study of Apoptotic Nuclear morphology by Fluorescent Microscopy

5.2.1 Introduction

Apoptosis is a fundamental feature of many biological processes. This mode of cell death culminates in early recognition (i.e., before plasma membrane rupture) of dying cells by phagocytes and appears to have been highly conserved throughout evolution. Apoptosis can be triggered by a diverse array of stimuli, and is characterized by a number of morphological changes. These include cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies (Guohong *et al.*, 1997).

A number of methods exist for detecting apoptotic cells. Loss of cell viability (failure to either exclude vital dyes or transform tetrazolium salts to colored products), DNA fragmentation (assayed by agarose gel electrophoresis or in situ terminal transferase labeling) and DNA condensation (detected by Hoechst dye staining of nuclear DNA) are some of the traits used to monitor apoptosis. Chromatin condensation, nuclear shrinkage and formation of apoptotic bodies can easily be observed under fluorescence microscopy, after appropriate staining of nuclei with DNA-specific fluorochromes. Both adherent cell lines and cells growing as suspension can be examined.

Fluorescence microscopy is a powerful tool for modern cell and molecular biologists. It provides a window into the physiology of living cells at sub-cellular levels of resolution. This allows direct visualization of the inner workings of physiological processes at a systems level context in a living cell or tissue.

5.2.2 Materials and Methods

(A) Collection of EAC cells:

The EAC cells collected from donor mice (Swiss albino) of 20-25 g body weight were suspended in sterile isotonic saline. A fixed number of viable cells (usually 3×10^6

cells/ml) were implanted into the peritoneal cavity of each recipient mouse, as described in the above experimental method. After 24 hours the tumor bearing mice were distributed into two groups. One of the groups of mice treated for five days with NNTL at a concentration 3.0 mg/kg/day and the remaining group was used as control. Mice in each group were sacrificed on the sixth day and the intraperitoneal tumor cells were harvested by normal saline.

(B) Observation of cell morphologic changes and nuclear damage:

Materials:

(i) Fluorescence Microscope (Olympus-iX71)

(ii) Hoechst 33342 dye

(iii) 10 mM Phosphate buffer saline (p^H 7.0)

(iv) Propidium Iodide.

Procedure:

Harvested EAC cells were collected and washed three times by 10 mM cold phosphate-buffered saline, pH- 7.0. Finally the cells were suspended in 1 ml of PBS and adjust the cell density to 1×10^6 cells/ml. For this assay a control and a NNTL treated sample of each 1 ml of cell suspension were taken, to which added 10 µl of Hoechst 33342 dye and mixed thoroughly. The mixtures were incubated separately at 37^0C for 15 minutes and then centrifuged at 1,000 rpm for five minutes at 4^0C. The precipitated cells were resuspended in 1,000 µl of 10 mM PBS. Then added 5 µl of propidium iodide (1mg/ml) solution to each 1 ml of cell suspension and incubated the cell mixture at 37^0C for 15 minutes, and avoid exposing to light.

After the incubation, the stained cells were analyzed by measuring fluorescence emission at ~460 nm emission of Hoechst 33342 dye and >575 nm emission of propidium iodide. Any morphological changes were confirmed by comparing the experimental and control cells under the fluorescent microscope.

5.3 Study of Apoptotic Related Genes Expression by RT-PCR

5.3.1 Introduction

Reverse transcription polymerase chain reaction (RT-PCR) analysis is increasingly becoming part of the diagnostic and prognostic evaluation for hematologic and oncologic disorders, Currently, different RNA isolation methods are used in the diagnostic laboratories.

Apoptosis:

Apoptosis is the process of programmed cell death (PCD), a crucial cellular event during development and tissue homeostasis. Apoptosis is necessary for normal tissue homeostasis (the balance between cell proliferation and cell loss) in self-renewing tissues and for the removal of DNA damaged, non-functional, or misplaced cells.

The effectors stage of apoptosis, in contrast to the initiation and degradation stages, is subjected to regulation by the family of *bcl-2* related proteins, and includes both death agonists (*bax, bak, bcl-Xs, bad, bik,* and *hrk*) and death antagonists (*bcl-2, bcl-*X_L*, bcl-w, mcl-1, bfl-1, brag-1* and *a1*). *Bax* heterodimerises with *bcl-2, bcl-*X_L*, mcl-1,* and *a1*, and also forms homodimers. The cellular concentrations of death antagonists and agonists (ratio of antagonists to agonists) and the competitive dimerisation between selective pairs of antagonists and agonists appear to determine a cell's susceptibility to apoptosis (Angelis *et al.,* 1998).

Apoptosis Regulating genes:

Genes that can influence cell viability versus cell death have been described, including genes belonging to the *bcl-2* family. Related genes of this large family encode proteins that regulate apoptosis both in a negative and, in some instances, positive fashion. In addition, alterations in the expression of these genes may cause aberrations in cell death and thus contribute to cancer (Angela Gradilone, *et al*., 2003).

The expression of apoptosis-related genes, such as *bcl-2*, *bcl-X*, and *bax*, has been evaluated by reverse transcriptase polymerase chain reaction (RT-PCR) in EAC cells implanted in vivo in mice.

Bcl-2:

The *bcl-2* oncogene, first described at a translocation breakpoint in B-cell lymphoma, has been shown to prevent apoptosis caused by a variety of physiologic, pathologic, and Pharmacologic stimuli (Angela Gradilone, *et .al*., 2003).

This gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes. Constitutive expression of *bcl-2*, such as in the case of translocation of *bcl-2* to Ig heavy chain locus, is thought to be the cause of follicular lymphoma.

Bcl-X:

Bcl-X, another member of the *bcl-2* family, is functionally similar to *bcl-2*; the gene product exists in two forms, *bcl-*X_L (long), which blocks apoptosis in many systems, and the spliced short form *bcl-Xs*, which acts as a dominant inhibitor of bcl-2. It has been studied that *bcl-2* and *bcl-*X_L gene expression increases with progression of malignant melanoma, and the expression of these two genes could reflect an increased malignant potential caused by an apoptosis inhibition and growth advantage for metastatic melanoma cells (Leiter *et al.*, 2000).

Bax :

Bax gene product, an intracellular partner of *bcl-2*, first identified by co-immunoprecipitation with *bcl-2*, is a 21-kd protein with 21% homology to *bcl-2*. Expression of *bax* does not block apoptosis; instead, it seems to inhibit *bcl-2* function, perhaps by forming *bcl-2* /*bax* heterodimers or by competing with other *bcl-2* targets.

5.3.2 Methods

5.3.2.1 Isolation of RNA using TRIzol Reagent:

Total RNA was extracted from EAC cells after treatment of mice with and without NNTL by using TRIzol method with slight modification as illustrated by the flow chart below:

Isolate RNA from EAC cell using the TRIzol Reagent by adding 1ml of TRIzol Reagent

↓ Incubated for 5 min. at room temperature (RT)

Added 0.2 ml of chloroform per 1 ml of initial Trizol

↓ Shaked vigorously and incubated for 2-3 min. at RT

Centrifuged at 10000 RPM for 15 min. at 4°C

↓ Clear supernatant were collected in a new tube

Precipitated by adding 0.5 ml of isopropanol

↓ Incubated at RT for 20 min.

Centrifuged at 10000 RPM for 15 min. at 4°C

↓ RNA forms a gel-like pellet, removed the supernatant

Washed the pellet once with 1ml of sterilized 70% ethanol

↓ Repeated the same washing method for twice

Added 50 µl of RNAse free water and stored at –20°C

5.3.2.2 cDNA transcription

According to manufacturer's instructions everything must be RNase free until cDNA is obtained. First-strand cDNA was synthesized from 3 µg of total RNA using 50 U of MuLV (Moloney murine leukemia virus) reverse transcriptase, in a final volume of 20 µl containing 100 pmole random hexamers. For the PCR reaction, cDNA template solution was amplified using three specific primers of apoptosis-related genes namely, *bcl-2*, *bax*, and *bcl-X* , where *β-actin* was used as control. The primer sets are listed in the Table-

Table 5.1 Oligonucleotide primer sequences

Primer	Sequences	Amplification product
Bcl-2 F	5′-GTGGAGGAGCTCTTCAGGGA-3′	0.304 kb
Bcl-2 R	5′-AGGCACCCAGGGTGATGCAA-3′	
BAX F	5′-GGCCCACCAGCTCTGAGCAGA-3′	0.479 kb
BAX R	5′-GCCACGTGGGCGTCCCAAAGT-3′	
Bcl -X F	5′-TTGGACAATGGACTGGTTGA-3′	0.78 kb and 0.591 kb
Bcl-X R	5′-GTAGAGTGGATGGTCAGTG-3′	
ß-Actin F	5′-ACCCACACTGTGCCCATCTACGA-3′	0.516 kb
ß- Actin R	5′-CAGGAGGAGCAATGATCTTGATCTTC-3′	

F (forward), R (reverse).

