ISOLATION, CHARACTERIZATION AND APPLICATION OF LECTINS FOR THE DETECTION OF CELL SURFACE MARKERS

Thesis submitted to the

UNIVERSITY OF CALICUT
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for the award of the degree of

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

by
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CERTIFICATE

This is to certify that the thesis entitled **"Isolation, Characterization and Application of Lectins for the Detection of Cell Surface Markers"** is a bonafide work of Mr. **Krishna Kumar,T.,** conducted in the Department of Life Sciences under my guidance and supervision. This thesis has not been previously formed the basis for the award of any other degree, diploma, associateship or any other similar title of any other university or society.

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CERTIFICATE

This is to certify that the thesis entitled **"Isolation, Characterization and Application of Lectins for the Detection of Cell Surface Markers"** is a bonafide work of **Mr. Krishna Kumar,T.**, conducted in the Department of Life Sciences under the guidance and supervision of **Dr. V.K. Sasidharan,** Professor of Biochemistry, Department of Life Sciences, University of Calicut during the period 1996-2001.

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DECLARATION

This thesis entitled **"Isolation, Characterization and Application of Lectins for the Detection of Cell Surface Markers"** is being submitted by me to the University of Calicut in partial fulfilment of the requirements for the award of degree of Doctor of Philosophy in Biochemistry in the Faculty of Science. The thesis in entirely the result of my work carried out in the Department of Life Sciences under the guidance and supervision of **Dr. VK Sasidharan,** Professor of Biochemistry, Department of Life Sciences, University of Calicut. This thesis or any part there of, has not been submitted for any other degree, diploma or associateship.

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Krishna Kumar,T.

CONTENTS

ABBREVIATIONS

µg	Microgram
µl	Microlitre
2,4-D	2,4- Dichlorophenoxy acetic acid
ABL	*Agaricus Bisporus* Lectin
AFP	Alpha Feto Protein
APS	Ammonium Persulphate
Ara.	Arabinose
BPA	*Bauhinia purpurea* Agglutinin
BSA	Bovine Serum Albumin
CD	Circular Dichroism
cm	Centimetre
Con A	*Concanavalia ensiformis*
CVL	*Canavalia virosa* Lectin
D-Gal P	D- Galactose-Phosphate
EDTA	Ethylene Diamine Tetra Acetic acid
ENL	*Euphorbia neriifolia* Lectin
FCS	Fetal Calf Serum
FDNB	Fluoro Dinitro Benzene
g	Gram
G	Gravity
Gal	Galactose
GalNAc	N-acetyl Galactosamine
GlcNAc	N-acetyl Glucosamine
h	Hour
HBSS	Hank's Balanced Salt Solution
HPA	*Helix Pomatia* Agglutinin

IAA	Indole Acetic Acid
JFL	Jack fruit lectin
kDa	Kilo Dalton
Lac.	Lactose
LCA	*Lens culinaris* Agglutinin
LCH	*Lens culinaris* haemagglutinin
M	Molar
MW	Molecular Weight
ManNAC	N- acetyl mannosamine
MBL	Mannose Binding Lectin
mg	Milligram
ml	Millilitre
mM	Millimolar
MS	Murashige Skoog
MTT	(3-[4,5-Dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue)
N	Normal
ng	Nanogram
nm	Nanometer
PAGE	Poly-Acrylamide Gel Electrophoresis
PAS	Periodic Acid Schiff's
PBS	Phosphate Buffered Saline
PNA	Peanut Agglutinin
RCA	*Ricinus communis* Agglutinin
SBA	Soybean Agglutinin
SCC	Squamous Cell Carcinoma
SDS	Sodium Dodecyl Sulphate

SRBC	Sheep Red Blood Cell
TEMED	Tetramethyl Ethylene Diamide
Tre.	Trehalose
TRIS	Trihydroxymethyl aminomethane
UEA	*Ulex europeus* Agglutinin
v/v	Volume/Volume
VVA	*Vicia villosa* Agglutinin
WGA	Wheat germ Agglutinin
WHO	World Health Organization

CHAPTER 1

Introduction and Review of Literature

1.1. Lectins- A historical overview

In 1888, a dissertation presented by Stillmark entitled 'Ricin' a toxic enzyme from seeds of *Ricinus communis* and some other euphorbiaceae at the medical school of Dorpat (now Tarta), in Estonia, commenced the era of lectins. In this paper Stillmark described 'interesting effects of ricinus (extract), on the blood' i.e., their ability to agglutinate erythrocytes without realising that ricin had binding sites for carbohydrate groups. Ehrlich (1891), compared ricin with the toxic abrin from *Abrus precatorius*, which initiated many fundamental advances in the knowledge of immunological reactions.

Hellin (1891), in his thesis described the agglutination and serum precipitating properties of the toxic extract of Jeriquintiy (*Abrus precatorius*), seeds. Later Elfstrand (1898), showed the differences in the properties of crotin towards different animal erythrocytes. It was he, who introduced the term 'haemagglutinin' for the first time for plant proteins that causes the clumping of cells. Landsteiner and Raubitschek (1907), described for the first time the presence of nontoxic lectins in the seeds of plants coming under fabaceae family and showed that raising the temperature to 50 °C can liberate the lectins attached to the red blood cells. They recognised that the intensity of haemagglutination by the same plant extract varied with the origin of the red cells tested i.e., plant agglutinins are species specific. They also found that, these plant agglutinin were soluble and nondialyzable protein, which could be salted out by electrolytes. In addition to this, they observed that agglutination of erythrocytes by ricin, abrin or bean extracts could be inhibited by porcine gastric mucin.

Raubitschek (1909), observed that heat inactivated serum has the capacity to inhibit haemagglutination by lectins. Schneider (1912), had studied the localization of lectins in certain structures of seeds and various other parts of plants. In 1919, Sumner reported the presence of two haemagglutinating constituents namely Concanavalin A (Con A) and Concanavalin B in the extracts of

jack bean *Canavalia ensiforms*. In earlier days, most of the haemagglutination properties were studied in crude saline extracts and it was Kobert (1913), who first isolated by deadsorption from erythrocytes with an acid solution. Later, Fugiwara (1923), purified soybean lectin and crotin by adsorption to alumina, kaloin and freshly prepared calcium phosphate. By using a similar technique, Karrer *et al.*, (1925), purified ricin and crotin and analysed its amino acid content partially.

Sumner and Howell (1936a), isolated Con A from *Canavalia ensiformis*, and that its haemagglutinating activity was inhibited by cane sugar. They suggested that haemagglutination by Con A might be the consequence of a reaction of the protein with carbohydrates on the surface of red blood cell, thus paving the way for the recognition that the plant agglutinins are sugar specific.

In 1945, Boyd reported that the agglutinin from lima bean (*Phaseolus lunatus Syn. limensis*), agglutinated red cells of human blood type 'A' but not type 'B', which is a significant discovery in the field of lectinology. Independently, Renkonen (1948) made similar observations, reporting several blood group specific extracts among 57 plant species belonging to 28 different genera.

As a result of these observations, Boyd and Shapleigh (1954), proposed the term 'lectins' (from the Latin word *'legere'*, to choose or to pick out), for agglutinins. Although, the name 'lectins' has been generally accepted, the term 'phytohaemagglutinins' are still used for plant lectins in general. In addition, the term phytohaemagglutinins (PHA), is commonly used to denote the crude or purified lectin from the red kidney bean *Phaseolus vulgaris*, which is widely employed in clinical laboratories.

1.2. Definition of lectins

In 1978 Goldstein and Hayes defined lectins as carbohydrate binding proteins that bind glycans of glycoprotein, glycolipids or polysaccharides with high affinity. Later they redefined lectins as sugar-binding and cell- agglutinating proteins or glycoproteins, of nonimmune origin. In contrast to antibodies, which are structurally similar, lectins vary in composition, molecular weight, subunit structure and number of sugar binding sites. Thus Kocourek and Horejsi (1981) proposed that 'lectins' are sugar binding proteins or glycoproteins of nonimmune origin which are devoid of enzymatic activity towards sugars to which they bind and have at least one binding site. This definition also includes the monovalent sugar binding protein. Dixon (1981) described lectins as carbohydrate binding proteins of nonimmune origin, which agglutinate cells or precipitate polysaccharides or glycoconjugates.

Barondes (1988) has proposed a less restrictive definition of lectin, that is, a carbohydrate binding protein other than an enzyme or an antibody. Franz in 1990 has also proposed the same definition to lectin.

This distinction may perhaps not be a real one; although lectins are believed to be devoid of any enzymatic activity. Hankins and Shannon in 1978 had shown that a highly purified lectin from mung bean (*Vigna radiata*), specific for ∝-galactosides also possesses ∝- galactosidase activity.

1.3. Detection and assay of lectins

The presence of lectin in a plant is readily detected by testing whether an extract of the plant agglutinates erythrocytes and by testing wether the agglutination is sugar specific i.e., inhibited by simple or complex saccharides. Haemagglutination occurs by the formation of multiple cross bridges between the opposing cells and the lectin molecules.

Nicolson in 1976 had reported that agglutinin is influenced by many factors like molecular properties of lectin, cell surface and metabolic state of the cell. Usually haemagglutination is tested with native or modified erythrocytes from human or other animals. Blood group specific lectins are identified with the aid of a panel of typed human erythrocytes.

The most common cell modification is mild digestion with trypsin or other proteolytic enzymes (Lis and Sharon, 1972), or with neuraminidase (Marikovsky *et al.*, 1976), an enzyme that removes sialic acid from complex carbohydrates. Such treatments leave the cell intact but render them more sensitive to agglutination. The cell may also be cross linked by glutaraldehyde or formaldehyde to stabilise them and to provide a standard cell preparation that can be used for long periods (Butler 1963; Liener, 1975). Cross-linking of soybean agglutinin (SBA) with glutaraldehyde increased the haemagglutinating activity by 100 to 200 fold and succinylation of Con A causes significant decrease in its haemagglutinating activity (Goldstein *et al.*, 1978).

The haemagglutinating activity of lectins is usually measured by serial dilution technique and the end point is determined either visually or photometrically (Sharon and Lis 1975). Agglutination may also be monitored automatically by using a fragilligraph (Danon *et al.*, 1969), or by aggregometer (Maca and Hoak, 1974). Under suitable conditions, purified lectins agglutinate erythrocytes at concentrations as low as 0.1 – 1 μg/ml and occasionally even lower (Lis and Sharon, 1981).

Lectin may also be detected by their ability to form precipitates with polysaccharides or glycoproteins either in liquid (capillary tubes), or in semisolid (agar gel) media (Goldstein, 1972). Such interactions also provide information regarding lectin specificity as well as on the constituent sugars and glycosidic linkages of the polysaccharide or glycoprotein precipitated.

Another method used for detecting lectins is affinity electrophoresis. This technique combines the principles of affinity chromatography and electrophoresis (Horejsi *et al.*, 1979). In this technique, proteins are subjected to electrophoresis on a matrix formed by copolymerisation of alkenylglycosides with acrylamide. Proteins having combining sites complementary to the ligand are retarded, whereas others are not retarded.

Later improved techniques were tried by various scientists to detect the presence of lectins. A method developed by Pongor and Ried (1983), utilized the ability of lectins to precipitate glycoconjugates and polysaccharides, which includes testing the ability of the extract to agglutinate polystyrene particles containing adsorbed glycoconjugates. Recently Kamesaki *et al.*, (1990), have introduced a new blotting technique for defining lectin activity. Kaul *et al.*, in 1991 has proved that latex agglutination method can be employed for screening crude plant extract for the presence of lectins.

1.4. Distribution of lectins

Lectins are widely distributed in plant and animal kingdom. They are seen in viruses, bacteria, algae, protozoa, slime molds, plants, invertebrates and higher animals.

1.4.1. Viruses

The oldest and perhaps the best characterized lectin - carbohydrate recognition system is the interaction of influenza viruses with their target cell (Paulson, 1985; Wiley and Skehel, 1987). The ability of the viruses to agglutinate erythrocytes has been known since 1941. It took more than a decade before it was shown that the human viruses bind to erythrocytes and other cells by recognising N-acetyl neuraminic acid, one of the sialic acids present on the cell surface and this

binding is a prerequisite for initiation of an infection. Subsequently the viral haemagglutinin (lectin), responsible for this binding was purified, crystallised and studied in detail, culminating in the elucidation of its reaction with N-acetyl neuraminic acid containing oligosaccharides. The subunit of this lectin is composed of two polypeptides HA 1 and HA 2 which are covalently linked by a disulphide bond and it associates non covalently to form a trimer that is located on the surface of the viral membrane (Sharon and Lis, 1989).

More than hundred strains of influenza viruses, mostly of the A and B types were examined for their ability to bind to enzymatically modified erythrocytes carrying terminal N-acetyl neuraminic acid attached to galactose either by an α 2→3 or 2→6 linkage. Vlasak *et al.*, in 1988 reported that some strains of influenza C viruses and corona viruses have sialic acid binding lectins. The detailed knowledge of the sialic acid-haemagglutinin interaction provides a possible basis for the design of antiviral drugs that would block viral attachment to cell, which renders protection from viral infections.

1.4.2. Bacteria

Many bacterial species have the ability to produce surface lectins. In enterobacteria (*Escherichia coli* and *Salmonella* Species) and in several other species, the lectins are commonly in the form of a submicroscopic hair like appendages known as fimbriae (pili), that protrude from the surface of the cells (Sharon, 1987; Mirelman, 1987). The best characterized are type I (mannose specific), fimbriae of *E. coli* which preferentially binds to oligomannose and hybrid oligosccharides of animal cell surface glycoproteins (Sharon and Lis, 1989).

Bacterial surface lectins play a key role in the initiation of infection by mediating bacterial adherence to epithelial cells of the host in the urinary and gastrointestinal tracts (Sharon, 1987). This has been well documented for type I fimbriated *E. coli* and *Klebsiella pneumoniae.*

The galactose specific lectins produced by oral actinomyces, such as *Actinomyces naeslundi* and *Actinomyces viscosus*, facilitate initial colonisation on epithelial surfaces of the mouth and teeth by mediating the attachment of the bacteria to galactose residues either on the surface of the epithelial cells or on the surface of other bacteria, which are adsorbed to the enamel of the teeth (Cisar, 1987). Ofed and Sharon in 1988 reported that lectin carrying bacteria bind readily to sugars on phagocytic cells like human polymorphonuclear leukocytes or human and mouse peritoneal macrophages, which results in metabolic activation of the phagocyte ingestion of bacteria and eventual bacterial death. This lectin mediated, nonopsonic phagocytosis is designated as lectinophagocytosis.

In 1991 Depierrer *et al.*, characterized a lectin from *Agrobacterium tumefaciens*. The haemagglutination activity of this lectin is inhibited by a polycaccharide, fucoidin. Javadekar *et al.*, in 2000 isolated and purified a cell surface lectin from the cell walls of a highly flocculent strain of *Saccharomyces cerevisiae* (NCIM 3528), by chromatography on DEAE-cellulose, which showed a molecular mass of 40 kilodalton (kDa) on SDS-PAGE. They also noted that this cell surface mannose-specific lectin probably plays an important role in flocculation.

1.4.3. Algae

Molecules with agglutinating activity have been found in a large number of marine algae, but few have been studied in detail. A few of the representative species, which have been investigated are *Pilota pumosa, Palmaria palmata, Codium fragile, Garcilaria verrucosa* (Ingram, 1985).

1.4.4. Protozoa

Among the numerous protozoa that infect humans and animals, the occurrence of lectins have been best documented in the pathogenic amoebae

Entamoeba histolytica, which causes dysentery in humans by disruption and invasion of the colonic mucosa (Ravdin, 1989). It has been observed that various saccharides inhibit amoebic adherence to erythrocytes and other mammalian target cell *in vitro*, which suggests that amoebic adherence, is mediated by lectin-carbohydrate interactions.

Mirelman (1987), identified two distinct lectins in *Entamoeba histolytica* one specific for $\beta 1 \rightarrow 4$ linked oligomers of N-acetyl glucosamine (GlcNAc) and the other for galactose. Facilitation of the adherence of amoebae to intestinal epithelial cells of the host is only one of the ways in which the lectins may enhance the pathogenicity of the parasite. In addition to the above role, the lectins enable the amoebae to bind to bacteria, carrying the appropriate sugars. The bound bacteria are subsequently ingested and serve as a source of nutrition for the parasite, increasing its virulence.

1.4.5. Slime molds

Aggregation of slime molds, a key event in the differentiation of these organisms from their single-cell vegetative form to an aggregated form was for more than a decade considered the most convincing example of the involvement of lectins in cell to cell recognition. Rosen and True in 1987 identified a lectin called discoidin in *Dictyostelium, discoideum*, which is involved in slime mold aggregation. This lectin apparently acts by a sugar independent mechanism in which it binds to the cell through a tripeptide Arg-Gly-Asp (Ruoslahti and Pierschbacher, 1987).

1.4.6. Plants

Lectins are of common occurrence in plants especially in leguminosae family (Toms and Western, 1971). The richest source of lectins in many plants are seeds, where they may comprise upto several percentage of the dry matter. In the

jack bean, Con A is 3-4% of the protein (Sumner and Howell, 1936a). Lectins are however, not always confined to a single part of the plant and the highest concentration is not always in the seeds. These are also present in other parts of plants such as roots, leaves, bark, pulp, tubers and bulbs which was proved by Bohloot and Schmidt (1974) and Hamblin and Kent (1973). Information about the cellular and subcelluar location of lectins are limited. High levels of lectin are found in cotyledon and embryo axis of the lentil (Rouge 1974a), Pea and red kidney bean (Rouge, 1975). A survey of various tissues conducted by Pueppkue *et al.*, in 1978 showed that in seeds, most of the lectin was present in the cotyledons. They also detected lectin in embryo axis and seed coat. In these studies, lectins were detected not only by their haemagglutinating activity but also by immunodiffusion or radioimmunoassay with antibodies against purified seed lectins.