5.3.2.3 RT- PCR

cDNA was amplified in a thermal cycle (BioRad, USA) in a solution containing 1X of taq polymerase buffer, 25 pmol each of forwarded and reverse primer, 2.5 mM of each dNTP and 0.25 U of platinum Taq polymerase. The PCR amplification was performed as follows: Initial PCR activation step was 3 min at 95^{o}C, followed by 35 cycles of 95^{o}C/1 min. 55^{o}C/1 min, 72^{o}C/1 min and a final extension of 72^{o}C/10 min for *β-actin*, and *bcl-2 gene.* The anealing temperature for *bax* and *bcl-X* was 54^{o}C instead of 55^{o}C.

Aliquots of the RT-PCR products were visualized after electrophoresis migration in a 1.0% agarose gel and GeneRular 1 kb DNA ladder (Fermentas, USA) was used as marker.

5.4 RESULTS

5.4.1 Antitumor activity

The number of viable cells in the control and NNTL treated samples in normal saline were counted by haemocytometer. Proliferation of EAC cells was effectively inhibited by the lectin. As presented in the figure 5.1, maximum proliferation was observed in the control (without NNTL). By taking it as 100%, it was found that the proliferated cell numbers were decreased significantly with increased concentration of NNTL. At the dose of 1.5 mg/Kg/day and 3.0 mg/Kg/day, the inhibition of EAC cells growth was found to be 56% and 76% respectively as compared with control

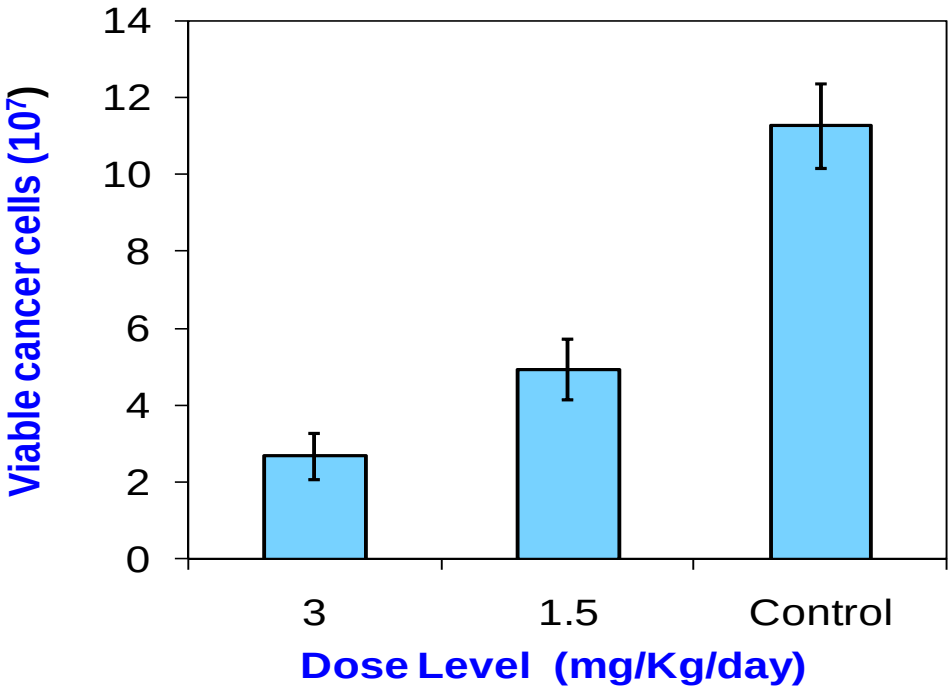


Figure 5.1 Number of EAC cells counted by light microscope in the presence and absence of NNTL in mouse.

5.4.2 Study of Apoptotic Nuclear morphology by Fluorescent Microscopy

The effect of NNTL on apoptotic morphological changes of EAC cells *in vivo* in mice were detected by fluorescence microscopy after staining with Hoechst 33342. In the control group, the nuclei in which DNA resides were round and homogeneously stained (Fig. 5.2A). Although no such morphological change and nuclear damage were observed in the cells treated with NNTL, but in NNTL treated sample the cells become cluster like form as compared to those were observed as disperse from in the control (Fig. 5.2B). The number dead cells were shown by high-red fluorescence (Fig.5.3 and 5.4).

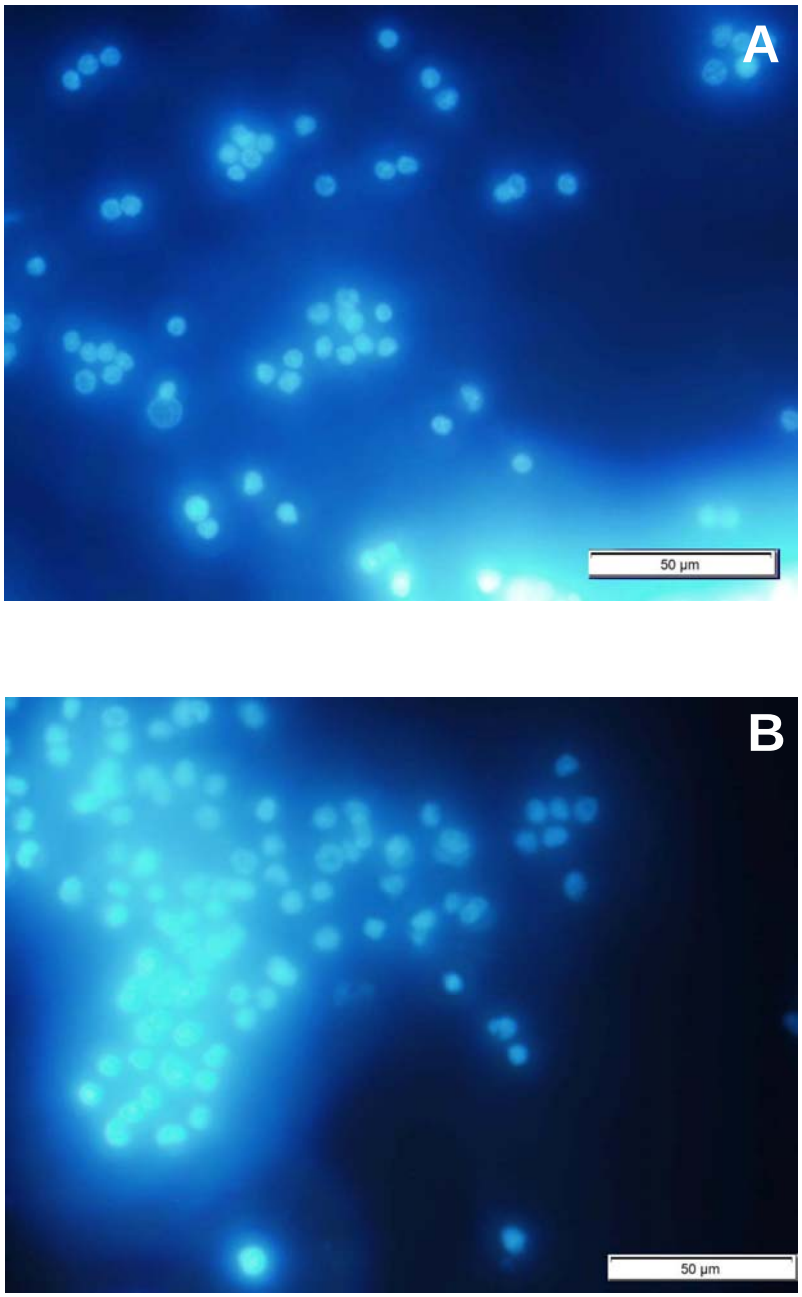


Figure 5.2 Morphology of EAC cells observed by fluorescence microscopy. (A) Control EAC cells, (B) NNTL treated EAC cells.

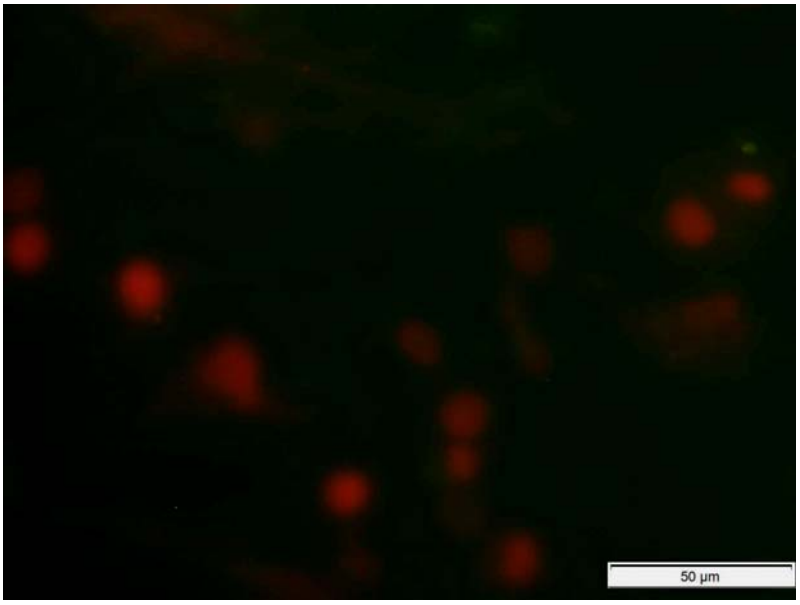


Figure 5.3 Observation of dead cells in control EAC cells in high-red fluorescence.

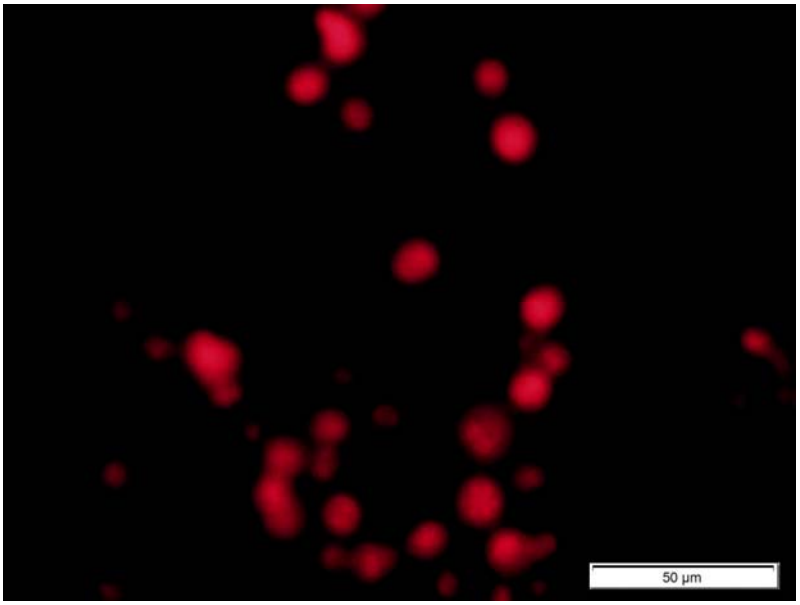


Figure 5.4 Observation of dead cells in the NNTL treated EAC cells in high-red fluorescence.

5.4.3 Observation of apoptotic gene expression by RT-PCR

The expression of the apoptosis-related genes *bcl-2, bax, bcl-X and ß-actin* was investigated by RT-PCR analysis in the EAC cells collected from mice treated with and without NNTL. It can be suggested from the Figure-5.5 that the amplification of *ß-actin* (both control and NNTL treated) confirmed the isolation of good quality of RNA from EAC cells for RT-PCR. It was found that the *bcl-2*, *bcl-X and bax* were expressed in the EAC cells treated with NNTL, as well as without NNTL. No change was observed for each of the genes both in the treated and untreated EAC cells.

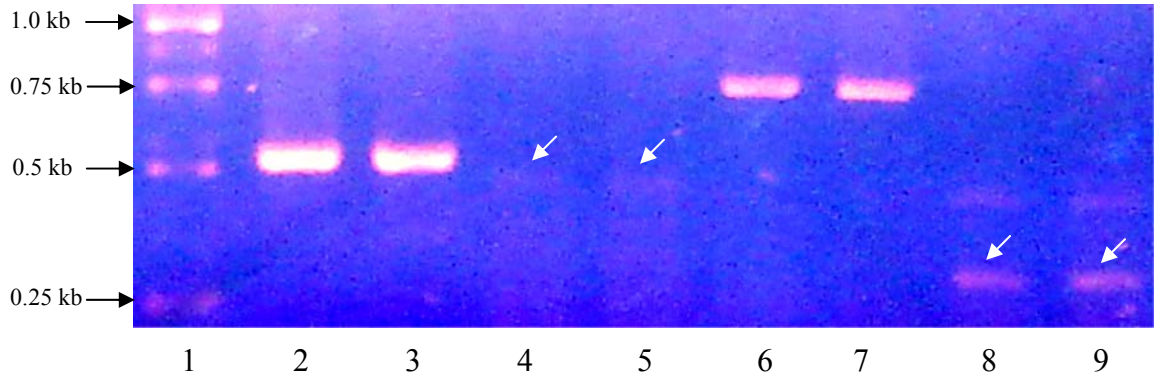


Figure 5.5 Expression of *bcl-2, bcl-X* and *bax* genes by PCR in 1% agarose gel

Lane 1: DNA Ladder (from up 1.0 kb, 0.75 kb, 0.5 kb and 0.25 kp)

Lane 2: β-actin control, Lane 3: β-actin treatment

Lane 4: *bax* control, Lane 5: *bax* treatment

Lane 6: *bcl-X* control, Lane 7: *bcl-X* treatment

Lane 8: *bcl-2* control, Lane 9: *bcl-2* treatment.

5.5 DISCUSSION

It is well established that lectin play a vital role to inhibit the multiplication of cancer cells due to the differences in their sugar specificity. Lectin molecules are bind to cancer cell membranes or receptors and exhibit cytotoxicity, apoptosis and inhibition of tumour growth (De Mejia & Prisecaru 2005). Several experiments were performed to study the anticancer effect of lectins on different cancer cells but only a few reported against EAC cells (Ahmed *et al.,* 1988, Roy *et al.,* 1993, Akev *et al.,* 2007). The present study had been carried out to detect the antitumor effect of NNTL on EAC bearing mice. The present data clearly demonstrated the NNTL effectively decreased the number of EAC cells *in vivo* in mice, and the growth rate decreased to 56% when EAC cells are treated with 3.0 mg/Kg/day for 5-days. EAC cell growth was also studied *in vivo* in mice by using Jackfruit seed lectin that inhibited 21.8, 40.2, 57.5 and 83% of growth when administrated as 50, 100, 150 and 200 µg/day respectively (Ahmed *et al.,* 1988). Similarly, lectin from *Kaempferia rotunda* Linn. tuberous rhizome (KRL) inhibited 51% and 67% at 1.25 and 2.5 mg/kg/day respectively (Kabir *et al*., 2011).

Antitumor activity was also found in many other plant lectins, such as lectin from *Curcuma amarissima* rhizomes also showed *in vitro* antitumor activity against a breast cancer cell line (BT 474) and showed the high antitumor activity with an IC_{50} of approx. 21.1 μg (Kheeree *et al*, 2010). Lectin from *Pleurotus ostreatus* (Wang *et al*., 2000) can inhibit tumor growth in *vivo*, while wheat germ lectin (Timoshenko *et al*., 2001) acts on lung cancer and *Concanavalin A* could be used as an anti-hepatoma therapeutic agent (Lei *et al*., 2009).