Many investigators have studied the appearance and disappearance of lectins during the life cycle of the plant. Jones *et al.*, (1967) have reported that in osage orange (*Maclura pomifera*), agglutinin begins to accumulate during the early development of the seed and reaches a maximum as the seed achieves maturity and decreases slowly during germination, but can still be detected in lower concentrations in six month old seedlings. In mature trees all the lectin activity was localised within the seed, none being found either in the seed coat or in the leaves. Cammue *et al.*, (1985) isolated a lectin from Couch grass (*Agropyrum repens),* which is a dimer composed of two identical sub units. This lectin showed no blood group specificity. They also noticed that the lectin content in leaves varies enormously in different seasons.

In lentil, red kidney bean and soybean, low levels of agglutinins were detected in the roots, stems and leaves of young seedlings (Howard *et al.*, 1972). In *Dolichos biflorus*, low levels of lectins were present in stems and leaves throughout the life cycle, but none was found in the roots of the plant at any stage of its development (Talbot and Etzler, 1978b).

As seeds germinate, there is a progressive decrease in lectin content (Talbot and Etzler, 1978a; Rouge, 1974a,b). This decrease parallels the decrease in reserve proteins. In castor bean, Youle and Huang (1976) reported that the disappearance of reserve proteins is much faster than that of the lectin. Developmental studies of the lectin in *Dolichois biflorus* conducted by Talbot and Etzler (1978a), has revealed the presence of small amounts of a material in leaves and stems that cross reacts with antibodies against the seed lectin and they postulated that the cross reacting material was a prolectin.

Kauss and Bowles (1976) showed that plants might also contain lectins, which are extractable, by detergents and chaotopic solvents. Such lectins have been found in cellular and subcellular membranes of various plants. Bowles *et al.*, (1976) detected high levels of lectin activity in the extracts of inner mitochondrial membranes of endosperm of *Ricinus communis*. However, Kohle and Kauss (1979) showed that Ricinus communis agglutinin (RCA), is bound to the inner mitochondrial membranes as an artifact during preparation of the mitochondria.

Of the many plant lectins that have been characterized extensively, most are secretory proteins, meaning that they enter the secretory system and subsequently accumulate either in vacuoles or in the cell wall and intercellular spaces. The well known lectins like phytohaemagglutinin, Con A, SBA and Pea lectin are present at relatively high levels and accumulate in vacuoles in the cotyledon and at lower levels in the embryonic axis of the seeds (Bowles *et al.*, 1979).

1.4.7. Animals

In animal kingdom lectins are distributed both in invertebrates and vertebrates. They have a variety of lectins which are membrane bound and soluble. The integral membrane lectins differ in their sugar specificities and physiological

properties and require detergents for their extraction (Ashwell and Harford, 1982). The soluble lectins recognize terminal sugars of glycoprotein and bind them.

Noguchi made the first report of agglutinin in invertebrate haemolymph in 1903. He identified an agglutinating material in the haemolymph of marine horseshoe crab *Limulus polyphemus*. Sialic acid binding lectins are ubiquitous among invertebrates. Arthropoda, mollusca and urocordata, a group of lower invertebrates are abundant sources of these lectins. Sialic acids play a pivotal role in receptors for viruses, peptide hormones and toxins and also in the social behaviour of cells (Lloyd 1975). In contrast to their receptor functions, sialic acids act as masking agents on antigens, receptors and other recognition sites of the cell surface (Schauer 1985). So, sialic acid binding lectins are potentially useful in the detection, quantitation, localization, purification and characterization of many biomolecules containing sialic acids.

Krupe and Pieper (1966) reported that extracts from 19 of 20 different species of snails contained haemagglutinins reacting either with human 'A' or 'B' red blood cells (RBC). Hammarstorm and Kabat (1969) purified and characterized a blood group specific lectin from the albumin gland of the snail *Helix pomatia*. An N-acetyl lactosamine specific lectin was isolated from larvae of a moth, *Phalera flavescens* by Umetsu *et al.*, in 1993. They reported a high concentration of the lectin in the haemolymph also. A 27- kDa lectin, tachylectin-P (TL-P), was newly identified in perivitelline fluid of the horseshoe crab *Tachypleus tridentatus* by Nagai *et al.*, (1999). TL-P preferentially agglutinated human A-type erythrocytes, and the activity was inhibited by N-acetyl group-containing monosaccharides.

A possible function of soluble lectins in invertebrates is immune surveillance, since they lack immunoglobulins. Yoshikaki (1990) isolated β-D-galactoside specific lectin from chick embryo thigh muscle. Many evidences demonstrate the existence of a large number of animal lectins. Recently obtained structural information makes it possible to organise the known lectins into several

categories, two of which are C-type (Ca^{++} dependent), animal lectins, which are structurally related to the asialoglycoprotein receptor and S-type (thiol dependent), animal lectins (Drickamer, 1988).

1.5. Isolation and purification

To date, over hundred lectins have been isolated in homogenous form and an increasing number are becoming commercially available. Isolation of lectins generally begins with the extraction of finely ground seed meal with saline or buffer. Preextraction with organic solvents (e.g.,: methanol or petroleum ether), are often employed to remove lipids and other interfering substances. In the past, the isolation of lectins was achieved by conventional protein fractionation. At present, virtually all lectin purification schemes employ affinity chromatography, based on the ability of lectins to bind to saccharides specifically and reversibly. Knowledge of the sugar specificity of a lectin can be obtained from sugar inhibition tests. Commercially available adsorbents are usually employed for isolation. In other cases mono or oligosaccharides or glycoproteins such as hog gastric mucin or desialylated fetuin coupled to Sepharose were used. Sometimes synthetic ligands are employed e.g., N-ε-aminocaproyl derivatives of galactosylamine for the purification of SBA. Since the adsorbents used for purification of lectins may also bind with glycosidases, which are frequently present in crude plant extracts, such enzymes may contaminate lectin purified by affinity chromatography.

In 1965, Agarwal and Goldstein applied affinity chromatography for the first time to isolate lectins. They observed that cross-linked dextran (Sephadex), can be used as a specific adsorbent for the isolation of Con A. Later *Pisum sativum* lectin was purified by affinity chromatography using Sehadex G-75 by Wauwe *et al.*, (1975). Lonngren *et al.*, (1976) have used cross-linked guaran for isolation of lectin form *Helix pomatia*, *Glycine max*, *Ricinus communis* and *Echinocystis lobata*. Horejsi and Kocourek (1973), copolymerised a series of

alkenyl glycosides with acrylamide, which was later employed in the affinity purification of lectins.

Glycopeptides were also used as ligand in the affinity purification of lectins. Yokoyama *et al.*, (1976) isolated a lectin form *Phytolocca americana* using affinity chromatography on a column of desialised human erythrocyte glycopeptide coupled to Sepharose 4B. Like this, Kilpatrick and Yeoman in 1978, purified *Datura stramonium* lectin using affinity chromatography on Sepharose–fetuin column. Ahmed and Chatterjee (1983) purified a α-D galactosyl binding lectin from the seeds of jackfruit on a melibiose-agarose column.

Sugars incorporated to epoxy activated Sepharose-6B have been widely used for the purification of lectins. Sharper *et al.*, (1973), isolated wheat germ agglutinin from an extract of wheat germ by affinity chromatography on 6-amino 1-hexyl-2-deoxy-β- D-glulcopyranoside-Sepharose 4B column. GalNAc and GlcNAc - Sepharose 4B column were used to purify SBA and WGA respectively (Vretblad 1976; Vijayakumar and Forrester, 1986).

Lectin from *Sechium edule* was purified on a fetuin-agarose column by Hampe *et al.*, in 1992. Lectin form *Parkia speciosa* seeds was isolated on Sephadex G-100 by Suvachittanont and Peutpuiboon in 1992. In this case, the lectins adsorbed to the column were desorbed by 0.1 M D-glucose. These affinity chromatographic techniques offer many advantages in terms of simplicity of isolation, purity and yield. Fetuin-Sepharose column was used for the purification of radish seed lectins (Pop *et al.*, 1994) and thyroglobulin-Sepharose column for the purification of *Arum maculatum* tuber lectin (Allen, 1995).

Ion exchange chromatography was also employed in the purification of lectins. Khang *et al.*, (1990) purified a lectin from the seeds of *Crotalaria striata* on DEAE cellulose column equilibrated with PBS. The adsorbed lectin was desorbed by increasing the ionic strength of NaCl.

Different matrices used in lectin purification.

Type of adsorbent	Matrix	Ligand	Source of lectin
Polysaccharide	Sephadex		Jack bean, lentil, garden pea and sweet pea
	Sepharose		*Abrus precatorius* and *Ricinus communis*
	Chitin		Wheat germ
Matrix bound glycoproteins	Sepharose	Thyroglobulin	*Phaseolus vulgaris*
		Ovomucoid	Wheat germ and potato
		Fetuin	Jack bean wheat germ and pea
Matrix bound monosaccharides	Sepharose	L-fucose	*Lotus tetragonolobus*
		Galactose	Soybean and peanut
	Epoxy-activated Sepharose	N-acetyl glucosamine	Wheat germ
	CH-Sepharose	3-O-methyl glucosamine	*Vicia faba*
		N-acetyl galactosamine	*Vicia cracca*
		Galactosamine	Soybean
		N-acetyl glucosamine	Wheat germ
	Biogel	Melibiose	*Bandeiraea simplicifolia*
	Starch	L-fucose	*Ulex europeus*
	Acrylamide gel	Lactose	*Ononis hircina*

1.6. Carbohydrate specificity

Lectins are sugar binding proteins and their sugar specificity is usually established by hapten inhibition technique, in which different carbohydrates are tested for their ability to inhibit either haemagglutination or polysaccharide or glycoprotein, precipitation by the lectin (Lloyd *et al.*, 1969). The specificity of a lectin is defined in terms of the best monosaccharide inhibitor. Sugars commonly employed in inhibition studies are in the pyranose (six-membered ring), form. For Con A, it has been found that fructose and arabinose in their furanose forms (five membered ring), also inhibits haemmagglutination, *albeit* not a very effective one (So and Goldstein, 1969).

On the basis of their carbohydrate binding specificities, lectins may be classified into the mannose/glucose binding lectins, the N-acetyl galactosamine (GalNAc)/galactose/lactose binding lectins, the N -acetyl glucosamine binding lectins, fucose binding lectin, sialic acid binding lectins with complex binding sites (Susmita *et al.*, 1997).

Although most plants contain one lectin (or isolectin) with single sugar specificity, examples are known of plants that contain two or more lectins, which differ in their sugar specificity. Two lectins have been isolated from the seeds of *Ulex europeus,* one specific for GlcNAc (Osawa and Matsumoto, 1972) and the other for α -L-fucose. (Horejsi and Kocourek, 1974a). Two lectins with distinct sugar specificities have also been isolated from *Bandeiraea simplicifolia*, one specific for GlcNAc and the other for ∝-galactosides (Shankar-Iyer *et al.*, 1976). Van Dame *et al.*, (1995) isolated lectins from the seeds of *Robinia pseudoacacia.* This lectin is different from its bark lectin in its sugar specificity and its molecular weight.

Binding of lectins to non-carbohydrate ligands has also been reported in several cases. Robert and Goldstein (1983) reported that legume lectins including con A bind to hydrophobic molecules such as indole acetic acid (IAA), 2,6-toluidonyl naphthalene sulphonic acid and its N-6 derivatives.

Lectins differ markedly with respect to the anomeric specificity. Some are specific for only one anomeric configuration, where as others interact equally well with both anomeric configurations. Con A (Smith and Goldstein, 1967) and lectins from *B. simplilcifolia* (Hayes and Goldstein, 1974) and *L. tetragonolobus* (Pereira and Kabat, 1974), exhibit pronounced specificity for the α-anomers of mannose (or glucose), galactose and L-fucose respectively. On the other hand, SBA (Lis *et al.*, 1970) and RCA are almost completely devoid of anomeric specificity (Nicolson *et al.*, 1974).

Many lectins tolerate some variation at the C-2 of the sugar to which they bind. Thus lectins that exhibit a primary specificity for mannose also bind to glucose and to a lesser extent to GlcNAc (Poretz and Goldstein, 1970). Some lectins tolerate variation at C-3 of the sugar to which they binds (Allen *et al.*, 1976). The 4'-hydroxyl group is critically involved in lectin binding, so that, lectins which bind with mannose (and glucose), do not interact with galactose and *vice-versa* (Makela, 1957).

For some lectins, no efficient monosaccharide inhibitors have been detected and they are inhibited only by oligosaccharides. These lectins include PHA, which is inhibited by a glycopeptide, released form human erythrocytes by trypsin at very low concentrations (Kornfeld and Kornfeld, 1971). Glycopeptides form erythrocyte membrane, fetuin and immunoglobulin A, inhibit the lectin from the commercial mushroom (*Agaricus bisporus*). The lectin from *Datura stramonium* is also inhibited by oligomers of GlcNAc but not by its monomer (Horejsi and Kocourek, 1978a). Hampe *et al.*, in 1992 reported that lectin from *Sechium edule* is strongly inhibited by chitin oligosaccharide but not by GlcNAc which is the

monomer of chitin. Lectins with a similar specificity towards monosaccharide may differ in their affinity for particular disaccharide, oligosaccharides or glycopeptides. For e.g., Con A and lentil lectin are both specifically inhibited by mannose and glucose derivatives, but they are inhibited to different degrees by glycopeptides from ovalbumin or transferrin (Young and Leon 1974). From the specificity studies, it is possible to draw conclusions about the size and shape of the saccharide binding sites of lectins (Kabat, 1978). Obviously, lectins that interact more strongly with oligosaccharides than with monosaccharides have more extended sites than those of that which do not.

Despite a wide diversity in the three-dimensional structures, and improbable binding sites among lectins, there are some features of interaction of protein with carbohydrate that appears to be common to all lectin. Both hydrogen and Van der Waals' interaction are involved in stabilising these interactions (Ouiocho, 1986). Lectins often display hydrophobic interactions with derivatives of glycosides that are stronger than with the glycoside alone. For e.g., N-dansyl galactosamine binds sixty times more strongly than GalNAc to *Erythrina crystagalli* lectin (Iglesias *et al.*, 1982).

The binding constants of specific inhibitors to lectins have been measured by equilibrium dialysis or by other methods. Typically, the association constants for monosaccharide inhibitors are in the range of $1 \times 10^3 - 3 \times 10^4\ M^{-1}$ (Lis and Sharon, 1977). In experiments with Con A and a series of sugars, it has been conclusively demonstrated by Lootiens *et al.*, (1975) that there is a direct correlation between the inhibitory capacity and the association constants in the range of $7 \times 10^1 - 6 \times 10^4\ M^{-1}$.

Yamashita *et al.*, (1988) studied the carbohydrate binding specificity of *Allomyrina dichotoma* lectin II by analysing the behavior of various complex types of oligosccharides. Basically, the lectin interacts with the Gal β-1→4 GlcNAc group. They have also shown that substitution of their terminal galactose residues

by N-acetylneuraminic acid (Neu-5-Ac) α-2→6 would enhance the affinity to lectin.

Mo *et al.*, (2000), purified a lectin from *Polyporus squamosus*. Based on the results obtained by different assays, they concluded that the *Polyporus squamosus* lectin binds β-galactosides as it has an extended carbohydrate-combining site that exhibits highest specificity and affinity towards the nonreducing terminal Neu-5-Ac-α-2, 6-Gal β1,4Glc/GlcNAc (6'-sialylated type II chain), of N-glycans (2000 fold stronger than towards galactose). The strict specificity of the lectin for α-2,6-linked sialic acid renders this lectin a valuable tool for glycobiological studies in biomedical and cancer research.

1.7. Physicochemical properties

1.7.1. Composition

Primary and secondary structures of lectin have revealed that there are no structural features common to all lectins. Many of these proteins are rich in aspartic acid, serine and threonine, which may comprise as much as 30% of their amino acid content and are low or completely devoid of sulphur containing amino acids (Lis and Sharon, 1981). Such a pattern of amino acids is characteristic of plant proteins. In contrast, lectins such as those from wheat germ, potato and pokeweed are rich in cysteine and most of which are in the form of cystine. The content of disulphide bonds endows the protein with high stability to heat, to proteolytic enzymes and to denaturing agents such as detergents, urea, alkali and acids (Rice and Etzler, 1975). The lectins from potato and *Datura stramonium* are rich in hydroxyproline, an amino acid which is confined to limited number of proteins (Lamport, 1967).

Most of the lectins however are glycoproteins with carbohydrate contents that can be as high as 50% (Lis and Sharon, 1978). They also noticed that few

lectins such as Con A, WGA and Peanut agglutinin (PNA) are devoid of covalently bound sugar.

Debray *et al.*, 1992 conducted the structural analysis of the lectin from *Viscum album* using spectroscopy. They determined two oligomannose type glycans in the ratio 4:1, one containing six mannose and the other containing five mannose units, both with two 2-acetamido-2-deoxy glucose units.