More importantly, the antitumor effect of NNTL was also studied to assess the apoptopic nuclear morphology of the EAC cells by Hoechst 33342 staining. From the morphological observation under fluorescent microscope, there was no so much nuclear

damage remarkably found in NNTL treated cells except some agglutination as compared with the control group.

Plant lectins, have been reported to induce programmed cell death (including apoptosis and autophagy) in many types of cancer cells. Recently, plant lectins such as Mistletoe lectins (ML-1, 2 and 3), *Concanavalin A* (ConA), and *polygonatum cyrtonema* lectin (PCL) have been widely reported to induce apoptosis in various types of cancer cell (Hoessli *et al*., 2008; Liu *et al*., 2009_a; Liu *et al*., 2009_b; Li *et al*., 2011).

Although a variety of plant lectins have been reported to induce apoptosis in cancer cells, beside this, ConA and PCL have also been found lead to induce autophagic cell death in cancers (Chang *et al*., 2008; Liu *et al*., 2009_c). Autophagy was only discovered when the updated investigations demonstrated that bioactive Con A was cytotoxic or inhibitory to typical hepatoma cells in autophagic pathway mediated via mitochondria; however, the typical caspase-dependent apoptosis was not yet observed in the studies (Lei *et al*., 2007 and 2009).

Apoptosis was also studied against EAC cells by using different proteins and plant extracts. It was reported that 'Ottelion A' a plant extract inhibited the EAC cells proliferation and causes apoptosis which mediated by increased of p53 level and $CD8^+$ (El-Missiry *et al*. 2012). A cytotoxic protein BMP1 from the aqueous extract of common Indian toad (Bufo melanostictus Schneider) skin arrested the growth of EAC cells by inducing apoptosis in the caspase dependent pathway (Bhattacharjee *et al*., 2011). Recently it was reported that Pea lectin inhibited the growth of EAC cells and induced apoptosis, by up-regulation of *bax* gene and down-regulation of *bcl-2* and *bcl-X* genes expression (Kabir *et al*., 2013).

In this study, we examined the expression of apoptosis regulating gene *bcl-2*, *bax*, and *bcl-X* in NNTL treated EAC cells by RT-PCR. From the present result, it might be

concluded that intensive *bcl-2* and *bcl-X* genes were expressed in the lectin treated and control EAC cells. On the other hand, expression of the apoptosis causing gene *bax* was slightly found both in the treated and control EAC cells. This suggests that NNTL is not associated with causing apoptosis of EAC cells *in vivo* in mice.

Many anticancer agents arrest cell cycle and then induce apoptotic cell death (Kessel and Luo 2000), however several lectins reported to induce G_1 or G_2/M accumulation mechanisms without causing apoptosis (Yan *et al*., 2009; Lyu *et al*., 2001; Siegle *et al*., 2001). *Viscuum album agglutinin*-1 (VAA-1) induced G_1-phase accumulation mechanisms without causing apoptosis in lung cancer cells A549 (Siegle *et al*., 2001).

From this study it can be concluded that although NNTL inhibit the growth of EAC cells *in vivo* in mice but could not induce the apoptosis of these carcinoma cells. However, the underlying mechanism for inhibiting the growth of EAC cell is not understood yet. In conclusion from the present data, it is an observation that NNTL might be used as an anticancer therapeutic drug.

CONCLUTION

Lectin have been isolated from *Nymphaea nouchali* tuber and purified from the crude extract through ion-exchange chromatography on DEAE-cellulose column, followed by hydrophobic chromatography on Hitrap Phenyl HP column and finally re-applying to anion-exchange chromatography on a Hitrap Q FF column. The homogeneity of the pure protein fractions obtained from different chromatographic stages were checked by SDS-PAGE on 15 & 12.5 % polyacrylamide gel. The purified lectin migrated with single band in SDS-PAGE and the apparent molecular mass was 27±1 kDa. Again in the presence and absence 2–mercaptoethanol lectin showed single band with the said molecular weight, which suggested that NNTL have no more subunit. NNTL gave a yellow-orange color in presence of phenol-sulfuric acid solution indicating that the lectin was a glycoprotein in nature and the sugar content was 8%. The presence of sugar in the lectin was further confirmed by staining with Periodic Acid-Schiff's **(**PAS) base, as gave red-purple color visible band. The lectin exhibited haemagglutinating activity towards different animal erythrocytes, and the minimum concentration for haemagglutination activity of rat erythrocytes was 8 µg/ml. Moreover NNTL did not show any human blood group specificity as it agglutinated all human blood types (A, B, O and AB groups).

The sugar specificity of NNTL was performed by inhibiting the agglutination of rat RBC against different mono- and oligosaccharides. The haemagglutination inhibition studies revealed that *O*-nitrophenyl-ß-D-galactopyranoside was the best inhibitor for NNTL and the minimum inhibitory concentration was 25 mM.

Thermal stability of NNTL was checked at different temperature and the activity was affected with the changes of temperature. The maximum activity was observed between the temperature 30 to 60ºC, and the activity of the lectin was decreased sharply above 60ºC and at 70ºC it becomes 50%. The NNTL lost its activity significantly beyond the

optimum pH value. NNTL showed the maximum haemagglutination activity (lectin activity) between the pH values ranging from pH 5.0 to 9.0. Again the lectin activity was observed to be higher at pH 10.0 than that of the pH 3.0, suggesting that the lectin may be more active at basic pH.

Treatment with DTT and urea lectin lost its activity 50% and 77.5% respectively. On the other hand, the NNTL did not show any agglutination activity when treated with EDTA in the absence of divalent ions. However after addition of 10 mM of each Ba^{2+}, Ca^{2+} and Mg^{2+} NNTL showed strong agglutination activity. The dematalization and remetalization of NNTL suggest that the lectin activity was dependent on the presence of metal ions.

The first 10^{th} residues of the N-terminal amino acids of NNTL were determined as –PEEADYLTE and homology sequence was searched by using BLAST. The sequence was not identical with any other lectins so far available and the result reveled that NNTL was a novel lectin. The amino acid compositions showed that the lectin contained higher amounts of leucine, methionine and glycine residues. The dependency of the divalent cations (especially Ca^{2+}) on the stability of NNTL was performed by the measurement of fluorescence spectra. In the presence of Ca^{2+} salt, the fluorescence intensity of NNTL was increased remarkably. Furthermore, on addition of *O*-nitrophenyl-ß-D-galactopyranoside to the lectin solution, the fluorescence intensity decreased remarkably as compared to that in presence of Ca^{2+} salt. From this observational change by fluorescence spectroscopy, it can be suggested that the NNTL is a metal ion dependent protein and its conformational changes is markedly affected upon binding to the Ca^{2+} salt and *O*-nitrophenyl-ß-D-galactopyranoside.

Like other plant lectins, NNTL is also toxic in nature as it exhibited the toxicity on brine shrimp nauplii, and the LC_{50} value was estimated to be 120± 29 µg/ml. The purified lectin exerted strong agglutination activity when subjected against several pathogenic bacteria.

The result showed that *Bacillus subtilis*, *Sarcina lutea*, *Shigella shiga*, and *Shigella sonnei* were more sensitive to NNTL and the minimum agglutination concentration were 0.42±0.17, 1.0±0.39, 1.1±0.43 and 2.2±0.86 µg/ml respectively. The lectin also responded well to the anti-tumor activity against EAC cells *in vivo* in mice. The NNTL decreased the EAC cells growth by 56% and 76% at a dose of 1.5 and 3.0 mg/kg/day respectively. This result was very significant when compared with other plant lectins. The anti-tumor effect of NNTL was also studied to assess the apoptotic morphological alternation of EAC cells by Hoechst 33342 staining. Observation under fluorescence microscopy there was no nuclear damage markedly found of NNTL treated EAC cells, which suggested that NNTL could not induce apoptosis in the EAC cells. It was also confirmed from the apoptotic related genes expression. *Bcl-X* and *bcl-2* genes expression were observed both in control and NNTL treated EAC cells. On the other hand no gene expression of *bax* was observed, which suggested that NNTL not associated with causing apoptosis of EAC cells in mice.

REFERENCES

Absar N., Yeasmin T., Raza M. S., Sarkar S.K. and Arisaka F. (2005). Single Step Purification, Char acterization and N-Terminal Sequences of a Mannose Specific Lectin from Mulberry Seeds. *The Protein J.* **24**: 369-377.

Ahmed H., Chatterjee B. P., Debnath A. K. (1988). Interaction and *in vivo* growth inhibition of Ehrlich ascites tumor cells by jacalin, *Journal of Biosciences.* **13:** 419–424.

Akev N., Turkay G., Can A., Gurel A., Yildiz F., Yardibi H., Ekiz E. E., Uzun H. (2007). Tumour preventive effect of *Aloe vera* leaf pulp lectin (Aloctin I) on *Ehrlich ascites* tumours in mice. *Phytotherapy Research* **21**: 1070–1075.

Angata T, Brinkman-Vander Linden E. (2002). I-type lectins. *Biochim. Biophys. Acta* **1572:** 294–316.

Angela Gradilone, Paola Gazzaniga, Diego Ribuffo, Susanna Scarpa, Emanuele Cigna, Fortunata Vasaturo, Ugo Bottoni, Daniele Innocenzi, Stefano Calvieri, Nicolo' Scuderi, Luigi Frati, and Anna Maria Agliano (2003). Survivin, bcl-2, bax, and bcl-X Gene Expression in Sentinel Lymph Nodes from Melanoma Patients *J Clin Oncol.* **21***:* 306-312.

Angelis De P.M., Stokke T., Thorstensen L., Lothe R.A., Clausen O.P.F. (1998). *J Clin Pathol:Mol Pathol.* **51**: 254–261

Kilpatrick D.C. (2002). Animal lectins: a historical introduction and overview. *Biochemica et Biophysica acta.* **1372:** 187-197.

Atkinson H. M., Trust T.J. (1980). Hemagglutination properties and adherence ability of Aeromonas hydrophila. *Infect Immun*. **27**: 938–946.

Banerjee S., Chaki S., Bhowal J. and Chatterjee B.P. (2004). Mucin binding mitogenic lectin from freshwater Indian gastropod *Belamyia bengalensis*: purification and molecular characterization. *Arch. Biochem. Biophys*. **421**: 125–134.

Barondes S.H., Cooper D.N.W., Gitt M.A. & Leffler H. (1994). Galectins. Structure and function of a large family of animal lectins. *J. Biol. Chem*. **269:** 20807-20810.

Bhattacharjee P., Giri B., Gomes A. (2011). Apoptogenic activity and toxicity studies of a cytotoxic protein (BMP1) from the aqueous extract of common Indian toad (Bufo melanostictus Schneider) skin. *Toxicon* **57**: 225–236.

Bhutia S.K., Mallick S.K., Maiti S., Maiti T.K. (2008a). Antitumor and proapoptotic effect of Abrus agglutinin derived peptide in Dalton's lymphoma tumor model. *Chem Biol Interact.* **174**: 11–18.

Bohlool B.B., and Schmidt E.L. (1974). Lectins: A possible basis for specificity in the rhizobium-legume root nodule symbiosis. *Science* **185**: 269-271.

Boyd W.C., Reguera R.M. (1949). Studies on haemagglutinins present in seeds of some representatives of the family Leguminoseae. *J Immunol.* **62**: 333-339.

Boyd W.C., Shapleigh E. (1954). Specific precipitating activity of plant agglutinins (lectins). *Science* **119**: 419.

Brewin N.J., and Kardailsky I.V. (1997). Legume lectins and nodulation by rhizobium. *Trends in Plant Science* **2**: 92-98.

Broekaert W.F., Mariën W., Terras F.R., De Bolle M.F., Proost P., Van Damme J., Dillen L., Claeys M., Rees S.B., Vanderleyden J. (1992). Antimicrobial peptides from Amaranthus caudatus seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins. *Biochemistry* **31**: 4308–4314.

Candy L, Van Damme E. J., Peumans W. J., Menu-Bouaouiche L., Erard M., Rougé P. (2003). Structural and functional characterization of the GalNAc/Gal-specific lectin from the phytopathogenic ascomycete Sclerotinia sclerotiorum (Lib.) de Bary. *Biochem. Biophys.Res.Commun* **308:** 396-402.

Chan H.S. and K.A. Dill (1993). The protein folding problem. *Physics Today* **46**: 4-32.

Chang C. P. and Lei H.Y. (2008). Autophagy induction in T cell-independent acute hepatitis induced by concanavalin A in SCID/NOD mice. *Int. J. Immunopathol. Pharamacol.* **21**: 817–826.

Chen J., Liu B., Ji N., Zhou J., Bian H., Li C., Chen F., Bao J. (2009). A novel sialic acid-specific lectin from *Phaseolus coccineus* seeds with potent antineoplastic and antifungal activities. *Phytomedicine* **16**: 352-360.

Chen S.J., Chen N.T., Wang S.H., Hsu J.C., Ding W.H., Kuo-Huang L.L., Huang R.N. (2009). Insecticidal action of mammalian galectin-1 against diamondback moth (Plutella xylostella). *Pest Manag Sci.* **65**: 923–930.

Cheng T.C., Marchlonis J.J., Vasta G.R. (1984). Recognition Proteins, receptors and Proves- invertebrates prog. *Clini. Biol res.* **157:** 1-15.

Cheng T.C., Marchlonis J.J., Vasta G.R. (1984). Role of molluscan lectins in recognition processes. *Prog Clin Biol Res.* **157**: 1-15.

Cheung A.H., Wong J.H., Ng T.B. (2009). Musa acuminata (Del Monte banana) lectin is a fructose-binding lectin with cytokine-inducingactivity. *Phytomedicine* **16:** 594–600.

Choi S.H., Lyu S.Y., Park W.B. (2004). Mistletoe lectin induces apoptosis and telomerase inhibition in human A253 cancer cells through dephosphorylation of Akt. *Arch Pharm Res.* **27:** 68–76.

Cooper D.N.W. (2002). Galectinomics: a lesson in complexity. *Biochim. Biophys. Acta* **1572:** 209-231.

Dahms N.M., Hancock M.K. (2002). P-type lectins. *Biochim. Biophys. Acta*. **1572:** 317–340.

Damico D.C.S., Freire M.G.M., Gomes V.M., Toyama M.H., Marangoni S., Novello J.C. and Macedo M.L.R. (2003). Isolation and Characterization of a Lectin from Annona muricata Seeds. *Journal of Protein Chemistry* **22:** 655-661.

De Mejia E. G., & Prisecaru V. I. (2005). Lectins as bioactive proteins: a potential in cancer treatment. *Critical Reviews in Food Science and Nutrition* **45:** 425–445.

Diaz C.L., Melchers L.S., Hooykaas P.J.J., Lugtenberg B.J.J., and Kijne J.W. (1989). Root lectin as a determinant of host-plant specificity in the rhizobium-legume symbiosis. *Nature* **338**: 579-581.

Dodd R.B. and Drickamer K. (2001). Lectin- loke proteins in model organisms: implications for evolution of carbohydrate-binding activity. *Glycobiology* **11:** 71R-79R.

Does M.P., Houterman P.M., Dekker H.L., Cornelissen B.J. (1999). Processing, targeting, and antifungal activity of stinging nettle agglutinin in transgenic tobacco. *Plant Physiol*. **120**: 421-32.

Drickamer K. & Taylor M. E. (1993). Biology of animal lectins, *Annu.Rev. Cell Biol.* **9:** 237-264.

Drickamer K. (1999). C-type lectin-like domains. *Curr Opin Struct Biol*. **9:** 585–590.

Dubois K., Gilles K.A., Hamilton, J.K., Rebers P.A., and Smith F. (1956). *Anal Chem.* **28**: 350-356.

Ehrlich P. and Apolant H. (1905). *Berl. Klin. Wochenschr*. **42**: 871.