1.7.2. Size and subunit structure

The molecular weight of lectins range from 36 kDa for WGA (Nagata and Burger, 1974), to 265 kDa for lima bean lectin (Galbraith and Goldstein, 1972). Some lectins exhibit a pronounced tendency to aggregate. Thus the molecular weight of Con A at pH below 6 is 51 kDa and at physiological pH it is 102 kDa (Kalb and Lustig, 1968). Lotan *et al.*, (1975b) reported that SBA (MW 120 kDa), upon storage at room temperature undergoes irreversible association of high molecular weight aggregates. Kurokawa *et al.*, (1976) reported that *Wistaria floribunda* lectin has a molecular weight of 68 kDa by PAGE in presence of Sodium Dodecyl Sulphate (SDS). On reduction with α- mercaptoethanol, it is dissociated into subunits with concomitant loss of haemagglutinating activity. They also reported that in air, the subunits reassociated into lectin molecules with haemagglutinating activity. The carboxy methylation of the subunits with iodoacetic acid prevented their reassociation on oxidation in air.

Most lectins consist of subunits, the number of subunits in plant lectins being two or four. The dissociation in to subunits may be irreversible or reversible. Various chemical modifications affect the subunit structure. Thus in physiological conditions Con A is a tetramer, succinylated Con A is a dimer (Gunther *et al.*, 1973). Rice and Etzler (1975), observed that acetylation or oxidation of tryptophan in WGA leads to the formation of subunits that possess sugar binding activity but cannot reassociate, and the subunits are devoid of haemagglutinating activity.

In most lectins the subunits are identical, but lectins comprised of nonidentical subunits are also known. SBA and the lectin from *Dolichos biflorus* (Carter and Etzler, 1975a), are tetramers, each consisting of two types of slightly different subunits. There are some reports that subunits of lectins differ markedly. In garden pea lectin there are two subunits having molecular weight 17 kDa and 5.8 kDa (Richardson *et al.*, 1978) and in lentil lectin the subunits are of 18 kDa and 6 kDa (Fliegerova *et al.*, 1974).

Usually each subunit of the lectin has one sugar binding site, but Nagata and Burger in 1974 have reported that WGA has two sugar binding sites per subunit. The subunits of the same lectin usually have the same sugar specificity and the binding sites are homogenous and noninteracting. However, exceptions are known in which subunits of lectin differ in sugar specificity. The anti-B lectin from *B. simplicifolia* consists of a family of five closely related proteins, each of which is a tetramer of one or two types of subunits. One of the subunits is specific for α-GalNAc (but also reacts with α-galactose), where as, the specificity of the other is confined to α-galactose (Goldstein *et al.*, 1977).

Bloch *et al.*, 1976 reported that in *Caragana arborescence*, the lectin is composed of two types of polypeptides with apparent molecular weight of 30 kDa. In the native molecule, the subunits are cross-linked by disulphide bonds to dimers. Hayes and Goldstein, 1974 isolated α-D-galactopyranoside binding lectin from *B. simplicifolia* seeds. In 1977, Murphy and Goldstein separated the five isolectins from the same plant. These isolectins are tetrameric structures composed of various combinations of two different subunits, designated as A and B. They also showed that the carbohydrate binding specificity of the two subunits differs significantly. The A subunit exhibits a primary specificity for α-D-GalNAc, whereas the B subunit showed a sharp specificity towards α-D-Gal-P residues. *Wistaria floribunda* lectin is a glycoprotein containing 3.2% carbohydrates. The carbohydrate moiety is composed of mannose, galactose and galactosamine in a

molar ratio of 1:2:1 and these sugars are linked as a single oligosaccharide chain to each sub unit of the protein (Kurokawa *et al.*, 1976).

Ricinus communis contains two proteins, a toxic protein (RCA 2 MW. 60 kDa) with an affinity for GalNAc and D-Galactose and RCA1 (MW. 120 kDa,) which has an affinity for D-Galactose only (Nicolson *et al.*, 1974). Jacalin consists of a heavy chain (α), having 133 amino acids and a light chain (β), of 20 amino acid residues (Mahantha *et al.*, 1992). Jacalin (MW. 42 kDa), is a tetramer having four apparently identical subunits (MW. 11.4 kDa.), joined noncovalently in the same molecule. It is also a glycoprotein, which contains 8% carbohydrates (Ahmed and Chatterji, 1986).

1.7.3. Metal ion requirements

All lectin are metalloproteins, with few exceptions. In some cases there are evidences showing the requirement of Mn^{++} and/or Ca^{++} for activity. Jaffe *et al.*, (1977), have reported that treatment of lectin with ethylenediamine tetraacetic acid (EDTA), at neutral pH did not remove the metal ions form Con A and SBA. The reversible removal of metal ions can, however be achieved under acid conditions.

The Mn^{++} in lectins can be replaced by a variety of transition metal ions without loss of their biological activity as demonstrated by Shoham *et al.*, (1973). In 1977 Jaffe *et al.*, replaced Ca^{++} in Con A by Cd^{++} but not by Ba^{++}. Doyle *et al.*, (1976) have demonstrated the role of metal ions in lectins. He proved that the metal confers a high degree of structural stability on Con A protecting the lectin against heat inactivation and hydrolysis by seven proteolytic enzymes. Barber and Carver (1975), conducted conformational studies using various physiochemical methods and concluded that the binding of Ca^{++} caused an alternation in the environment of the transition metal binding site of lectin, which in turn is important for the creation or stabilisation of the saccharide binding site. This shows that metal ions are required in lectin binding in some cases.

1.7.4. Primary sequences

Primary sequences of Con A by Cunninghan *et al.*, in 1975, have shown an unusual distribution of certain amino acids. The charged residues are concentrated in the N-terminal half of the polypeptide chain. They also observed that six of the seven tyrosine residues are located in the N-terminal half of the molecule and all of the eleven phenylalanine aminoacids are between the third residue and C-terminus. Many of the aromatic residues are located in hydrophobic regions in the 3D structure of the protein.

The comparison of the first twenty five residues of the N-terminal amino acid sequences of β-chain of the lentil, Pea and the ε-subunits of PHA has indicated the presence of homologies (Etzler *et al.*, 1977). Amino acid composition of *Wisteria floribunda* lectin has showed that it was rich in hydroxy amino acids and low in sulphur containing amino acids (Kurokawa, 1976). The above homologies suggested that lentil, pea and Con A may have evolved form a common ancestor. It is possible that the former lectins are synthesized as a single polypeptide chain and subsequently cleaved into two or possibly three fragments. Two of these would be the α and β subunits and the third fragment homologous to the residues 1-70 of Con A and the latter fragment may be further degraded post synthetically or lost during preparation of lectin.

Homologies also have been noticed between different lectins obtained from a single plant family, the legumes strongly suggest a common genetic origin for these proteins. It appears therefore that lectins may be grouped in to families, which have conserved their primary structure. Moreover, the homologies suggest a common ancestry for the genes coding for these lectins.

Ruffet *et al.*, (1992) determined the amino acid sequence of α- subunit and the slightly larger α^1- subunit of jacalin. The α^1- and the α- subunits are identical in their primary structure except for the substitution of one valine with isoleucine at position 113.

Calvete *et al.*, (2000), established the primary structure of a lectin isolated from the red algae *Bryothamnion triquetrum* by combination of Edman degradation of sets of overlapping peptides and mass spectrometry. They observed that the lectin contains 91 amino acids and two disulphide bonds. The primary structure of the lectin does not show amino acid sequence similarity with known plant and animal lectin structures. Hence, this protein may be the paradigm of a novel lectin family.

Mo *et al.*, (2000), purified a lectin from the carpophores of the mushroom *Polyporus squamosus.* The N-terminal amino acid sequencing indicated that the native lectin, designated as polyporus squamosus agglutinin, is composed of two identical subunits of 28 kDa,which are associated by noncovalent bonds.

1.7.5. Secondary and tertiary structure

Only limited information is available on the conformation of lectin in solution. Circular dichroism studies by Hertmann *et al.*, (1978) in the far ultraviolet, of several lectins, suggest a β-pleated sheet as the primary structural feature. This was confirmed by the X-ray crystallographic studies on Con A. Privat *et al.*, (1974c) have studied the conformational changes upon binding of sugars to lectin by ultraviolet spectral studies and by rapid reaction techniques.

X-ray crystallographic studies of lectin are still their infancy and Con A is the only one of these proteins for which the complete three-dimensional structure based on a high-resolution map is available. But now many researchers are carrying CD spectral and X-ray crystallographic studies. These studies of Con A

showed that the subunit is dome or gum drop- shaped approximately 42 A° high, 40 A° wide, and 30 A° thick (Edelman *et al.*, 1972). The monomers are paired base to base by an exact two-fold symmetry axis to form ellipsoidal dimers. The dimers in turn are paired by additional two-fold axis to form roughly tetrahedral tetramers.

The general distribution of polar and nonpolar side chain in Con A is similar to that found in other proteins with polar side chains usually on the surface and the hydrophobic groups predominantly in the interior. X-ray crystallography has also shown that the metal in Con A are bound to two separate sites designated as S1 and S2 near the top of the monomer and these sites are 4.6 A° apart.

Elucidation of the detailed 3D structure of WGA has been hampered by the nonavailability of the primary structure of lectin. An electron density map obtained at 2.2 A° resolution shows that the WGA dimer (dimensions of 40 X 40 X 70 A°), contains two closely associated subunits of 164 amino acids residues each, centred around the crystallographic two fold axis (Wright, 1980).

Dhanaraj *et al.*, (1988) prepared and characterized two crystal forms of jacalin, one orthorhombic and the other monoclinic. They contain more than one molecule in the asymmetric unit. Rajan in 1996, studied the crystal structure of jacalin with methyl α-D-galactose, which revealed that each sub unit has a three fold symmetric β prism fold made up of three four stranded β sheets.

1.8. Biological role

Lectins exert a variety of effects on cells, the most extensively studied being agglutination and mitogenic stimulation. Other remarkable effects include redistribution of cell surface components, insulin-like activity on fat cells, modification of the activity of membrane enzymes, blocking of fertilization of ova

by spermatozoa, toxicity *in vitro* and *in vivo*, and inhibition of fungal growth. These various biological effects, however, are not exhibited by all lectins.

1.8.1. Binding of cells

In order to act on cells, lectins must bind to their surfaces. Although, agglutination can be taken as an evidence for binding to the cells, lack of agglutination does not mean that binding has not occured. Binding is best demonstrated with the aid of lectins labelled with radioactive isotopes or with compounds that are visible under a microscope, such as fluorescein, ferritin, peroxidase and haemocyanin (Sharon and Lis, 1975).

The surface structures to which lectins bind are the carbohydrate moieties of glycoproteins or glycolipids that protrude from the cell surface. The conclusion is based primarily on the fact that binding is inhibited by the sugars, which are specific to the lectins. The fact that lectin binding is affected by modification of cell surface sugars by glycosidases, glycosyltransferases and galactose oxidase provides direct evidence for this conclusion. Thus, unmasking the subterminal galactose residues of erythrocytes or lymphocytes by removing the terminal nonreducing sialic acid with neuraminidase, increased the amount of PNA bound to the cells to 20-40 fold. (Novogrodsky *et al.*, 1975; Carter and Sharon, 1977).

In contrast, oxidation of galactose residues on the surface of mouse thymocytes by treatment of the cells with galactose oxidase, decreased the amount of PNA binding by 70-80% (Kornfeld and Kornfeld, 1969). They also reported that the removal of glycopeptides from cell surfaces by proteolytic enzymes sometimes markedly reduces lectin binding, and the released glycopeptides are powerful inhibitors of the lectins tested.

1.8.2. Cell agglutination

Agglutination is the most easily detectable manifestation of the interaction of a lectin with cells. For agglutination to occur, the bound lectin must form multiple crossbridges between apposing cells. Lectins may, however, bind to cells without causing agglutination. This complicates the interpretation of agglutination experiments in terms of the structure and architecture of the cell surface.

Most studies on the agglutination of cells by lectins have been carried out with intact animal cells. Other cells, as well as viruses (Becht *et al.*, 1973; Okada and Kim, 1972; Stewart *et al.*, 1972) and subcellular particles such as nuclei and mitochondria (Nicolson *et al.*, 1972), are also agglutinated by lectins.

Interest in agglutination by lectins has been greatly stimulated by observations on the marked differences in agglutinability between normal and malignant cells, between embryonal and adult cells, and between mitotic and interphase cells (Burger, 1973: Rapin and Burger, 1974; Nicolson, 1976a,b). Moreover, cells that are not agglutinated by low concentrations of lectin frequently become agglutinable after very mild proteolysis, although, as a rule such treatment does not affect significantly the total number of lectin-binding sites.(Sharon and Lis, 1975).

Chemical or enzymatic modification of lectins under conditions that do not abolish their sugar-binding ability may have pronounced effects on their biological activities. Thus cross-linking of SBA with glutaraldehyde increases the haemagglutinating activity of the lectin to human erythrocytes by 100–200 fold (Lotan *et al.*, 1973). Succinylation decreases the haemagglutinating activity of Con A by more than 500-fold (Wang and Edelman, 1978a), but has no effect on the activity of WGA (Rice and Etzler, 1975). Whereas, acetylation decreased the activity of both the lectins by 30-50 fold. On the other hand, modification of the carbohydrate moieties of glycoprotein lectins has no effect on their

haemagglutinating activity. SBA, from which more than half of the mannose residues have been destroyed by mild periodate oxidation (Lotan *et al.*, 1975b) and *Dolichos biflorus* lectin from which up to 40% of the mannose residues have been removed by treatment with α-mannosidase (Biroc and Etzler, 1978), retain their ability to agglutinate erythrocytes. These experiments, as well as the fact that several lectins are not glycoproteins, seem to indicate that carbohydrate moieties of lectins are not required for their biological activity.

1.8.3. Lymphocyte stimulation

One of the most dramatic effects of the interaction of lectins with cells is the triggering of quiescent, non dividing lymphocytes into a state of growth and proliferation, known as mitogenic stimulation (Lis Sharon, 1977). The first mitogenic agent to be described was PHA (Nowell, 1960). In 1960s, three additional mitogenic lectins were reported: pokeweed mitogen (Farnes et al., 1964), a lectin from *Wisteria floribunda* (Baker and Farnes, 1967) and Con A (Douglas *et al.*, 1969).

Most mitogenic plant lectins stimulate on the thymus-dependent set of lymphocytes (T cells) and are inactive or inhibitory for mitosis of the other class of lymphocytes, the thymus-independent (or B), cells. An exception is the pokeweed mitogen Pa-I, which stimulates the T and B cells of mice and humans (Wazdal and Basham, 1974; Basham and Wazdal, 1975). The stimulation of lymphocytes by lectins (or other mitogens), results in the release of soluble substances with wide variety of biological activities, including lymphokines such as the macrophage migration inhibitory factor, lymphotoxins, and interferon (Granger *et al.*, 1975). In certain cases, an increase in immunoglobulin production is also observed (Melchers and Andersson, 1973).

The proliferative response of cell population from human peripheral blood by purified jacalin was studied by Pineau *et al.*, (1990). They showed that the

lectin is mitogenic through an interaction with lymphocytes by its lectin binding sites. But the lectin failed to stimulate B cells to proliferate and to undergo plasma cell maturation.

Although, extensively studied, the mechanism of mitogenic stimulation is unknown. It is generally accepted that the initial step or 'first signal' is binding of the lectin to cell surface sugars. Binding may lead to modification of membrane structure and function, resulting in the generation of a trigger or 'second signal', the transmission of which to the interior of the same cell initiates a series of biochemical events culminating in cell growth and proliferation (Lis and Sharon, 1977).

1.8.4. Stimulation of plant cells

Con A and PHA stimulate pollen germination *in vitro* by reducing the lag period before emergence of the pollen tube (Southworth, 1975). However, when the effect of SBA and PHA on the growth of soybean root and tobacco pith segments were examined *in vitro*, no increase or induction of cell division was observed (Vasil and Hubbell, 1977). A slight transient increase in mitotic activities reported with PHA (Nagl, 1972a,b), might have been due to the presence of plant growth substances in the lectin preparations used.

1.8.5. Inhibition of fungal growth

WGA binds specifically to hyphal tips and hyphal septa of *Trychoderma viride* and inhibition of hyphal growth as measured by the incorporation of radioactive acetate. (Mirelman *et al.*, 1975). Potato lectin acts in a similar way on *Botrytis cineria* (Callow, 1977). WGA, PNA and SBA inhibit incorporation of radioactive acetate, GlcNAc and galactose into young hyphae of *Aspergillus ochracus* (Barkai-Golan *et al.*, 1978). Incorporation is not affected when the lectins are pre-incubated with their specific inhibitors. The inhibition of

incorporation of precursors in to the fungal hyphae by lectins of different specificities seem to be the result of inhibition of nutrient uptake caused by the coating of the hyphal surface with lectins. Growth inhibition by lectins tested was indicated also by the effect on fungal germination. Inhibition of spore germination probably occurs at a very early stage of germination process, i.e., after the spore swells and before initiation of detectable germ tubes. This inhibition is mainly expressed by prolongation of the latent period, which precede germination.

1.8.6. Cytotoxicity of lectins

Several lectins like Con A, WGA, and PHA are toxic to mammalian cells. Toxic lectins are generally selective in their action on cells. In particular, transformed cells are frequently much more sensitive to the cytotoxic effects of lectins than normal cells (Nicolson *et al.*, 1974).

It is not surprising that therefore, attempts have been made to inhibit tumor growth *in vivo* by lectins. Injection of hamsters with Con A following injection with transformed cells, caused a significant inhibition of tumor growth. This effect was abolished by simultaneous injection of methyl α-mannoside (Shoham *et al.*, 1970). Tumor inhibition by Con A has been observed in other systems as well. Unfortunately at the concentrations of Con A required for tumor suppression, the lectin is also toxic. Experiments on tumor cell growth suppression have also been carried out with wax bean agglutinin and RCA (Lin *et al.*, 1971).