El-Missiry M.A., Othman A.I., Amer M.A., Mohamed E. (2012). Ottelione A inhibitedproliferation of Ehrlich ascites carcinoma cells in mice. *Chemico-Biological Inter-actions* **200**: 119–127.

Fairbanks G., Steck T.L., Wallach D.F. (1971). *Biochemistry* **10:** 2606–2617.

Fang E.F., Lin P., Wong J.H., Tsao S.W., Ng T.B. (2010). A lectin with anti- HIV-1 reverse transcriptase, antitumor, and nitric oxide inducing activities from seeds of Phaseolus vulgaris cv. extralong autumn purple bean. *J Agric Food Chem.* **58:** 2221–2229.

Finney D. J. (1947). Probit Analysis : A statistical treatmentof sigmoid response curve. *Cambridge University Press* 333.

Gaidamashvili M., Ohizumi Y., Iijima S., Takayama T., Ogawa T. and Muramoto K. (2004). Characterization of the yam tuber storage proteins from *Dioscorea batatas* exhibiting unique lectin activities. *J. Biol. Chem*. **279**: 26028–26035.

Goldstain I.J., Hughes R.C, Monsigny M., Osawa T., and Sharon N. (1980). What should be called a lectin? *Nature (London)* **285**: 66.

Guillot J, Konska G, (1997). Lectins in higher fungi. *Biochem Syst Eco* **25**: 203-230.

Guohong Zhang, Vanessa Gurtu, Steven R. Kain and Guochen Yan1 (1997). *BioTechniques* **23**: 525-531.

Hamblin J., and Kent S.P. (1973). Possible role of phytohaemagglutinin in *Phaseolus vulgaris* L. *Nature New Biology* **245**: 28-30.

Hansen J.E., Nielsen C.M., Nielsen C., Heegaard P., Mathiesen L.R., Nielsen J.O. (1989). Correlation between carbohydrate structures on the envelope glycoprotein gp120 of HIV-1 and HIV-2 and syncytium inhibition with lectins. *AIDS* **3**: 635–641.

Hiromasa Tsuda1, Zhao Ning1, Yoko Yamaguchi and Naoto Suzuki (2012). *Journal of Oral Science* **54**: 137-149,

Hirsch A.M., Brill L.M., Lim, P.O., Scambray J., and Van Rhijn P. (1995). Steps toward defining the role of lectins in nodule development in legumes. *Symbiosis* **19**: 155- 173.

Hoessli D.C, Ahmad I (2008). Mistletoe lectins: carbohydrate-specific apoptosis inducers and immunomodulators. *Curr Org Chem*. **2:** 918–25.

http://inbar.int/wiki/index.php/ Medicinal plants

http://plants.usda.gov/java/profile?symbol=NYLO

***http://www.ansci.cornell.edu/plants/index.html*.**

http://*www.ncbi.nlm.nih.gov/blast*.

***http://www.ucmp.berkeley.edu/protista/slimemolds.html*.**

Kabir S.R., et al., Pea lectin inhibits growth of Ehrlich ascites carcinoma cells by inducing apoptosis and G2/M cell cycle arrest in vivo in mice. *Phytomedicine* (2013), http://dx.doi.org/10.1016/j.phymed. **2013.06.010**.

Kabir S.R., Hossen M.A., Zubair M.A., Alom M.J., **Islam M.F., Hossain M. A. and Kimura Y. (2011)**. A New Lectin from the tuberous Rhizome of *Kaempferia rotunda*: Isolation, Characterization, Antibacterial and Antiproliferative Activitiesthe. *Protein Pept Lett*. **18**: 1140-1149.

Kabir S.R., Islam M.F., Alom M.J., Zubair M.A., Absar N. (2012). Purification, characterizations of a snake guard seeds lectin with antitumor activity against Ehrlich ascites carcinoma cells in vivo in mice. *Protein Pept Lett.* **19**: 360-8.

Kaur A., Singh J., Kamboj S.S., Sexena A.K., Pandita, R.M. and Shamnugavel M. (2005). Isolation of an N-acetyl-D-glucosamine specific lectin from the rhizomes of *Arundo donax* with antiproliferative activity. *Phytochemistry* **66**: 1933-1940.

Kaur M., Singh K., Rup P. J., Kamboj S.S., Saxena A.K., Sharma M., Bhagat M., Sood S. K. and Singh J. (2006$_a$). A tuber lectin from *Arisaema jacquemontii* Blume with anti-insect and anti-proliferative properties. *J. Biochem. Mol. Biol*. **39:** 432–440.

Kaur M., Singh K., Rup P.J., Saxena A.K., Khan R.H., Ashraf M.T., Kamboj S.S., Singh J. (2006$_b$). A tuber lectin from *Arisaema helleborifolium* Schott with anti-insect activity against melon fruit fly, Bactrocera cucurbitae (Coquillett) and anti-cancer effect on human cancer cell lines. *Arch Biochem Biophys*. **445**: 156–165

Kaur N., Dhuna V., Kamboj S.S., Agrewala J.N., Singh J., (2006c). A novel antiproliferative and antifungal lectin from *Amaranthus viridis* Linn seeds. *Protein Pept Lett.* **13**: 897-905.

Kessel D., Luo Y. (2000). Cells in cryptophycin-induced cell-cycle arrest are susceptible to apoptosis. *Cancer Lett.* **151**: 25–29

Keyaerts E., Vijgen L., Pannecouque C., Van Damme E., Peumans W., Egberink H., Balzarini J., Van Ranst M. (2007). Plant lectins are potent inhibitors of coronaviruses by interfering with two targets in the viral replication cycle. *Antivir Res.* **75**: 179–187.

Kheeree N., Sangvanich P., Puthong S. and Karnchanatat A. (2010). Antifungal and antiproliferative activities of lectin from the rhizomes of *Curcuma amarissima* Roscoe. *Appl. Biochem. Biotechnol*. **162**: 912–925.

Khin M.M., Hua J.S., Ng H.C., Wadstrm T. and Ho B. (2000). Agglutination of Helicobacter pylori coccoids by lectins. *World Journal of Gastroenterology* **6**: 202-209.

Kobayashi T, Kuroda J, Ashihara E, Oomizu S, Terui Y, Taniyama A, Adachi S, Takagi T, Yamamoto M, Sasaki N, Horiike S, Hatake K, Yamauchi A, Hirashima M, Taniwaki M. (2010). Galectin-9 exhibits anti-myeloma activity through JNK and p38 MAP kinase pathways. *Leukemia*. **24**: 843–850.

Konozy E.H.E., Bernardes E.S., Rosa C., Faca V., Greene L.J. and Ward R. J. (2003). Isolation, purification and physicochemical characterization of a D-galactose-binding lectin from seeds of *Erythrina speciosa*. *Arch. Biochem. Biophys.* **410**: 222–229.

Konska G. (1985). Lectins in higher fungi. Thesis, Faculty of Pharmaceutical Sciences, Cracow (in Polish).

Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lam S.K., Ng T.B., (2010a). First report of a haemagglutinin-induced apoptotic pathway in breast cancer cells. *Biosci Rep*. **30**: 307–317.

Le Minor L., Tournier P., Chalon A.M.(1973). The agglutination by concanavalin A of certain gram negative bacilli. A study into the relationship with the somatic antigens of *Salmonella* serotypes. *Ann. Microbiol.***124A:** 467-476.

Lei H.Y., Chang C.P. (2007). Induction of autophagy by concanavalin A and its application in anti-tumour therapy. *Autophagy* **3**: 402– 404.

Lei H.Y., Chang C.P. (2009). Lectin of Concanavalin A as an antihepatoma therapeutic agent. *J. Biomed. Sci.* **16**: 1-12.

Leiter J., Schmid R.M., Kaskel P. (2000). Antiapoptotic bcl-2 and bcl-xL in advanced malignant melanoma. *Arch Dermatol Res.* **292**: 225- 232.

Leonand A.S., Sauer, Arlene P.H., Martin and Elmer (February 1960). Cytochemical fractionation of the letter-Eharlich Ascites tumour, *Cancer Res*. **20**: 251-256.