As mentioned below, the molecular basis of cytotoxicity of the plant toxin ricin and abrin has been clarified by Olsnes and Phil, (1977) and it is likely that modecin acts by similar mechanism. These toxins consist of two chains joined by disulphide bonds. The heavier B-chain possesses carbohydrate-binding site, whereas, the lighter A-chain inhibits protein synthesis in cell free systems thus representing the toxic moiety of the molecule. Only the intact molecule is active on cells. Subsequent to binding to cell surface galactose, residues *via* the B chain, the

toxin is taken up by the cell where the A-chain inhibits protein synthesis by interfering with the peptide chain elongation on polyribosomes. The modulatory potency of the β-galactoside specific lectin from mistletoe extract (Iscador), on the host-defence system *in vivo* in rabbits were studied by Hajto *et al.*, (1989). They found that the injection of mistletoe lectin in rabbits increased the natural killer cytotoxicity of large granular lymphocytes and phagocytic activity of granulocytes.

Hybrid molecules have been prepared in which the carbohydrate binding fragment of the diphtheria toxin is replaced by Con A resulting in highly toxic compounds (Gilliland *et al.*, 1978).

Parslew *et al.*, (1999), studied the antiproliferative effect of lectin from the edible *Agaricus bisporus*, which binds to the disaccharide galactosyl β-1,3-GalNAc. This is expressed in keratinocytes as an O-linked chain on CD44, a polymorphic membrane glycoprotein. Many lectins are mitogens. However, *Agaricus bisporus* lectin (ABL) reversibly inhibits proliferation of colonic cancer cell lines without cytotoxicity and thus has therapeutic potential in situations such as psoriasis, where proliferation is increased.

Mistletoe lectin I (ML-I), is a major active component in plant extracts of *Viscum album* which is widely used in adjuvant cancer therapy. ML-I exerts potent immunomodulating and cytotoxic effects, although its mechanism of action is largely unknown. Bantel *et al.*, (1999), showed that treatment of leukemic T- and B-cell lines with ML-I induced apoptosis. They also observed that ML-I enhances the cytotoxic effect of chemotherapeutic drugs. So, these data may provide a molecular basis for clinical trials using ML-I in anticancer therapy.

Ueno *et al.*, (2000), modified Con A with 2,4-bis[O-methoxypoly(ethylene glycol),]-6-chloro-s-triazine, activated PEG2, to form PEG-Con A. PEG-Con A had a complete reduction of the immunogenicity in mice and prolonged the clearance time in blood. Although the mitogenic activity of Con A towards murine

spleen cells was reduced by the conjugation with activated PEG2, the administration of PEG-Con A to mice enhanced the antitumor cytotoxicity of peripheral lymphocytes against melanoma B16 cells.

1.9. Nutritional significance

The fact that the lectins are found in legumes such as beans and peas, which are important in dietary protein for much of the world's population, raises the question of the nutritional significance. Lectins have been implicated in the toxicity and impaired growth rates observed in experimental animals fed nonheated plant proteins. Moreover, in many plants, closely related toxins accompany nontoxic lectins. Indeed, association of the lectins from the castor bean and from *Abrus precatorius* with ricin and abrin respectively has been known for a long time. However, not all lectins are toxic or even growth inhibitory when fed to animals. Thus selective removal of lectin from soybean extract by adsorption to Con A did not improve the rate of growth or the protein efficiency ratio in animals ingesting raw soybean proteins (Turner and Liener, 1975). The toxic effect of lectins present in edible plants can be generally eliminated by proper heat treatment. Although in practice, their complete destruction may not always be achieved.

Jaffe in 1969, had proposed that the toxic effects of lectins when ingested orally may be due to their ability to bind to specific receptor site on the surface of the intestinal epithelial cells. Support for this hypothesis comes from the studies showing that a number of different lectin react with the crypts and /or villi of the intestine, but at different regions of the intestine depending on the specificity of the lectins (Etzler, 1979). Since surface-bound lectins may produce profound physiological effects on cells, they could impair seriously the ability of the intestinal cells to adsorb nutrients from the gastrointestinal tract.

1.10. Applications

The first application of lectins was in immunological research. With the passage of time, they have become a widely used, invaluable tool in many other areas chemical and biological research as well as clinical medicine.

1.10.1. Isolation of glycoproteins and glycopeptides

Because of the analogy of lectin-saccharide interaction to antibody-antigen reactions, the application of lectins either in solution or more frequently in immobilized form for the purification of glycoproteins and glycopeptides are employed. Although, the specificity of lectins is confined to sugars, their application offers many advantages. Firstly, lectins can be easily purified in large quantities. Secondly, the specifically precipitated or adsorbed glycoproteins or glycopeptides can frequently be eluted with readily available monosaccharides; and thirdly, elution can be carried out at neutral or near neutral pH with minimal deleterious effects on the glycoprotein.

Affinity chromatography on lectins is being employed for the purification of both soluble glycoproteins and membrane glycoproteins. Lectins are also used for detecting glycoproteins on gel electropherograms (Burridge, 1978). Affinity chromatography using lectins is also useful for the fractionation of glycoproteins and glycopeptides, which differ only slightly in their carbohydrate composition or in the structure of their oligosaccharide units. For example, several molecular variants of α-fetoprotein from calf serum and rat amniotic fluid, all of which are glycoproteins, have been resolved by chromatography on Con A-Sepharose and on RCA- Sepharose column (Lai and Lorscheider, 1978).

1.10.2. Isolation of glycosylated nucleic acids

An interesting application of lectins has been in the isolation of t-RNA species containing glycosylated bases (Okada *et al.*, 1977). They employed Con A-Sepharose column to isolate t-RNA^{asp} from rabbit liver, rat liver and rat ascites hepatoma, while t-RNA^{tyr} from the same source was purified by affinity chromatography on RCA-Speharose column.

1.10.3. Structural studies on carbohydrates

The contribution of lectins to our knowledge of the chemical structure of the 'A' 'B' and 'O' blood group determinants in humans has been extensively reviewed (Watkins, 1972). An example is utilization of specificity of *Lotus tetragonolobus* lectin for mono or di-L-fucosyl derivatives of Gal-β-(1→4), GlcNAc for establishing structures of certain di-substituted blood group oligosaccharide (Rovis *et al.*, 1973).

1.10.4. Characterization of cell surfaces

Studies with radiolabelled lectins provide information on the lectin receptor sites on the cell surface and their homogeneity, as well as on the strength of binding of the lectin to the cells. Some insight into the relative disposition of the receptor sites for different lectins may also be obtained from competition experiments (Sharon and Lis, 1975). Generally, the bound lectin is determined by measuring radioactivity, but it can be estimated by autoradiography as well. In addition, microscopic methods have been used for quantitative determinations of lectin receptor sites. Thus individual molecules of ferritin and haemocyanin are easily distinguishable in the electron microscope and can be counted. In the peroxidase method, a peroxidase-Con A complex is bound to the cell and quantitated by measuring the activity of the bound enzyme.

The number of receptor sites for lectins on lymphocytes is usually in the range of 10^6 - 10^7 per cell, regardless of the lectin used, and on erythrocytes it is frequently 10^5 – 10^6 (Lis and Sharon, 1977).

Experiments with labelled lectins and intact cells or isolated membranes have shown that lectin receptors are found exclusively on the outer membrane surface (Nicolson and Singer, 1971; 1973). These results are consistent with the findings obtained by Benedetti and Emmelot, (1967); Gahmberg and Hokamori, (1973), which provide strong evidence for the asymmetry of surface membranes.

Asymmetric distribution of lectin receptors has been found on intracellular membranes. Characterization of lectin binding sites on subcellular particles has been less unequivocal. Thus, on the basis of studies on the radioactively labelled lectins, it has been claimed that the cytoplasmic surface of the nuclear envelope (Kaneko *et al.*, 1972), of the mitochondrial outer membrane and chromaffin granule membrane of adrenal gland cells (Meyer and Burger, 1976), have lectin binding sites. On the other hand, results obtained with Con A-peroxidase indicate an abundance of Con A receptors both on the cytoplasmic and cisternal surfaces of the nuclear envelope (Virtanen and Wartiovaara, 1978).

Using fluorescent lectin derivatives with the optical microscope or ferritin peroxidase- conjugated lectins with the electron microscope (Nicolson, 1978), lectin receptors were found to redistribute on the cell surface by lateral movement in the plane of the membrane (Sharon and Lis, 1975). Rates of such movements are evaluated by a technique in which a cell labelled with a fluorescent lectin derivative is photo bleached by a laser beam over a small selected area after which the recovery of fluorescence in the bleached area is measured. The kinetics of recovery of fluorescence is governed by the rate of diffusion of the unbleached fluorophores into the bleached area and can therefore be used to calculate the diffusion coefficient of the fluorophore and thus the fluidity of the membrane (Shinitzky and Inbar, 1976).

1.10.5. Cell separation

The use of lectins to separate intact cells bearing different surface carbohydrates is a recent development. Most of this work has been done with mammalian cells, particularly lymphocytes. Two approaches have been used for this purpose, in one, cells were fractionated by differential agglutination with soluble lectins and in the second by affinity chromatography on insolubilised lectins.

The first approach was originally used to separate leucocytes from erythrocytes by agglutination of the latter by PHA and their removal from the mixture (Li and Osgood, 1949). In 1976, Reisner *et al.*, fractionated mouse thymocytes to medullary (mature) and cortical (immature), sub populations using PNA. For cell separation by affinity chromatography, lectins immobilized on a solid support, either by adsorption or by covalent attachment is employed. Mouse thymocytes were separated from mouse erythrocytes with the aid of nylon fibers derivatised with con A (Edelman and Rutishauser, 1974). The bound cells could not be removed by specific sugar because of strong nonspecific interaction with the surface of the fiber.

1.10.6. Lectin resistant cell surface variants of eucaryotic cells

A large number of mutants of Chinese Hamster ovary and baby hamster kidney cells, resistant to ricin as well as to various other lectins including Con A, WGA, PHA and lentil lectin have been isolated by Briles and Kornfeld, (1978). These mutants provide a new tool for studying the biological role of cell surface carbohydrates. Stanley *et al.*, (1979), isolated mouse 3T3 cells resistant to lectin from *B. symplicifolia*. The cells retain substantial resistance even when cultured for several months in the absence of the lectins and are therefore apparently stable genetic mutations with low reversion rates.

There are two general ways in which lectin receptor- oligosaccharides might be structurally modified so as no longer to serve as lectin binding sites. The residues involved in lectin binding may be masked or sterically hindered by additional sugar residues or they may be deleted from oligosaccharide structure. Oligosaccharide deletions of varying degrees have been reported for lectin resistant cells. These extensive deletions have been found in wheat germ resistant Chinese hamster ovary cells characterized by sialic acid deficiency in their oligosaccharides (Briles *et al.*, 1977). As a result of these variants decreased ability to bind WGA and an increased number of exposed terminal galactose residues to which ricin may bind. Thus studies with lectin resistant mutants provide insight into the biological role of cell surface sugars. Lectin resistant variants may also be useful in furthering our understanding of the relationship between cell surface and neoplasia.

1.11. Clinical uses

Mitogenic lectins are being employed in clinical medicine to recognise congenital and acquired immunological deficiencies, to detect sensitisation caused by infectious diseases, to monitor the effects of various immunosuppressive and immunotherapeutic manipulations and in the diagnosis of genetic diseases with chromosomal defects (Oppenheim *et al.*, 1975).

In blood banks, lectins are used to detect 'secretors' (humans who secrete blood group substance A, B, or H in saliva or other body fluids) and to type blood. Thus the lectins of *Lotus tetragonolobus* and *Ulex europeus* serve to identify O cells, mainly because of the nonavailability of natural anti-H (O), antibodies. The lectins from *Dolicos biflorus* serves to identify A1 and A2 subtypes of the A determinant and *V. graminae* lectin to distinguish between M and N cells.

Another application of lectins is in the differential diagnosis of 'polyagglutination,' a condition accompanying certain bacterial and viral infections

in which human erythrocytes become agglutinable by antibodies present in the sera of all human adults. One type of polyagglutinability is due to the appearance of human erythrocytes T-antigen by the action of neuraminidase of the infective bacteria or viruses. In the past, time consuming procedures were required to detect this antigen. With the discovery that PNA was specific for T antigen, determination of T-polyagglutinability became greatly simplified.

Ricin, a highly toxic glycoprotein expressed in the endosperm of castor seeds is composed of a galactose binding lectin. Chemically modified ricin has been conjugated to monoclonal antibodies and used for targetted therapy of cancer and autoimmune diseases.

1.12. Role in nature

The ubiquitous occurrence of lectins in plants (as well as in other organisms) and their ability to discriminate among different saccharides in solution and on cell surface have prompted speculations on their physiological role. Unfortunately none of these is well found, so that the role of lectins in nature is still an open question (Sharon, 1979).

There are increasing indications that lectins function in recognition phenomenon, both intracellular and extracellular. Such recognition implies distinction between self and non self. An extension of this idea is the suggestion that lectins play a role in host-parasite relationships, both in animals and in plants, and that they serve in the defence mechanism of the plants against pathogenic microorganisms. Recognition by lectins may also be the basis of the association between legumes and the symbiotic nitrogen-fixing bacteria.

1.12.1. Plant defence

Maarten *et al.*, (1991), suggested the role of lectin in plant defence. They recognised that when dry seeds imbibe water, vacular proteins especially lectins are released into the imbibed water. This results in, presence of lectin in the vicinity of the germinating seed, where they can interact with potential pathogens. Secondly, when seeds or other plant organs are eaten by animals, lectin will be released from the disrupted cellular structures of the plant tissues which will then come into contact with the glycoproteins that line the intestinal tracts of predators, possibly inhibiting absorption of nutrients. Lectins also show antifungal activity by inhibiting further hyphal growth.

1.12.2. Binding of nitrogen fixing bacteria to legumes

The association between legumes and nitrogen fixing bacteriae such as rhizobia are specific. Legume species or cultivars, which are nodulated by some rhizobium isolates, are not nodulated by others. The basis of this specificity may involve the capacity of the bacterial cell to be recognised and bound by some component, possibly a lectin in the roots of the plants. The evidence implicating lectins is the finding that a lectin from a particular legume binds only to the corresponding rhizobial species and not the bacteria that infect other legumes (Bohloot and Schmidt, 1974).

A different mode of action of lectins in plant symbiont attachment has been proposed on the basis of studies on the interaction between *Rhizobium trifolii* and white clover (Dazzo, 1978). According to this proposal, the lectin serves as a bridge between common or similar carbohydrate structures present on the surfaces on both the tips and the bacteria. Not withstanding the above observations, a variety of findings cast serious doubts on the validity of the proposal, that lectins serve as recognition in host-symbiont interaction in leguminous plants. No

evidence was found for the preferential binding of Con A to rhizobia strains capable of nodulating jack bean (Dazzo and Hubbell, 1975 b).

1.13. Lectin histochemistry

Lectins can be used as a potential histochemical markers of different cell types differentiation, of maturation and of neoplastic changes (Damjanov, 1987). Lectins are also used in carbohydrate cytochemistry and their validity as probes for *in situ* visualization of sugar sequences in oligosaccharides of glycoconjugates in cells and tissues, have been unequivocally demonstrated (Walker, 1989). The binding of lectins to sugar residues can be detected microscopically by using appropriate markers. These are fluorescent dyes and peroxidase for light microscopy and ferritin, peroxidase, haemocyanin, latex beads and polysaccharide iron complexes for electron microscopy (Etzler and Branstrater, 1974; Walker 1984a), The peroxidase method gives a non specific background staining. Biotinylated lectins were also used to ensure ultrastructural localization (Jones and Stoddart, 1986).

Lectin histochemistry has several areas of application. It has proven to be a valuable tool in the investigation of the molecular differences between tumor cells and normal cells by their ability to detect distinct changes in the cell surface glycoproteins. Lectins from peanut, soybean and *Helix pomatia* have mainly been used for this purpose (Sharon, 1984). One of the most useful diagnostic application of lectins is the identification of specific cells UEA-1 binds specifically to human endothelium, which is a reliable marker. Holthofer *et al.*, (1982) first described the use of UEA-1 and subsequently several other workers used it for the study of vascular lesions and tumors (Walker, 1985).

Mucin histochemistry involves the use of oxidation-reduction sequences and enzymes. More (1991) has combined galactose oxidase with lectins to determine the structures of secretory glycoproteins of the respiratory tract.

Mitchel *et al.*, (1990) have noticed that affects of alcohol on the alternation in the lectin binding of rat gastric mucosa. Bhattathiri *et al.*, (1992) studied the lectin-binding pattern of epidermoid cancers of the oral cavity and soft palate before and after radiotherapy. They found that proportion of binding to cells by jackfruit lectin (JFL) decreased with the increase in dose of radiation. Remani *et al.*, (1994) showed that JFL, have weak binding in the membrane as well as cytoplasm of normal cells, whereas carcinomatous cells showed strong binding towards JFL.

Shanti *et al.*, (1994) studied the distribution of glycoconjugates in sixteen colonic lesions comprising eight polyps and eight adenocarcinomas with PNA, RCA, SBA, UEA-1, WGA and Con A. They found that adenocarcinomas bound to Con A, RCA, PNA, UEA-1 and WGA in decreasing order of intensity of binding. Muto *et al.*, in 1999 studied the binding of human mannose-binding lectin (MBL) to human colon adenocarcinoma cell lines and leukemia cell lines by flow cytometry employing specific antibodies against MBL. MBL binding was observed in colon adenocarcinoma cell lines (Colo205, Colo201 and DLD-1), but not in leukemia cell lines tested. The binding of MBL to these cell lines is sugar-specific and calcium-dependent, since it is almost completely inhibited in the presence of 10 mM EDTA or 50 mM mannose. They also noted that the degree of MBL binding was well correlated with the expression of Lewis A and Lewis B antigens on these cell lines. These results suggest that MBL could bind to some human colon adenocarcinoma cell lines through their Lewis A and Lewis B moieties.