Li W.W., Yu J.Y., Xu H.L., Bao J.K. (2011). Concanavalin A: a potential anti-neoplastic agent targeting apoptosis, autophagy and anti-angiogenesis for cancer therapeutics. *Biochem. Biophys. Res. Commun*. **414**: 282−286.

Li Y.R., Liu Q.H., Wang H. X. and Ng, T. B. (2008). A novel lectin with potent antitumor, mitogenic and HIV-1 reverse transcriptase inhibitory activities from the edible mushroom Pleurotus citrinopileatus. *Biochim. Biophys. Acta (BBA)* **1780**: 51-57.

Liener I.E., Sharon N. and Goldstein I.J. (1986). The Lectins; Properties, Functions and Applications in Biology and Medicine. *Academic, Orlando, Fla*. **600**.

Lin J., Zhou X., Pang Y., Gao H., Fei J., Shen G.A., Wang J., Li X. Sun X. and Tang K. (2005). Cloning and characterization of an agglutinin gene from Arisaema lobatum, *Biosci. Rep*. **25:** 345–362

Lin P., Ye X., Ng T. (2008). Purification of melibiose-binding lectins from two cultivars of Chinese black soybeans. *Acta Biochim Biophys Sin (Shanghai).* **40**: 1029–1038.

Lis H. and Sharon N. (1998). Lectins: Carbohydrate-specific proteins that mediate cellular recognition. *Chem. Rev*. **98**: 637-674.

Liu B., Cheng Y., Bian H.J., Bao J.K. (2009c). Molecular mechanisms of Polygonatum cyrtonema lectin-induced apoptosis and autophagy in cancer cells. *Autophagy* **5**: 253–255.

Liu B., Cheng Y., Zhang B., Bian H.J., Bao J.K. (2009_b). Polygonatum cyrtonema lectin induces apoptosis and autophagy in human melanoma A375 cells through a mitochondria-mediated ROS-p38-p53 pathway. *Cancer Lett.* **275**: 54−60.

Liu B., Li C.Y., Bian H.J., Min M.W., Chen L.F., Bao J.K. (2009_a). Antiproliferative activity and apoptosis-inducing mechanism of Concanavalin A on human melanoma A375 cells. *Arch Biochem Biophys* **482**, 1–6.

Liu, B., Bian, H. J. and Bao, J. K. (2010). Plant lectins: potential antineoplastic drugs from bench to clinic. *Cancer Lett*. **287**: 1–12.

Loewenthal, H. and Jahn, A. (1932). *Z. Krebsforsch*. **37**: 439.

Lowry O.H., Rosebrough N.J., Farr A.L., and Randall, R.J. (1951**)**, Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **193**: 265-275.

Lyu S.Y., Park W.B, Choi K.H., Kim W.H. (2001). Involvement of caspase-3 in apoptosis induced by *Viscum album* var. *coloratum*agglutinin in HL-60 cells. *Biosci. Biotechnol. Biochem.* **65**: 534–541.

Majumder P., Mondal H.A. and Das S. (2005). Insecticidal activity of *Arum maculatum* tuber lectin and its binding to the glycosylated insect gut receptors. *J. Agric. Food Chem*. **53**: 6725-9.

Manpreet Kaur, Kuljinder Singh, Pushpinder Jai Rup, Sukhdev Singh Kamboj, Ajit Kumar Saxena, Madhunika Sharma, Madhulika Bhagat, Sarvesh Kumar Sood and Jatinder Singh (2006). A Tuber Lectin from Arisaema jacquemontii Blume with Anti-insect and Anti-proliferative Properties. *Journal of Biochemistry and Moleculer Biology*. **39**: 432-440.

McLaughlin J. L. (1991). Crown gall tumours in potato discs and brine shrimp lethality: Two simple bioassays for higher plant screening and fractionation. In: Methods in Plant Biochemitry. *Academic Press, London* **6**: 1-31.

Meyer B.N., Ferrigni N.R., Putnam J.E., Jacobsen L.B., Nichols D.E. and McLaughlin J.L. (1982). Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Med*. **45**: 31-34.

Millar D.J., Allen A.K., Smith C.G., Sidebottom C., Slabas A.R. and Bolwell G.P. (1992). Chitin-binding proteins in potato (*Solanum tuberosum* L.) tuber Characterization, immunolocalization and effects of wounding. *Biochem. J*. **283**: 813-821.

Moreira R.A., Monteiro A.C.O., Horta A.C.G., Oliveira J.T.A., & Cavada B.S. (1996). Isolation and characterization of Dioclea altissima var. megacarpa seed lectin. *Phytochemistry* **46**: 139–144.

Müller W.E., Renneisen K., Kreuter M.H, Schröder H.C., Winkler I. (1988). The D-mannose-specific lectin from Gerardia savaglia blocks binding of human immune-deficiency virus type I to H9 cells and human lymphocytes in vitro. *J Acquir Immune Defic Syndr*. **1**: 453–458.

Nakagawa R., Yasokawa D., Ikeda T. and Nagashima K. (1996). Purification and Characterization of two lectins from callus of *Helianthus tuberosus***.** Biosci. Biotechnol. Biochem. **60**: 259- 262.

Naughton P.J., Grant G., Bardocz S., Pusztai A. (2000). Modulation of Salmonella infection by the lectins of *Canavalia ensiformis* (Con A) and *Galanthus nivalis* (GNA) in a rat model in vivo. *J. Appl. Microbiol*. **88:** 720-727.

Nelson D.L. and Cox M.N. (2001). The three dimentional structure of proteins. Lehninger's Principles of Biochemistry, pp.159-202. *Macmillon Worth Publishers, New York.*

Ng TB, Ngai PH, Xia L (2006). An agglutinin with mitogenic and antipro- liferative activities from the mushroom. *Flammulina velutipes. Mycologia.* **98**: 167–171

Ochoa, J.L. (1979). The mechanism of lectin-mediated cell agglutination. *Pathologie-Biologie* 2**7: 193-113.**.

Ohizumi Y., Gaidamashvili M., Ohwada S., Matsuda K., Kominami J, Nakamura-Tsuruta S., Hirabayashi J., Naganuma T., Ogawa T., Muramoto K. (2009). Mannose-binding lectin from yam (*Dioscorea batatas*) tubers with insecticidal properties against Helicoverpa armigera (Lepidoptera: Noctuidae). *J Agric Food Chem*. **57**: 2896–2902.

Oliveira M.D.L., Andrade C.A.S., Santos-Magalha N.S., Coelho L.C.B.B., Teixeira J.A., Carneiro-da-Cunha M.G., Correia M.T.S. (2008). Purification of a lectin from Eugenia uniflora L. seeds and its potential antibacterial activity. *Lett. Appl. Microbiol.* **46:** 371-376.

Pajic I, Kljajic Z, Dogovic N, Sladic D, Juranic Z, Gasic MJ, (2002). A novel lectin from the sponge Haliclona cratera: isolation, characterization and biological activity. Comp Biochem Physiol C Toxicol Pharmacol. 132: 213-21.

Park W.B., Lyu S.Y., Kim J.H., Choi S.H., Chung H.K., Ahn S.H., Hong S.Y., Yoon T.J., Choi M.J. (2001). Inhibition of tumor growth and metastasis by Korean mistletoe lectin is associated with apoptosis and antiangiogenesis. *Cancer Biother Radiopharm*. **16**: 439–447.

Perssone G. (1980). Proceeding of the International Symposium on Brine shrimp. *Artemia salina*, Volumes **1-3**, Univ.Press. Witteren, Belgium.

Popovic M.M., Bulajic A., Ristic D., Krstic B., Jankov R.M., Gavrovic-Jankulovic M. (2012). In vitro and in vivo antifungal properties of cysteine proteinase inhibitor from green kiwifruit. *J Sci Food Agric*. **92**: 3072-8.

Powell L.D., Varki A. (1995). I-type lectins. *J Biol Chem*. **270**: 14243–14246.

Pramod S.N. and Venkatesh V. (2006). Utility of pentose colorimetric assay for the purification of potato lectin, an arabinose-rich glycoprotein. *Glycoconjugate Journal* **23**: 481-488.