Hormia *et al.*, (1988) found DBA to selectively react with the mast cells in connective tissues. Kirkpatric *et al.*, (1988) showed that Con A, LCA and WGA, all of which detect N-linked glycoprotein, also bind to mast cells. In lymphoid organs, lectins showed differential binding to different cells and have been used for histochemical mapping (Hsu and Ree, 1983). Gabius and Bardosi (1990) observed a marked regional difference in the lectin binding with cortex, hippocampus, basal ganglia and thalamus of adult human brain.

Immunohistochemical and histochemical comparative study was carried out by Lin *et al.*, (1998), in benign and malignant colorectal tissues, with and without schistosomiasis. This includes a quantitative determination of PNA-binding sites and proliferating cell nuclear antigen. They found that the presence of PNA-binding sites in mucin containing tumors of the colorectal carcinoma associated with schistosomiasis, group was increased, compared with that in the same subtype in the colorectal carcinoma without schistosomiasis. So their findings suggest a close relationship between mucin-containing colorectal carcinomas and schistosomiasis.

Lectins have also been widely used to examine glycoprotein alterations in diseased conditions. Yoshiaki (1989) studied the profiles of Con A and LCH by affinity immunoelectrophoresis for serum α-feto protein (AFP) from patients with yolk-sac tumors and carcinomas of the gastrointestinal tract. The results indicated that AFP binding to LCH is weakened if a fucosylated sugar chain has, in addition, a bisect - GlcNAc attached to α-linked mannose. Alroy *et al.*, (1984) have suggested the use of lectins in examining lysosomal storage diseases such as α-mannosidosis, α-fucosis and sialadosis. Fraggiana *et al.*, (1981) have studied the value of BSA-I and RCA-I in Fabry's disease. Changes in colorectal mucin can be detected in Crohn's disease with a less fucose-binding sites in the left colon and GalNAc residues in the descending colon (Jacobs and Huber, 1988).

In neuropathology lectins have been used to compare normal and pathologically altered neurons (Estruch and Damjanov, 1986). The two GalNAc binding lectins *Helix pomatia* agglutinin(HPA) and *Maclura pomifera* agglutinin bound selectively to altered nerves but are not pathogenic for any specific type of injury. Banshek *et al.*, (1988) have applied lectins to distinguish between plaques and neurofibrillary tangles in Alzheimer's disease. Mann *et al.*, (1992) have reported the lectin histochemistry of cerebral microvessels in aging, Alzheimer's disease and Down's Syndrome.

Shue *et al.*, (1993) investigated the tissue distribution of carbohydrate structures reactive to *Vicia villosa* agglutinin (VVA), *Bauhinia purpurea* agglutinin (BPA) and PNA by using lectin histochemistry in gastric and colonic cancer and in their normal counterparts. VVA showed a higher affinity to gastric and colonic cancer when compared to that of the cells of their normal counterparts. But BPA failed to show significant binding differences between neoplastic and normal cells. Yoshihiro *et al.*, (1994) studied the binding pattern of *Helix pomatia* agglutinin in gastric cancer patients. They reported that the positive staining correlated well with lymphatic invasions and metastasis. They also noticed that patients with gastric cancer, positive to HPA staining of their tumor tissues, lived for a shorter time than did those with a negative staining even in the absence of metastasis to the lymph nodes. So, careful follow up and aggressive therapy is required post operatively for patients with an HPA-positive gastric cancer.

Altered lectin binding pattern can be found in some skin conditions such as psoriasis (Kariniemi *et al.*, 1983). Raju and Lee (1988) could not find any apparent difference in the lectin binding between male and female breast and no discriminate sugars were identified among normal gynaecomastia and carcinoma of the male breast.

Tsambaos *et al.*, (1998) studied the lectin binding pattern in the normal laryngeal epithelium and in laryngeal squamous cell carcinomas (SCCs). The lectin-binding pattern of well-differentiated SCCs was comparable to that of the spinous cells of the normal laryngeal epithelium. In the less differentiated SCCs, staining of the keratinocyte plasma membrane with lectins was either reduced or absent, indicating a decline in the glycosylation of cell surface glycoconjugates. This indicates that the lectin can be used in the rapid assessment of less-differentiated areas within a laryngeal SCC, but they cannot be regarded as reliable markers of laryngeal keratinocytes undergoing malignant transformation.

Lectin histochemistry has been employed more in the area of glycoconjugate alterations related to malignancy to explore changes in glycosylation that can occur with malignancy and relate them to tumor behavioral characteristics (Krogerus and Andersson, 1990; Matsumoto *et al.*, 1992; Narita and Nano, 1992).

Sivridis *et al.*, (2000), reported that Canavalia ensiformis agglutinin (Con A) and *Lens culinaris* agglutinin (LCA), reacted strongly with the luminal borders. The cytoplasmic staining in epithelial cells of normal and benign endometrial tissues were confined to the apical and the basal aspect of the cells. In endometrial carcinomas and in some atypical hyperplasias, the lectin binding was also observed in the lateral cytoplasm.

1.14. Aims and objectives

The occurrence of lectins as seed proteins is fairly well established for a number of plant species. To date, the number of purified lectin has been increased from a dozen to well over hundred, and the number of the purified lectins is rapidly growing. These new lectins share different characteristics and properties and have been used for different purposes in biochemical as well as in biological research. Eventhough, India is the home of large number of tropical plants, only few plant lectins have so far been isolated. The primary objectives of this study is to isolate and characterize these lectins and finally to investigate the usefulness of these new lectins.

In the preliminary screening, we came across two new plant lectins i.e., from *Euphorbia neriifolia* Linn. and *Euphorbia splendens*. The lectin present in the exudate of *E. neriifolia* was not studied earlier, thus the lectin was studied in detail and its usefulness was evaluated.

CHAPTER 2

Screening of Plants for Lectins

2.1. INTRODUCTION

Plant seeds constitute convenient sources of lectins, many of which are now commercially available. Each lectin is unique in its biological, biophysical and biochemical properties. Therefore, the wider the range of available lectins, the better will be their utility in biological studies. The great diversity of flora in the Indian subcontinent offers a rich material for undertaking search for novel lectins. The positive findings of earlier workers are suggestive of the wide occurrence of phytolectin in Indian plants especially in Kerala. Though this is the condition, only a few plant lectins have been studied in our country. Therefore, the screening of plants for lectin activity in locally available plants is inevitable. So, in this study a screening attempt was made to detect the presence of haemagglutinin in the seeds of 101 locally available plants in Kerala. In addition to seeds, the exudates of nine plants were also screened for lectin activity.

2.2. MATERIALS AND METHODS

Fully matured seeds of wild/cultivated plant species and stem exudates of plants were collected from different regions of Kerala. The plants were identified with the help of standard keys available in the Department of Botany, University of Calicut. The list of plants tested for lectin activity is given in table- 2.1.

2.2.1. Preparation of seed extracts

The seeds were dried, dehulled and powdered. The flour was defatted twice with petroleum ether (1:5 w/v). 10 g of defatted dry powder was then homogenised in 100 ml phosphate buffered saline (PBS pH 7.2), using a blender and kept overnight at 4 °C with constant stirring. After clearing the homogenate through several layers of surgical gauze, it was centrifuged at 10,000 X G for 30 minutes at 4 °C and the supernatant was subjected to haemmaglutination and sugar inhibition assays as described in section 3.2.6 and 3.2.7 respectively. The haemmaglutination

assays were done in U-shaped microtitre wells against erythrocytes collected form human 'A', 'B', 'O' blood groups and also from four other mammals i.e., rabbit, rat, mice and goat.

2.2.2. Exudate extraction

Exudate (10 ml) from stems were collected by making a cut on the stem. Then it was diluted twice with PBS and stirred well using a magnetic stirrer at 4 °C till all the white sticky substances are removed by using a glass rod. Then the extract was centrifuged at 15,000 G at 4 °C and the haemagglutination and sugar inhibition tests were carried out by the same method as in the case of seeds.

Table 2.1. List of plants screened for the presence of lectin

Scientific Name	Common/Local Name	Family
Mucuna pruriens	Cowhage	Fabaceae
Crotalaria pallida	----	Fabaceae
Crotalaria brevifolia	----	Fabaceae
Crotalaria juncea	----	Fabaceae
Pterocarpus marsupium	----	Fabaceae
Pongamia glabra	Indian beech	Fabaceae
Cymopsis psoraloides	Blester beans	Fabaceae
Erythrina subumberans	Murikk	Fabaceae
Tephrosia wynadensis	----	Fabaceae
Tephrosia diffusum	----	Fabaceae
Canavalia virosa	Wild sowrd bean	Fabaceae
Clitoria biflora	-----	Fabaceae
Clitoria ternatea	Clitoria	Fabaceae
Cajanus indicus	Pigeon pea	Fabaceae
Psophocarpus tetragonolobus	Winged bean	Fabaceae
Caesalpinia pulcherima	Peacock's pinde	Caesalpiniaceae
Caesalpinia bonducella	Kazhanji	Caesalpiniaceae

Scientific Name	Common/Local Name	Family
Tamarindus indica	Tamarind	Caesalpiniaceae
Bauhinia racemosa	Mountain ebony	Caesalpiniaceae
Bauhinia acuminata	Mandaram	Caesalpiniaceae
Cassia fistula	Indian laburnum	Caesalpiniaceae
Cassia tora	Foetid cassia	Caesalpiniaceae
Delonix regia	Poomaram	Caesalpiniaceae
Saraka asoka	Asoka tree	Caesalpiniaceae
Psidium gujava	Guva	Myrtaceae
Eugenia jambolana	Black plum	Myrtaceae
Adenanthera pavonia	Coral wood	Mimisae
Entada pursnetha	Gardal	Mimosae
Glosriossa superba	Mendonni	Liliaceae
Zizyphus jujuba	Elantha	Rhamnaceae
Zizyphus oenoplea	Makai	Rhamnaceae
Cardiospermum halicabum	Winter Chery	Sapindaceae
Guazuma ulmifolia	Rudraksha	Sterculiaceae
Helecteres isora	Potum	Sterculiaceae
Clerodendrum infortunatum	Peragu	Verbenaceae
Lantana camera	Aripoo	Verbeneceae
Myristica frangrens	Jathikka	Myristicacae
Momordica charantia	Bitter gourd	Cucurbitaceae
Momordica cochinchinensis	Karkadasrinkiri	Cucurbitaceae
Trichosanthus cucumerina	Padavalam	Cucurbitaceae
Benincassa hispid	White gourd melon	Cucurbitaceae
Citrullus vulgaris	Water melon	Cucurbitaceae
Luffa cylindrica	Looth gourd	Cucurbitaceae
Cucumis sativus	Cucumber	Cucurbitaceae
Lawsonia alba	Henna	Lythraceae
Anacardium occidentale	Cashew nut	Anacardiaceae

Scientific Name	Common/Local Name	Family
Spondias mangifera	Hog palm tree	Anacardiaceae
Lucuma nervosa	Egg fruit	Sapotceae
Mimusops elangi	Elangi	Sapotceae
Archas sapota	Sapotta	Sapotceae
Bassia latifolia	Butter tree	Sapotceae
Moringa olefera	Muringa	Moringaceae
Areca catechu	Betel palm	Palamae
Coffea arabica	Coffee	Rubiaceae
Anthocephalus chinensis	Kodambu	Rubiaceae
Ixora coccinea	Jungle jeranium	Rubiaceae
Canthium didynum	Garbha	Rubiaceae
Calophyllum inophyllum	-----	Guttifera
Garcina cambogia	Kodapully	Guttifera
Hibiscus esculentus	Ladies finger	Malvaceae
Hibiscus canabinus	Brown Indian hemp	Malvaceae
Hibiscus sabdrifa	Roselli	Malvaceae
Gossipium herbaceum	Cotton	Malvaceae
Sida acuta	Katu kurunthoti	Malvaceae
Mirabilis jalapa	4 '0' clock plant	Nyctaginaceae
Melia azadirechta	Neem	Meliaceae
Cipadessa baccifera	Kattuvepu	Meliaceae
Phyllanthus emblica	Gooseberry	Euphorbiaceae
Phyllanthus niruri	keezharnelli	Euphorbiaceae
Jatropha curcas	Kattu avanakka	Euphorbiaceae
Havea brasiliensis	Rubber	Euphorbiaceae
Physalis minima	Chinese lantern	Solanaceae
Datura stramonium	Ummath	Solanaceae
Passiflora edulis	Passion fruit	Passiflorceae
Passiflora foetida	Pottalli	Passiflorceae

Scientific Name	Common/Local Name	Family
Leucas aspera	Thumba	Labiatae
Oscimum sanctum	Tulsi	Labiatae
Sesamum indicum	Gingellyl	Pedalaceae
Annona retilculata	Ramapazham	Annonaceae
Annona squamosa	Custard apple	Annonaceae
Cananga odorata	Lang-lang	Annonaceae
Citrus acida	Acid lime	Rutaceae
Citrus aurantium	Orange	Rutaceae
Eupetorium odoratum	Communist pachha	Compositae
Nerium odorum	Sweet oleander	Apocyanaceae
Nerium indicum	--------	Apocyanaceae
Rauvolfia tetraphylla	Kattu sarpagandhi	Apocyanaceae
Vinca rosea	Shavam naari	Apocyanaceae
Cerbera odollam	Othalam	Apocyanaceae
Hoppea parviflora	-----	Dipterocarpaceae
Vecteria indica	White dammer tree	Dipterocarpaceae
Terminalia chebula	Ink-nut tree	Combretaceae
Casurina equisetifolia	Beet wood tree	Casurinaceae
Carica pappaya	Pappaya	Caricaceae
Zea mays	Maize	Graminae
Sorhum vulgare	Great millet	Graminae
Hordeum vulgare	Barley	Graminae
Artocarpus integrifolia	Jack fruit	Moraceae
Artocarpus hirsuta	Ayini	Moraceae
Bamboosa arundinacea	Bamboo	Poaceae
Vitis vinifera	Grapes	Vitaceae

Latex

Scientific Name	Common/Local Name	Family
Achras sapota	Sapotta	Sapotaceae
Vinca roseae	Savamnaari	Apocyanaceae
Plumeria subra	-----	Apocyanaceae
Nerium indicum	-----	Apocyanaceae
Ervatamia coronaria	-----	Apocyanaceae
Cryptostegia grandiflora	-----	Asclepiadeceae
Calotropis gigantia	Errikku	Asclepiadeceae
Jacqumentia cerolia	-----	Convolvulaceae
Euphorbia neriifolia	-----	Euphorbiaceae

2.3. RESULTS

Out of the 101 plant seeds screened, 30 plants showed the presence of haemagglutinins against the RBCs tested. In addition to this, exudates of two plants also showed haemagglutinating activity. Some of the seeds included in this study are used as food material while others are utilized in the indigenous system of medicine. Table-2.2 shows the scientific names of plants that showed haemgglutination activities, their titre against each kind of RBCs and their respective sugar specificity. Lectins present in the screened plants displayed differences as well as similarities in their biological activities i.e., their abilities to agglutinate human and animal erythrocytes.

Of the plants which showed haemagglutination, *Artocarpus hirsuta* showed maximum titre against all the kind of RBCs tested, which is followed by *Artocarpus integrifolia, Trichosanthus cucumerina, Crotalaria junceae, Canavalia virosa* respectively. Most of the lectins showed a lower titre against animal RBCs than human RBCs, but *Artocarpus hirsuta* showed almost equal titres against all RBCs tested. Out of 30 plants that showed haemagglutination, 14 showed

haemagglutinating activity against all the tested RBCs, which are *Artocarpus hirsuta, Artocarpus integrifolia, Phyllanthus neruri, Trichosanthus cucumerina, Tamarindus indica, Hibiscus canabinus, Garcinia cambojia, Lucuma nervosa, Vecteria indica, Lawsonia alba, Moringa oleferae, Areca catechu, Saraka ashoka* and *Momordica cochinchinsis*. Of the plants that showed haemagglutination, none was found to be blood group specific except *Mirabilis jalapa* which agglutinated only goat RBC. *Datura stramonium and Canavalia virosa* did not show haemagglutination against goat and mouse erythrocytes respectively.

The sugar specificity of each lectin differs, but some share common specificity. *Artocarpus hirsuta, Artocarpus integrifolia, Crotalaria pallida, E. neriifolia* and *Psophocarpus tetragonolobus* lectins are specific for GalNAc, while that of *Bassia latifolia, Crotalaria junceae, Tamarindus indica, Garcinia cambogia* and *Saraka ashoka* are specific to galactose. The haemgglutination of *Bamboosa arudinaceae, Phyllanthus niruri, Mirabilis jalapa, Zyzpus oenoplea, Hibiscus canabinus, Lucuma nervosa, Lawsonia alba, Vecteria indica, Lawsonia alba* and *Areca catechu* was not inhibited by any of the sugars tested. *Canavalia virosa* is specific to mannose.

Lactose is the specific sugar that inhibited haemagglutination of *Clerodendron infortunatum, Trichosanthus cucumerina, Erythrina sabumbrans, E. splendens* and *Mormordica cochinchinsis,* while arabinose was specific to *Canthium didinum* and *Vitis vinifera*. The haemagglutination of *Datura stramonium, Moringa olefera* and *Cardiospermum haliccabum* was strongly inhibited by GlcNAc.

The treatment of RBC with trypsin increased the haemagglutination titre in most of the cases, but in *Adananthera pavonia* and *Bassia latifolia* decreased haemgglutination titre was observed. In *Saraka ashoka, Areca catechu, Lawsonia alba, Vecteria indica, Psophocarpus tetragonolobus* and *Tamarindus indica*, there was no change in haemagglutination titre.

Table 2.2. List of plants showing haemagglutination activity with their titres and specific sugars.

Sl. No	Scientific names	Human blood group			Mouse	Goat	Rabbit	Rat	Trypsinized	Specific sugar
		A	B	O					B group	
1	*Adenanthera pavonia*	1024	256	256	------	-----	128	-----	128	Tre.
2	*Artocarpus hirsuta*	16384	8192	8192	8192	16384	16384	8192	32768	GalNAc.
3	*Artocarpus integrifolia*	8192	8192	8192	4096	8192	8192	8192	16384	GalNAc
4	*Bamboosa arudinaceae*	16	64	128	-----	------	8	-----	64	*
5	*Bassia latifolia*	128	256	512	------	64	64	-----	64	Gal.
6	*Cardiospermum haliccabum*	256	512	256	64	128	-----	-----	1024	GlcNAc.
7	*Canthium didynum*	512	1024	128	------	512	512	256	2048	Ara.
8	*Clerodendrum infortunatum*	128	512	8192	64	128	-----	-----	512	Lac.
9	*Psidium gujava*	256	512	8192	------	----	256	-----	16384	ManNAc
10	*Phyllnthus niruri*	128	2048	4096	128	256	512	256	8192	*
11	*Vitis vinifera*	64	256	512	------	----	64	256	2048	Ara.
12	*Zizyphus oenoplea*	64	128	128	------	----	----	----	1024	*
13	*Trichosanthus cucumerina*	8192	8192	4096	256	512	1024	512	8192	Lac.
14	*Crotalaria junceae*	8192	8192	4096	2048	----	2048	2048	16384	Gal.
15	*Crotalaria pallida*	4906	8192	4096	------	----	----	1024	16384	GalNAc.
16	*Erythrina sabumberans*	2048	2048	1024	------	----	512	----	4096	Lac.

17	Datura stramonium	1024	1024	512	256	-----	512	512	4096	GlcNAc
18	*Tamarindus indica*	512`	512	512	512	512	512	256	512	Gal.
19	*Hibiscus canabinus*	2048	1024	2048	1024	2048	1024	1024	2048	*
20	*Garcina cambogia*	1024	512	1024	2048	4096	2048	1024	2048	Gal.
21	*Canavalia virosa*	4096	4096	4096	-----	2048	2048	2048	2048	GalNAc.
22	*Momordica cochinchinensis*	1024	512	512	512	1024	1024	512	1024	Lac.
23	*Lucuma nervosa*	256	256	256	512	512	512	512	512	*
24	*Psophocarpus tetragonolobus*	128	128	128	----	64	32	----	128	GalNAc.
25	*Mirablilis jalapa*	-----	-----	-----	-----	512	----	-----	----	*
26	*Vecteria indica*	512	512	256	512	256	512	128	512	*
27	*Lawsonia alba*	128	128	128	128	64	64	64	128	*
28	*Moringa olefera*	1024	1024	1024	512	512	2048	2048	2048	*
29	*Areca catechu*	512	512	256	256	256	1024	512	512	*
30	*Saraka asoka*	256	512	512	512	128	256	256	256	Gal.
31	*Euphorbia neriifolia* **	1024	1024	1024	-----	512	512	-----	2048	GalNAc.
32	*Euphorbia splendens***	128	64	512	-----	64	64	-----	1024	Lac.

* Not inhibited by any of the tested sugars. ** Latex.

Plate 2.1 ***Euphorbia neriifolia*** **plant.**

In *Psidium guajava*, the specific sugar is N-acetyl D-mannosamine, and the lectin showed a drastic increase in the titre value against trypsin treated RBCs.

In *E. neriifolia* latex, presence of lectin was not reported earlier. The titre value showed that, there is a significant amount of lectin in the latex. So, this lectin was purified and subjected to detailed study.

2.4. DISCUSSION

The presence of lectin has been detected in large number of plant species seen in India, but very few lectins have been separated in pure form and these attempts were mainly done in North Indian flora. So, the present study demonstrates the wide occurrence of lectins in locally available plants. The plants that showed haemgglutination come under different families. This suggests that the lectins were not confined to a particular family. However, Rajindar sandu (1990) during their screening of Indian plants for the presence of lectin had noted that lectins are mainly present in fabaceae (leguminosae), which supports the findings of Cazal and Lalaurie (1952) and Krupe and Engraber (1958). There are many reports about the presence of lectins in nonleguminous plants also.

Table 2.2 shows that haemgglutination titre of lectins vary with the type of the RBCs used. This was due to the difference in the expression of carbohydrate moieties (antigen), on the surface of RBCs. This was supported by Monique *et al.*, (1983), in which they demonstrated that the haemgglutination titre for Pisum sativum lectin was high in blood group 'O' than in 'A' and 'B' blood groups.

The inhibition of haemagglutination by simple sugars proved that the lectin binds on the carbohydrate moities on RBCs. The specificity of a lectin is defined as the best monosaccharide inhibitor. Sugar specificity of lectins vary from lectin to lectin but some have common inhibitory sugars. The sugar specificity varies within the family or within the genera. For example *Crotalaria junceae* and

Crotalaria pallida have sugar specificity towards galactose and GalNAc respectively. *Erythrina subumbrans* and *Psophocarpus tetragonolobus,* which comes in the family fabaceae, have different sugar specificities i.e., the former is specific to lactose and the latter to GalNAc (Kalb, 1968). He also reported that *Lotus tetragonolobus* (winged pea) and *Phaseolus lunatus* (red kidney bean), which comes under the same family fabaceae, have different sugar specificity i.e., towards L-fucose and GalNAc respectively. Moreover, Osawa and Matsumoto (1972), have reported two lectins from *Ulex europeus*, one specific for GlcNAc and the other to L-fucose.

Trypsinized RBCs showed higher haemagglutination titre than the untreated cells in most of the cases. This is because of the mild trypsin digestion resulted in the exposure of more lectin binding sites. Lis and Sharon (1981), also detected this in many lectins.

In some cases like *Bamboosa arudinaceae*, *Phlyllanthus niruri*, *Zyzypus oenoplea*, *Mirabilis jalapa*, *Hibicus canabinus*, *Luccuma nervosa*, *Lawsonia alba*, *Vecteria indica* and *Areca catechu* none of the sugars tested inhibited haemagglutination. So, this may be due to the lectin like activity of these extracts, as they do not confirm the characteristics of lectins. Lectin like substances have been reported in may plant as well as animal species. Meade *et al.*, (1980) detected lectin like activity in Avacado peer. Sreelekha *et al.*, (1991) demonstrated that the lectin like activity attributed by two plants were due to the polysaccharides present in the extracts. The lectin like material, so detected were found to bind with Sephadex or Sepharose gels, but can be eluted from it only by using urea solution at high concentrations.

The sugar specificity gives a primary indication of the nature of the haemagglutinin present in the extracts, which helps in the purification process. Once the best inhibitory sugar was detected, a column immobilized with that specific sugar, can be employed in the purification of that lectin. In the present

study, GalNAc was found to be the best inhibitor, which is followed by galactose. In view of the results of the screening, the isolation, purification and biochemical studies were carried out on the lectin from *E. neriifolia* (Plate-2.1).

CHAPTER 3

Isolation and Characterization of *E. neriifolia* Lectin

3.1. INTRODUCTION

Screening studies revealed the presence of considerable amount of lectin in the exudate of *E. neriifolia* . This plant is a member of euphorbiaceae family and is a xerophytic plant seen usually in tropical and subtropical areas. Isolation, purification and biological properties of this lectin is discussed in this chapter.

3.2. MATERIALS AND METHODS

3.2.1. Partial purification of lectin

100 ml of exudate was collected in 100 ml of PBS(pH 7.4) and stirred well at 4 °C for 1 h and the white sticky substance was separated by a glass rod. The partial purification was carried out by fractional precipitation of proteins at 30%, 60% and 90% of $(NH_4)_2$ SO_4. To 100 ml of the latex-PBS mixture, 16.4 g of $(NH_4)_2$ SO_4 (30%), was added and dissolved gently with a magnetic stirrer for 20 min. The precipitate formed was separated by centrifugation at 20,000 X G for 20 min at 4 °C. The protein in the supernatant was precipitated successively by the addition of 18.1 g (60%) and 20.1 g (90%), of $(NH_4)_2$ SO_4 and the collected precipitate in each step was redissolved in minimum volume of PBS. These protein fractions were dialysed against PBS to remove $(NH_4)_2$ SO_4. The dialysed fractions were centrifuged to obtain a clear protein solution. The fractions were stored at –15 °C for further investigation. Each fraction was analysed for protein content by the method of Lowry et al., (1951) and lectin titre was determined as described in section 3.2.6.

The specific activity of lectin was calculated by the formula of Ahamed and Chatterjee, (1986).

Specific activity = Agglutination titre/mg protein/ml

3.2.2. Preparation of blood cell suspension

Samples of blood 'A', 'B', and 'O' groups were collected from healthy human volunteers in to equal volume of Alsever's solution. Goat blood was collected from a local butchery in Alsever's solution. Rat, mice and rabbit blood was collected from our animal house in the same way. All the blood after collection was stored at 4 °C. The erythrocytes were washed four times in PBS and finally resuspended in sufficient PBS to yield a standard erythrocyte suspension with an absorbance of 2 at 620 nm ($\approx 3.5 \times 10^8$ cells / ml).

3.2.3. Formalinization of erythrocytes

The isolated erythrocytes were washed thrice in 0.07 M Sorensen's saline, at pH 7.2-7.4 and suspended at a concentration of 10% in Sorensen's saline containing 3% formalin and kept stirring gently for 18 h on a magnetic stirrer. The cells were then washed thrice in Sorensen's saline to remove formalin and stored at 4 °C as 30% cell suspension, which is to be used over a period of several weeks. For bioassay, 2% cell suspension was prepared by suspending the pellet in PBS.

3.2.4. Trypsinization of erythrocytes

This was prepared on the day of the assay. To 10 ml of washed 4% RBC in PBS, 1 ml of 1% trypsin was added and the mixture was incubated at 37 °C for 1 h. Then the cells were washed 3-4 times with PBS and resuspended in PBS to give a final 2% RBC solution.

3.2.5. Neuraminidase treatment

RBCs were treated with neuraminidase by the method of Chatterjee and Varth (1979). 1 ml of citrated blood was centrifuged and the plasma was decanted. RBCs were washed with PBS and then washed twice with acetate buffered saline

(pH 5.6). 0.5 ml of packed RBCs were suspended in 1-2 ml of neuraminidase (1 IU/ ml). It was shaken well and incubated at 37 °C for 30 min. Then the cells were washed thrice with PBS and resuspended in PBS to give a 2% RBC suspension.

3.2.6. Haemagglutination assay

The haemagglutination assays were performed in micro-titre plates having 96 U-shaped wells by the method of Poretz et al., (1979). To each well, an equal volume (50 µl) of extract and cell suspension were added. The plate was mixed well and incubated at 37 °C for 30 min and then kept at 4 °C for 2-3 h to stabilize the agglutination reaction. Then the wells were examined visually for haemagglutination. The minimal lectin concentration required for haemagglutination was determined by serial double dilution of the extracts in PBS as described by Pueppkue (1979). The degree of haemagglutination was evaluated and the haemagglutination titre was calculated as the highest dilution capable of inducing agglutination of about 50% of RBCs.

3.2.7. Sugar inhibition test

Haemagglutination inhibition assay, to test the sugar specificity of lectins, was performed by the method of Anderson and Mclure, (1973). Various sugars, their derivatives and related compounds that are given below, were tested to determine the agglutination inhibition. The amount of each lectin was kept constant at twice the lowest concentration, which causes haemagglutination of the test cells as determined through serial double dilution technique. To 50 µl of 100 mM sugar solution, an equal volume of the extract was added and incubated at 37 °C for 1 h. To this mixture, 100 µl of 2% RBCs suspension was added and again incubated for 30 min. The restoration of red button formation in the presence of a sugar indicates inhibition of haemagglutination by that specific sugar. The efficacy of any hapten to inhibit haemagglutination by lectin was studied by determining its lowest

effective concentration, as determined through serial double dilution of the stock sugar solution.

The list of sugars and derivatives tested in agglutination inhibition assay were lactose, sucrose, maltose, glucose, mannose, galactose, melibiose, fucose, D-fructose, D-galactosamine, GalNAc, and N-acetyl glucosamine (GlcNAc).

3.2.8. Affinity purification of lectin using Sepharose 4B column

Sepharose 4B column (2 X 20 cm), which was previously equilibrated with PBS was prepared. 2 ml of 60% $(NH_4)_2SO_4$ extract was charged on to the column and was washed with PBS to remove the unbound protein at a flow rate of 6 ml/h. The washing was continued until the fraction had an absorbance less than 0.05 at 280 nm. The bound lectin was eluted with PBS containing 100 mM of galactose at the same flow rate, and 2 ml fractions were collected. The optical density and haemagglutination of each fraction was also determined. The protein containing fractions were pooled and dialysed extensively against PBS to remove galactose and then lyophilized.

3.2.9. Estimation of carbohydrates

The total sugar content of the lectin was estimated by phenol sulphuric method (Dubois *et al.*, 1956) using glucose as standard. 1 ml of lectin solution (600 µg/ml), 1 ml of glucose solution (50 µg/ml) and 1 ml of distilled water were taken in separate test tubes. To all the solutions, 50 µl of 80% aqueous phenol solution was added, which was followed by rapid addition of 2.5 ml concentrated H_2SO_4. The tubes were incubated at room temperature for 30 min. The optical density was read at 480 nm. The percentage of sugar content present in the lectin was estimated from the results obtained.

3.2.10. Elecrophoretic procedures

3.2.10.1. Native Poly-Acrylamide Gel Electrophoresis (PAGE)

Native PAGE or nondenaturing simple PAGE is used to analyse the purity of the lectin. This was carried out according to the method of Laemmli (1970). A discontinuous buffer system with two gel components was used.

3.2.10.1.1. Stock solutions

1. Electrophoresis buffer

   Tris-hydroxy methyl amino methane (TRIS) – 6 g / l

   Glycine - 28.8 g / l

   pH - 8.3

2. Stacking gel buffer

   0.5 M Tris-HCl

   pH 6.8

3. Separating gel buffer

   1.5 M Tris-HCl

   pH 8.8

4. Acrylamide-Bisacrylamide

   Acrylamide- 30 g

   Bisacrylamide-0.8 g

   Distilled water- 100 ml

   The solution was filtered and stored at 4 °C in the dark.

5. Sample buffer (3X)

   Glycerol -1.875 ml

   250 mM EDTA- 0.3 ml

   0.5 M Tris-HCl pH 6.8- 7.5 ml

0.5% Bromophenol blue- 0.3 ml

The solution was made up to 10 ml.

6. Water saturated butanol solution

   100 ml of n-butanol with 5 ml of distilled water was shaken well. The top layer was used for overlaying the gel.

7. Separating gel 10%

   Acrylamide-Bisacrylamaide - 11.4 ml

   Separating gel buffer - 8.75 ml

   Distilled water- 14.5 ml

   TEMED- 35 µl

   10% Ammonium persulphate (APS) – 360 µl

   APS solution should be prepared freshly.

8. Stacking gel

   Acrylamide-Bisacrylamide - 1.3 ml

   Stacking gel buffer- 2.5 ml

   Distilled water- 6 ml

   TEMED- 10 µl

   10% APS –100 µl

9. Coomassie blue stain (stock solution)

   Coomassie blue -1 g

   Methanol - 250 ml

   Distilled water- 250 ml

10. Working dye solution

    Stock dye solution – 50 ml

    Methanol - 250 ml

    Acetic acid - 37.5 ml

    Distilled water- 162.5 ml

11. Destaining solution

Methanol - 25 ml

Acetic acid - 37.5 ml

Distilled water- 437.5 ml

3.2.10.1.2. Casting of separating gel

Glass plates were assembled properly. The separating gel ingredients except TEMED and APS were mixed in a conical flask and deaerated. After deaeration, TEMED and APS were added and mixed gently. Then it was pipetted in to the casting mould to the required level. The resolving gel was overlaid by water saturated butanol and kept for 30 min at room temperature for polymerization. After polymerization, the top of the resolving gel was washed with distilled water and the water was removed completely.

3.2.10.1.3. Casting of stacking gel

Reagents were mixed and deaerated as in the case of separating gel. The mixture was poured on the separating gel and the comb was inserted into it and allowed to polymerise for 30 min at room temperature.

3.2.10.1.4. Preparation and loading of samples

50 µl of aqueous solution of lectin (3 mg/ml) was mixed with 100 µl sample buffer and mixed well.

3.2.10.1.5. Loading and running the gel.

The combs were carefully removed from the gels without disturbing the well dividers. The wells were washed with distilled water and excess water was

removed. The lower tank of the electrophoretic unit was filled with the running buffer. The gel was clamped to the electrophoretic chamber. Then the upper tank was filled with the same buffer. Each sample was loaded to separate wells. After loading the samples to appropriate wells, the unit was connected to the power supply (150 V for 1.5 - 2 h) and the gel was run.

3.2.10.1.6. Staining and destaining the gels

After running the gel, it was removed from the glass plates and put in the staining solution for 30 min. and then it was transferred to destaining solution with several changes until clear bands were seen.