Qiaojuan Yan, Yanxia Li, Zhengqiang Jiang, Yan Sun, Lifen Zhu, Zufeng Ding (2009). Antiproliferation and apoptosis of Human tumor cell lines by a lectin (AMML) of *Astragalus mongholicus*. *Phytomedicine* **16:** 586-593.

Raszeja S. (1958). Über spezifische Phytagglutinine aus *Laccaria laccata* varietas *proxima*. *Z Arztl Fortbild* **14**: 801-803.

Roy S., Ahmed H., Basu S., Chatterjee B.P. (1993). Purification, characterization, and carbohydrate specificity of the lectin of *Ficus cunia*. *Carbohydr Res.* **242**: 247-263.

Rüdiger H., Gabius H.J. (2001). Plant lectins: Occurrence, biochemistry, functions and applications. *Glycoconjugate J.* **18**: 589-613.

Rutkowski A. (1970). The feed value of rapeseed meal. *Journal of the American Oil Chemists' Society* **48:** 863–868.

Sá R.A., Santos N.D., da Silva C.S., Napoleão T.H., Gomes F.S., Cavada B.S., Coelho L.C., Navarro D.M., Bieber L.W., Paiva P.M. (2009). Larvicidal activity of lectins from Myracrodruon urundeuva on Aedes aegypti. *Comp Biochem Physiol C Toxicol Pharmacol*. **149**: 300–306.

Sallay I., Tojo S., Nomiyama K., Kouzuma Y., Kimura M. and Yamasaki N. (2001). Calcium ions stabilize a protein structure of hemolytic lectin CEL-III from marine invertebrate *Cucumaria echinata*. Biosci. *Biotechnol. Biochem.* **65**: 1347–1352.

Sarinya Charungchitrak , Amorn Petsom, Polkit Sangvanich, Aphichart Karnchanatat (2011). Antifungal and antibacterial activities of lectin from the seeds of Archidendron jiringa Nielsen. *Food Chemistry* **126**: 1025–1032.

Schultze B., Zimmer G., Herrlere G. (1993). Viral lectins for the detection of 9-O-Actylated Sialic acid on glycoproteins and glycolioids. *Lectins and Glycobiology*, 158-174. Gabius HJ, Gabius S, (Eds) Springer-Verlag Berlin, Heidelberg.

Semiglazov V.F., Stepula V.V., Dudov A., Schnitker J., Mengs U. (2006). Quality of life is improved in breast cancer patients by Standardised Mistletoe Extract PS76A2 during chemotherapy and follow-up: a randomised, placebo-controlled, double-blind, multicentre clinical trial. *Anticancer Res.* **26**: 1519–1529.

Sequeira L., Graham T.L. (1977). Agglutination of avirulent strains of *Pseudomonus solanacearum* by potato lectin. *Physiol Plant Pathol.* **11**: 43-54

Sharon N. (1984). Surface carbohydrates and surface lectins are recognition determinants in phagocytosis. *Immunol.Today* **5**: 143-147.

Sharon N. (1987). Bacterial lectins, cell-cell recognition and infectious disease. *FEBS Lett.* **217**: 145-157.

Sharon N. and Lis H. (1989). Lectins. *Chapman and Hall, London* **127**.

Sharon N. and Lis H. (1993). Carbohydrate in Cell Recognition. *Scientific American,* (page.82-89).

Sharon N. and Lis. H. (1990). Legume lectins - a large family of homologous proteins. *FASEB J*. **4**: 3198-3208.

Shewry P.R. (2003). Tuber Storage Proteins. *Annals of Botany* **91**: 755-769.

Siegle I., Fritz P., McClellan M., Gutzeit S., Murdter T.E. (2001). Combined cytotoxic action of *Viscum album* agglutinin-1 and anticancer agents against human A549 lung cancer cells. *Anticancer Res*. **21**: 2687–2691.

Sitohy M., Doheim M., Badr H. (2007). Isolation and characterization of a lectin with antifungal activity from Egyptian *Pisum sativum* seeds. *Food Chem.* **104**: 971-979.

Sopit Wongkham, Patcharee Boonsiri, Chusri Trisonthi, Sonthaya Simasthiansophon, Chaisiri Wongkham, Kanit Atisok (1995). Studies of Lectin from Thai plants. *J.Sci.Soc.Thailand* **21**: 27-36.

Stillmark H. (1888). Über Ricin ein giftiges Ferment aus den Samen von *Ricinus communis* L. und einige anderen Euphorbiaceen, *Inaugural Dissertation Dorpat*, Tartu.

Summer J.B. and Howell S.F. (1936). The identification of the hemagglutinin of the Jack bean with Concanavalin A. *Journal of Bacteriology* **32**: 227-237.

Suseelan K.N., Mitra R., Pandey R., Sainis K.B. and Krishna T.G. (2002). Purification and characterization of a lectin from wild sunflower (*Helianthus tuberosus* L.) tubers. *Arch. Biochem. Biophys*. **407**: 241–247.

Tian Q., Wang W., Miao C., Peng H., Liu B., Leng F., Dai L., Chen F., Bao J. (2008). Purification, characterization and molecular cloning of a novel mannose-binding lectin from rhizomes of *Ophiopogon japonicus* with antiviral and antifungal activities. *Plant Sci*. **175**: 877-884.

Timoshenko A.V., Andre S., Kayser K., Gabius H.J. (2001). Lectin (WGA)-dependent superoxide anion release by neutrophils as a prognostic factor in lung cancer. *Anticancer Res.* **21:** 3453-3456.

Tronchin G., Esnault K., Sanchez M., Larcher G., Marot-Leblond A., Bouchara J.P. (2002). Purification and partial characterization of a 32-kilodalton sialic acid-specific lectin from *Aspergillus fumigatus*. *Infect Immun.* **70**: 6891-6895.

Van Damme E.J.M., Barre A., Rouge P. and Peumans W.J. (2006). Potato lectin: an upated model of a unique chimeric plant protein. *The Plant Journal* **37**: 34-45.

Van Damme, E.J.M., Peumans W.J., Barre A., Rougé P. (1998). Plant lectins: A composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. *Crit. Rev. Plant Sci.* **17**: 575-692.

Van Eijsden R.R. (1995). Sugar-binding activity of pea (*Pisum sativum*) lectin is essential for heterologous infection of transgenic white clover hairy roots by *Rhizobium leguminosarum* biovar viciae. *Plant Molecular Biology* **29**: 431-439.

Van Parijs J., Broekaert W.F., Goldstein I.J., Peumans W.J. (1991). Hevein: an antifungal protein from rubber-tree (Hevea brasiliensis) latex. *Planta*.**183**: 258–264.

Varki A., Cummings R., Esko J.D., Freeze H.H., Stanley P., Bertozzi C.R., Hart G.W., and Etzler M.E. (2009). Essential of Glycobiology, 2nd Edition, Cold Spring Harbor (NY), *Cold Spring Harbor Laboratory Press*.

Velkov V.V., Medvinsky A.B., Sokolov M.S., Marchenko A.I. (2005). Will transgenic plants adversely affect the environment? *J Biosci.* **30**: 515-548.

Voet D, Voet JG. Biochemistry. New York: *J Wiley* **(1990).** 1223 p.

Wang H. and Ng T.B. (2006). A lectin with some unique characteristics from the samta tomato. *Plant Physiol. Biochem*. **44**: 181-185.

Wang H., Gao J., Ng T.B. (2000). A New Lectin with Highly Potent Antihepatoma and Antisarcoma Activities from the Oyster Mushroom *Pleurotus Ostreatus*. *Biochem. Biophys. Res. Commun.* ***275*****:** 810-816.

Wang J.H., Ng T.B. (2005). Isolation and characterization of a glucose/mannose/ rhamnose-specific lectin from the knife bean *Canavalia gladiata*. *Arch Biochem. Biophys.* ***439*****:** 91-98.

Xia L., Ng T.B. (2006). A hemagglutinin with mitogenic activity from dark red kidney beans. *J Chromatogr B Analyt Technol Biomed Life Sci*. **844**: 213–216.

Zhao J.K., Wang H.X., Ng T.B. (2009). Purification and characterization of a novel lectin from the toxic wild mushroom Inocybe umbrinella. *Toxicon* **53**: 360–366