3.2.10.2. Sodium Dodecyl Sulphate – PAGE (SDS- PAGE)

The molecular weight of a protein can be determined by SDS-PAGE, both, in presence and absence of α-mercaptoethanol by the modified method of Laemmli (1970).

Reagents

1. 10% SDS

   10 g of SDS was dissolved in 100 ml of distilled water.

2. Running buffer (5X)

   Tris-base - 30 g

   Glycine - 144 g

   10% SDS - 100 ml

   pH 8.3-8.4

   made up to 2 l

All other reagents used are same as the reagents for PAGE.

Composition of separating and stacking gels:

Reagents	Separating Gel (ml)	Stacking Gel (ml)
Distilled water	3.020	2.24
1.5 M Tris HCl	2.25	-----
0.5 M Tris HCl	-----	1
10%SDS	0.9	0.04
Glycerol	0.46	-----
Acrylamide-Bisacrylamide	3.15	0.667
TEMED	0.006	0.004
APS 10%	0.06	0.18

Gel casting, sample preparation and electrophoretic running was same as PAGE. Molecular weight markers used were phosphorylase b (rabbit muscle) 97.4 kDa., bovine serum albumin (BSA) 68 kDa., ovalbumin 43 kDa., carbonic anhydrase 29 kDa., soybean trypsin inhibitor 20kDa. and lysozyme 14.3 kDa. The preparation of standard was same as that of sample preparation.

3.2.10.2.1. Reduction with α- mercaptoethanol

Mixed 50 µl of sample (3 mg/ ml) with 150 µl sample buffer containing 5 µl α-mercaptoethanol and heated at 100 °C for 1-2 min and cooled. Then, it was electrophoresed as done earlier.

3.2.11. Periodic Acid -Schiffs (PAS) staining

PAS staining was carried out by the method of Segrest and Jacoson (1975) for detecting the presence of carbohydrate moiety in lectin. For this, the electrophoresed gel was fixed overnight in 100-200 ml of PAS fixative solution. The gel was then treated with periodic acid solution for 2-3 h followed by treatment with sodium metabisulphite for 2-3 h with one change after 30 min. The gel was

then put in Schiff's reagent and incubated at room temperature for 12-14 h. The gel was stored at 4 °C.

Reagents

1. PAS fixative

Ethanol - 40 ml.

Glacial acetic acid - 5 ml.

Distilled water – 55 ml.

stored at room temperature.

2. Schiff's reagent

Dissolved 10 g of basic fuschin in 2 l of distilled water with heating. It was cooled and 200 ml of 1 N HCl, 17 g of sodium metabisulfite was added. The solution was mixed until it was decolourised. The solution was stored in a brown bottle at 4 °C.

3. Periodic acid solution

Dissolved 1.4 g of periodic acid in 200 ml of 5% acetic acid.

4. Sodium metabisulphite

Dissolved 0.4 mg of sodium metabisulphite in 200 ml of 5% acetic acid.

3.2.12. Sephadex G-150 gel filtration

This was performed by the method of Andrews (1965). Sephadex G-150 column (50 X 1.9 cm), was preequilibrated with PBS (pH 7.4) was prepared. Then 1 ml of molecular weight standards (5 mg/ml) was added to the column. Standard molecular weight markers contained chymotrypsinogen 25 kDa, ovalbumin 45 kDa, BSA 68 kDa, SBA 120 kDa and bovine γ- globulin 150 kDa. 2 ml fractions were collected at a flow rate of 8 ml/h. The absorbance of each fraction was read at

280 nm. Then the column was washed thoroughly and 500 µl of lectin solution in PBS 1 mg/ml was added and the elution volume was determined at the same flow rate. The protein peak obtained was compared with the standard graph and the molecular weight of the lectin was determined by comparing its elution volume with those of standards (Rinderle *et al.*, 1989).

3.2.13. Physical properties

3.2.13.1. Thermal stability

The thermal stability of the purified lectin was done by the method of Chowdhary *et al.*, (1987). 0.5 ml of ENL (100 µg/ml), in PBS (pH 7.4) was incubated separately for 15 and 30 min respectively in a range of temperatures between 20-100 °C with an increment of 5 °C. Aliquots of 50 µl were withdrawn, cooled and haemagglutination titre was determined against 'A' blood group cells as described earlier in section 3.2.6. The thermal stability of demetalized lectin for 15 min was also carried out in the same way.

3.2.13.2. pH stability

The pH stability of ENL was studied by the method of Yamada and Akela (1982). Haemagglutination titre of ENL was carried out at different pH from 3, with an increment of one up to 13 using different buffers like Citrate-phosphate buffer (pH 3-7), TRIS-HCl(pH 7.4-9), Borax-NaOH buffer (pH 9.3-10), $NaHCO_3$–NaOH (pH 10-11) and KCl–NaOH (pH 12-13). 0.5 ml of ENL (100 µg/ml), was mixed with an equal volume of buffer at different pH. After incubation for 1 h, 50 µl of the sample was taken and haemagglutination titre was determined as described in section 3.2.6. pH stability of demetalized lectin was also carried out in the same way against 'A' blood group cells.

3.2.13.3. Metaperiodate oxidation

Periodate oxidation studies were carried out by the method of Reuban *et al.*, (1975). 10 mg of the lectin was dissolved in 2 ml of 0.2 M sodium acetate buffer (pH 4.5). 2 ml of 0.2 mM of sodium metaperiodate in the same buffer was then added to the above lectin solution and stirred in dark at 4 °C. With an interval of every two hours, 200 µl of the sample was taken (0-24 h) and 100 µl of ethylene glycol was added to the lectin periodate mixture to destroy excess periodate present. This mixture was dialysed against PBS (pH 7.4) and later subjected to haemmagglutination.

3.2.13.4. Metal analysis

Metal analysis was done by the method of Ahmed and Chatterjee (1989). Purified lectin (50 mg), was dissolved in 4ml of demineralized water and was refluxed for 30 min. with a mixture of concentrated HNO_3 and 70% $HClO_4$ (1:1). The material was allowed to cool and was filtered through whatman 40 filter paper. Metal contents were measured by atomic absorption spectrophotometer (Varian AA 20), using air acetylene flame and hollow cathode lamp.

3.2.13.5. Demetalization of lectin

Demetalization of the lectin was performed by using the method of Galbraith and Goldstein (1970). 500 mg of lectin was dissolved in 50 ml of 0.1 M acetic acid saturated with EDTA (10%) and dialysed ten times against 0.1 M acetic acid solution which was saturated with EDTA at 4 °C and finally dialysed against distilled water. The final solution was lyophilized. Its temperature and pH stability was carried out as described in the section 3.2.13.1 and 3.2.13.2 respectively.

3.2.13.6. Ion dependence in ENL binding to RBC

The role of ions in lectin binding was determined by the method of Teichberg *et al.*, (1988). Trypsin treated RBC ('B' group) was washed five times using 0.9% NaCl and the cells were resuspended as 20% cell suspension in 2% glutaraldehyde solution in PBS at pH 7.4. After 30 min of incubation at room temperature, the cells were washed thrice with 10 volumes of 0.1 M glycine in PBS/packed ml of cells and stored at 4 °C as 10% cell suspension in 0.02% sodium azide in PBS. Before use, the cells were washed once with 0.1 M glycine in PBS and three times with deionized water and finally resuspended in deionised water as 4% cell suspension. The agglutination of RBC by lectin was studied using photometric assay.

Aliquots of the erythrocyte suspension were mixed with the lectin solution in deionised water to obtain 0.8% cell suspension in a volume of 0.8 ml. The lectin was used at a concentration capable of agglutinating the erythrocyte suspension within 30 min i.e., 0.3 μg/ml. After the mixture was left to stand at room temperature for 30 min, a 0.4 ml aliquot from the upper part of the tube was removed and added to 1.6 ml of PBS solution containing 0.05 M galactose, that specifically blocks the lectin binding activity. The optical activity of this suspension was read at 620 nm using a spectrophotometer. To study the dependence of NaCl and $CaCl_2$ in lectin binding, NaCl and $CaCl_2$ at different concentrations (20 mM, 40 mM . . . . 120 mM and 2 mM, 4 mM . . . .10 mM respectively) were added to the reaction mixture separately. When the lectin-induced agglutination was studied as a function of salt concentration, a value of 100% agglutination was assigned to the optical density in the sample with maximal agglutination, whereas the optical density in the tube containing an erythrocyte suspension without lectin was assigned a value of 0% agglutination.

3.2.13.7. Modification of erythrocyte cell surface with ethylenediamine

In order to decrease the number of negative charges present on the erythrocyte cell surface, the cells were modified by incorporation of ethylenediamine residues. For this purpose, 1 ml of packed trypsin treated erythrocytes were mixed with 1 ml of 0.16 M N,N'- dicyclohexyl carbodiimide in water and 2 ml of 0.1 M ethylenediamine and left at room temperature for 15 h. The cells were then washed four times with ten volumes of deionised water and resuspended in deionised water.

3.2.13.8. UV absorption spectra

UV absorption spectrum of the lectin was done by the method of Bhattacharyya (1986). 1 ml of lectin in PBS (0.8 mg/ml) was taken in three test tubes. To the first, 1 ml of PBS was added and immediately the absorbance of the solution was recorded from 240-300 nm. To the second and third test tubes, 0.4 M galactose and 0.4 M GlcNAc was added and the absorbance was recorded from 240-300 nm immediately after mixing.

3.2.14. Immunological studies

3.2.14.1. Production of antibody against ENL

A sterile solution of the lectin (500 μg/ml) in PBS at pH 7.4 was thoroughly mixed with an equal volume of Freund's complete adjuvant using a leveuer lock syringe. Two groups of six animals each (mice) were used for this work. From the control group, blood was collected by sacrificing the animals. In the test group, 50 μg of the lectin was administered intramuscularly at different sites. Two booster doses were also given with an interval of three weeks, followed by a booster dose in Freund's incomplete antigen. After three weeks, the animals

were sacrificed, the blood was collected and Ouchterlony double diffusion was performed to identify the antibody production against the lectin.

3.2.14.2. Ouchterlony double diffusion

Ouchterlony double diffusion was carried out by the method of Ouchterlony (1953). 2-3 ml of molten 1% agarose in 0.05 M barbitone buffer (pH 8.6) was poured on to a clean microscopic slide. After solidification of the agarose, the slides were kept at 4 °C for 1 h. Wells were punched equidistantly from the central well (5 mm apart), using a well cutter. ENL (300 µg/ml) was loaded in the central well and the antiserum against the lectin was added at different concentrations in the surrounding wells. To confirm lectin-antibody binding, the lectin was preincubated with specific sugar for 1 h and this was allowed to diffuse against the antiserum. The plates were incubated at 4 °C in a moist chamber till optimal precipitin bands were formed i.e., for 12-48 h.

After the development of optimal precipitin bands, the slides were submerged in 0.15 M NaCl solution with several changes to remove the nonprecipitated protein and finally the salt was removed by keeping the gel in distilled water for 1 h. The gels were then fixed in methanol for 30 min and dried at 37 °C. After drying, the protein bands were detected by staining the gel with Coomassie brilliant blue for 30 min. Then the gel was destained using 3% acetic acid until a clear precipitin arc was visible.

3.2.14.3. Cross reaction of ENL with other lectins and serum constituents

The affinity of the lectin towards the serum constituents (serum of rat, rabbit and human) and different lectins like PNA, Con A, lentil, *Helix pomatia and Tetragonolobus purpureas* were carried out by Ouchterlony double diffusion technique as described above.

3.2.15. Purification of *Canavalia virosa* lectin

3.2.15.1. Conjugation of ENL to Sepharose 4B

ENL was conjugated to Sepharose 4B by the modified method of Cuatrecasas (1970). 50 ml Sepharose 4B beads were washed thoroughly with 0.2 M $NaHCO_3$ and the beads were suspended in equal volume of 0.2 M $NaHCO_3$ and were activated with 7.5 g of cyanogen bromide at 20 °C for 3 min at pH 11-11.5 (pH was adjusted with 4 M NaOH). The beads were washed rapidly with 500 ml of cold 0.2 M $NaHCO_3$. Then the lectin solution (100 mg in 50 ml $NaHCO_3$) was mixed with the activated beads and placed overnight at 4 °C on a rotary shaker. The beads were then washed with 1-2 l of 0.2 M $NaHCO_3$ and 500 ml of 0.1 M glycine. After incubation at 4 °C in the same buffer for 16-20 h, the beads were washed in 1 l of cold 0.25 M NaCl containing 0.025% sodium azide in 0.15 M phosphate buffer (pH 7.2) and stored at 4 °C.

3.2.15.2. Purification of CVL with ENL-Sepharose 4B column.

Extracts of *Canavalia virosa* was prepared as described in section 2.2.1. 1 ml of 60% $(NH_4)_2SO_4$ fraction (25 mg/ml), after dialysing against PBS was charged into a Sepharose 4B-ENL column (0.5 X 10 cm) equilibrated with 0.05 M PBS (pH 7.4). Then the column was washed with PBS at a flow rate 4 ml/hr and 2 ml fraction was collected. The protein concentration of each fraction was checked by measuring O.D. at 280 nm. When the fraction showed no protein, the column was eluted by 0.2 M galactose in PBS and the flow rate was kept constant. The protein containing fractions were pooled and dialysed against distilled water to remove all galactose and salts, which was later concentrated using a lyophilizer. The concentrate was subjected to haemagglutination and the purity of the protein was determined by gel chromatography using sephadex G-150.

3.2.16. Biological activity

3.2.16.1. Lectin toxicity

Mice (Balb/c), weighing 25 g were divided into three groups of 6 animals each housed in three different cages in an environmentally controlled room at 22-28°C with 12 h light/dark cycle. All the mice were fed *ad libitum*. Group I was considered as the control group and group II and III were treated as test batches and were force fed with purified lectin at a concentration of 2 mg and 1 mg/100 g of the body weight of test animals respectively. After nine days of feeding, the mice were sacrificed by decapitation. During autopsy duodenum and proximal jejunum portions were removed and taken in PBS and the tissues were fixed in 10% formalin. The histology of the tissues was carried out according to the standard procedures as described by Mc Manus and Mowry (1964). 5-6 μ thick sections were taken and stained with haematoxylin and eosin. The stained cross sections were examined under a microscope.

3.2.16.2. Jerne's plaque assay

The assessment of immunopotentiating action of ENL was studied *in vitro* by Jerne's plaque assay. When lymphoid cells of animals, immunised with Sheep Red Blood Cells (SRBC) were used in the presence of complement and SRBC, it resulted in the lysis of red blood cells due to the production of antibodies. These lysed cells create an empty space known as haemolytic plaque, which will be counted and represented. Every plaque appears as a result of an antibody producing cell.

3.2.16.2.1. Preparation of Sheep Red Blood Cells

10 days old SRBC, stored in Alsevier's solution was used for the plaque assay. SRBC was washed in HBSS (Hank's Balanced Salt Solution), four times and a suspension of 20% was prepared in HBSS for immunization.

3.2.16.2.2. Immunization and treatment schedule

Three groups of mice (6 animals in each group) were injected intraperitoneally with 0.25 ml of 20% SRBC suspension. After 30 min, the ENL (20 mg/Kg), was given to the first group interaperitoneally. The second group was given levamisole (20 mg/Kg) intraperitoneally as positive control and the third group was kept as negative control. On 3^{rd}, 4^{th}, 5^{th} and 6^{th} day of immunisation, the mice were sacrificed from each group by cervical dislocation and the spleen was taken asceptically and single cell suspension was prepared.

3.2.16.2.3. Preparation of single cell suspension from spleen

The mice were killed by cervical dislocation and the spleen was removed asceptically in to a petridish containing cold RPMI-1640 medium supplemented with 10% foetal calf serum. The spleen was teased and then gently passed through a sieve with a plastic plunger using a disposable syringe in presence of cold medium. The cells were washed thrice with HBSS and resuspended in a tube containing cold medium and left at 4 °C for 45 min. The cells were collected and the RBCs present in it was lysed by treating with cold Tris-ammonium chloride buffer pH 7.2 at 4 °C for 10 min. The cells were then washed in the medium containing 10% FCS and viability of the cells were checked by trypan blue exclusion method.

3.2.16.2.4. Plaque forming cell assay

After checking the viability of lymphocytes, the number of lymphocytes was adjusted to 8 X 10^6 cell/ml. 0.5% agarose was prepared in HBSS and kept in a water bath at 45 °C. 50 µl of the lymphocyte suspension and 50 µl of 7% SRBC was mixed in the tube containing 0.5 ml of 0.5% agarose solution and kept at 45 °C in a water bath. After mixing the contents in the tube, it was immediately poured onto a grease free slide and spread into an area of 1" X 2" and allowed to solidify. The slides were covered with Whatman filter paper moistened with complement (fresh rabbit serum diluted at 1:10 in PBS) and incubated at 37 °C for 1 h in a moistened chamber. The number of plaques were counted. The number of plaques/millions spleen cells were calculated by

$$\frac{\text{Number of plaques formed}}{\text{Number of cells added}} \times 10^6$$

3.2.16.2.5. Mitogenic activity

Spleenic lymphocytes obtained from Balb/c mice were washed and suspended in RPMI-1640 medium supplemented with streptomycin (100 µg/ml), penicillin (10 µg/ml) and 10% heat inactivated foetal calf serum. The viability of the cells was checked by trypan blue exclusion method. 1 ml of cell suspension (1 X 10^6 cells/ml) was incubated with 4, 8, 12, 16 and 20 µg of ENL and Con A seperately in 96 well culture plates and incubated for 48 h at 37 °C in 5% CO_2 humified air. After 48 h incubation, 10 µl of MTT stock solution (5 mg/ml in PBS) was added to the culture wells and incubated at 37 °C for 4 h. Then, 100 µl of acid isopropanol (0.04 N HCl in isopropanol), was added directly to the wells. It was mixed well and kept for 5 – 10 minutes at room temperature. Read on a multicell spectrophotometer using a test wavelength of 570 nm and a reference wavelength of 630 nm.

then put in Schiff's reagent and incubated at room temperature for 12-14 h. The gel was stored at 4 °C.

Reagents

1. PAS fixative

Ethanol - 40 ml.

Glacial acetic acid - 5 ml.

Distilled water – 55 ml.

stored at room temperature.

2. Schiff's reagent

Dissolved 10 g of basic fuschin in 2 l of distilled water with heating. It was cooled and 200 ml of 1 N HCl, 17 g of sodium metabisulfite was added. The solution was mixed until it was decolourised. The solution was stored in a brown bottle at 4 °C.

3. Periodic acid solution

Dissolved 1.4 g of periodic acid in 200 ml of 5% acetic acid.

4. Sodium metabisulphite

Dissolved 0.4 mg of sodium metabisulphite in 200 ml of 5% acetic acid.

3.2.12. Sephadex G-150 gel filtration

This was performed by the method of Andrews (1965). Sephadex G-150 column (50 X 1.9 cm), was preequilibrated with PBS (pH 7.4) was prepared. Then 1 ml of molecular weight standards (5 mg/ml) was added to the column. Standard molecular weight markers contained chymotrypsinogen 25 kDa, ovalbumin 45 kDa, BSA 68 kDa, SBA 120 kDa and bovine γ- globulin 150 kDa. 2 ml fractions were collected at a flow rate of 8 ml/h. The absorbance of each fraction was read at

3.2.16.2.6. Stimulation of pollen germination

Stimulation of pollen germination by ENL was studied by the method of Darlene (1975). Matured pollen grains were collected from *Coleus species*. The pollens were germinated in 2.5 ml of germination medium in six different flasks (1.27 mM $Ca(NO_3).4H_2O$, O.99 mM KNO_3,O.16 mM H_3BO_3 and 0.29 M sucrose). To two flasks, 1mg/ml and 0.5mg/ml ENL was added seperately. To the third, Con A (1mg/ml) was added, which was considered as positive control. To the fourth flask, 1 mg/ml ENL preincubated with 0.2 M galactose for 1 h was added and to the fifth flask, 0.2 M galactose was added. The sixth flask contained only the germinating medium (control). All the flasks were kept at 37 °C with intermittent shaking. Aliquots of germinating medium along with the Pollen was taken from each flask in hourly intervals and the germinated pollens were counted microscopically.

3.2.16.2.6. Statistical anaysis

Statistical anaysis were done using one way ANOVA.

3.3. RESULTS

3.3.1. Purification and haemagglutination studies

The amount of protein in the crude extract of *E. neriifolia* latex was 18.2 mg/ml which showed a titre of 1024 against human 'A' group erythrocytes. The crude extract had a specific activity of 56 and the purification fold of this crude extract is considered to be one. Fractionation of the crude extract with solid $(NH_4)_2 SO_4$ at different concentrations (30%, 60% and 90%), gave three fractions. Of these three, the protein content and the titre was high in 60% fraction, which was followed by 90% and 30% $(NH_4)_2 SO_4$ fractions. The purification fold of the lectin by fractional precipitation at 30%, 60% and 90% was 2, 30 and 11 respectively. These results showed that fractional precipitation of the extract was an effective method for partial purification of the lectin. Of the three fractions, 60% fraction showed high titre, specific activity and purification folds as shown in table-3.1. Thus, this fraction was used for the purification of the lectin using affinity chromatography.

The haemagglutination titre of crude and different fractions are given in the table –3.3. 'A' and 'B' blood group cells showed higher titre than 'O' blood group cells. Of the different fractions, 60% $(NH_4)_2SO_4$ fraction gave high titre than other fractions in all the three blood groups. Haemagglutination titre was increased considerably by trypsin and neuraminidase treatment. The minimum amount of pure lectin that was required to agglutinate 'A' group RBCs was 0.15 µg/ml.

Sugar inhibition studies showed that the lectin was specific for GalNAc. The minimum inhibitory concentration of GalNAc was 3.125 mM. Galactose, lactose and galactosamine also inhibited haemagglutination (Table-3.2). Other sugars tested, failed to inhibit haemagglutination even at 100 mM concentration.

Table 3.1. Purification of ENL.

Fraction	Protein mg/ml	Titre using 'A' group	Specific activity*	Purification fold
Crude	18.2	1024	56	1
30%$(NH_4)_2SO_4$ fraction	0.6	64	106	2
60%$(NH_4)_2SO_4$ fraction	9.6	16384	1707	30
90%$(NH_4)_2SO_4$ fraction	1.6	1024	640	11
Affinity purified	0.6	4096	6827	122

*Specific activity= titre/mg protein/ml

Table 3.2. Sugar inhibition of haemagglutination of human 'A' erythrocytes by ENL.

Sugar	Minimum inhibitory concentration (mM)
Fucose	NI
Glucose	NI
Galactose	6.25
Sucrose	NI
Lactose	12.5
Maltose	NI
Fructose	NI
Arabinose	NI
Trehalose	NI
D-galactosamine	25
N-acetyl-D-galactosamine	3.125
N-acetyl-D-glucosamine	NI

NI - not inhibited at 100 mM

Table 3.3. Haemagglutination titre of *E. neriifolia* lectin against untreated, trypsin treated and neuraminidase treated human erythrocytes.

Fraction	Protein mg/ml	Blood group								
		Untreated			Trypsin treated			Neuraminidase treated		
		A	B	O	A	B	O	A	B	O
Crude	18.2	1024	1024	512	2048	2048	1024	2048	2048	512
30%$(NH_4)_2SO_4$ fraction	0.6	64	32	32	256	256	128	256	256	128
60%$(NH_4)_2SO_4$ fraction	9.6	16384	16384	8192	32768	32768	16384	65536	32768	8192
90%$(NH_4)_2SO_4$ fraction	1.6	1024	1024	1024	2048	2048	2048	4096	2048	2048

Affinity purification of the lectin was carried out using a Sepharose 4B column. The elution profile of the lectin is shown in figure-3.1. The lectin adsorbed on to the column was eluted by 0.1 M galactose. The elution profile showed that the lectin was eluted as a single peak. 10 mg of ENL was purified in a single run.

The estimation of total carbohydrate by phenol sulphuric method showed that the lectin contained 9.3 $\pm$ 0.31% of carbohydrates. The presence of carbohydrates in lectin was confirmed by periodic acid Schiff's staining. In PAS staning the lectin appeared as a dark pink band. Plate 3.1

3.3.2. Homogeneity of the lectin

The homogeneity of the purified lectin was determined by PAGE. In PAGE, lectin appeared as a single blue band, when the gel was stained with coomassie blue. The gel chromatography of the lectin using Sephadex G-150 showed that the lectin was eluted as a single peak. No other peak was obtained before or after the elution of the lectin.

3.3.3. Molecular weight

The molecular weight of the lectin was determined by SDS-PAGE and by gel filtration using Sephadex G-150. In SDS-PAGE, the purified lectin migrated as a single band (Plate 3.2). The mobility of the lectin band was 2.3 cm (Figure-3.2). So, by comparing the mobilities of the molecular weight markers, the molecular weight of the lectin was determined to be 60 kDa. On reduction with α-mercaptoethanol, the lectin appeared as a single band but the molecular weight of the reduced lectin was determined to be 30 kDa, which is half the molecular weight of the native lectin.

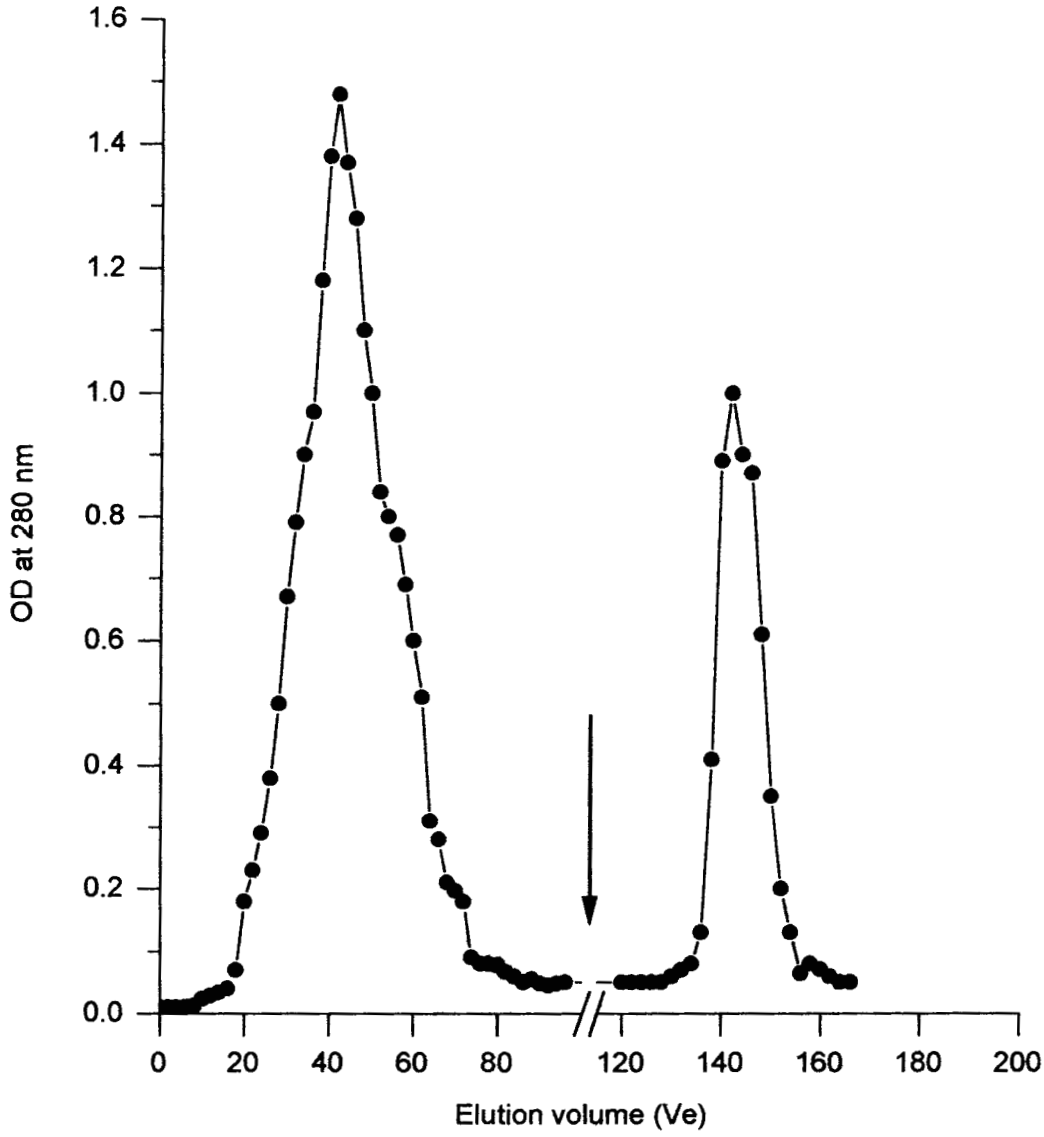


Figure -3.1 Elution profile of affinity chromatography of partially purified ENL on a column of Sepharose 4B (2 x 20cm). Arrow indicates the application of 0.1 M galactose. 2 ml fractions were collected.

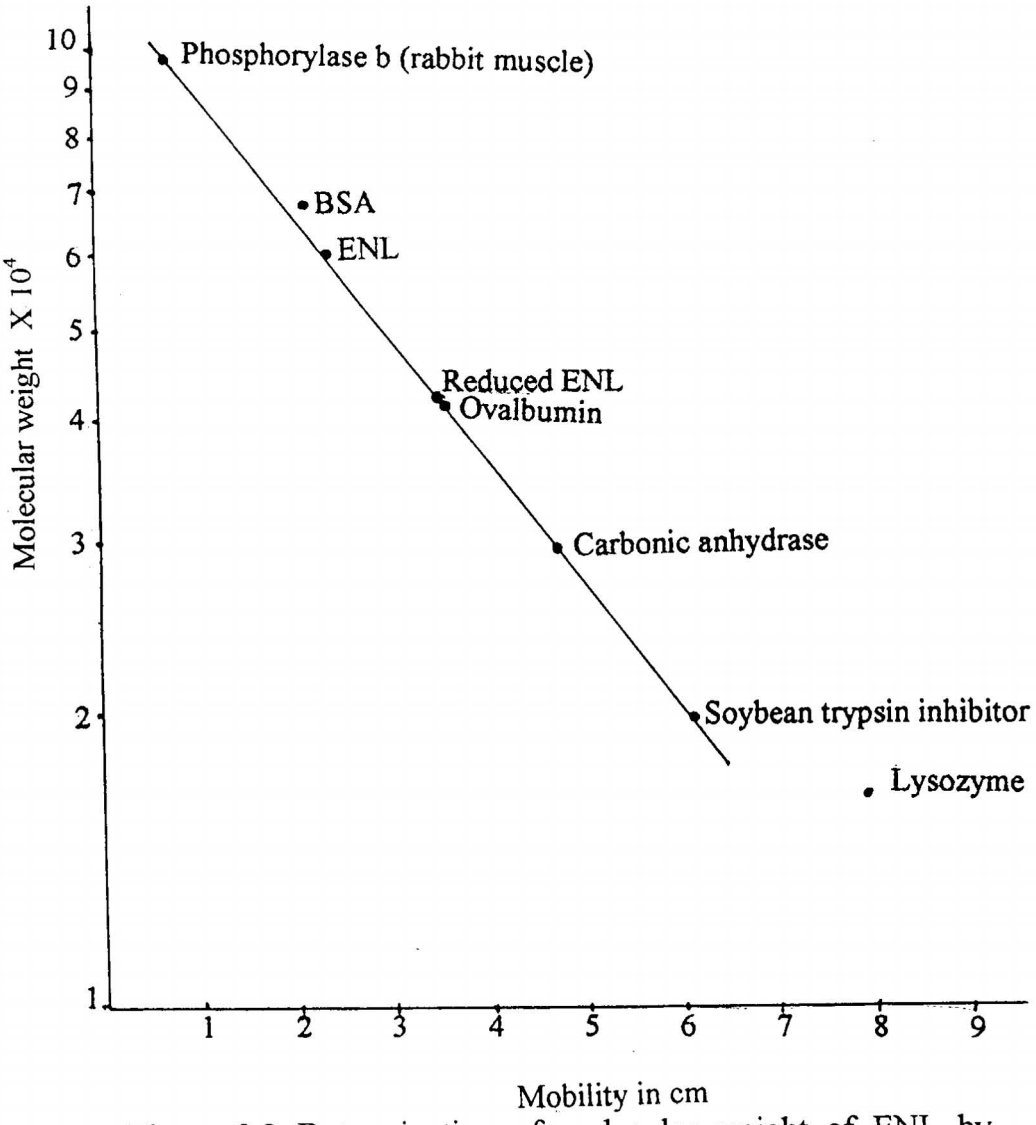


Figure-3.2 Determination of molecular weight of ENL by SDS-PAGE under reduced and nonreduced conditions.

Plate-3.1

Periodic acid Schiff's stained gel showing ENL after β-mercaptoethanol reduction.

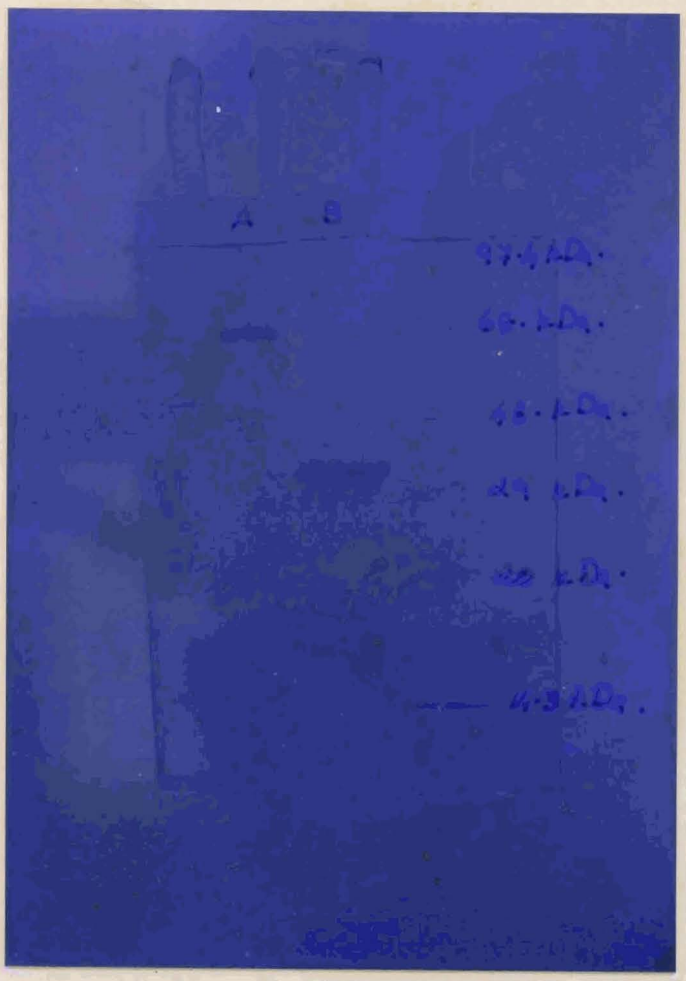


Plate-3.2

SDS-PAGE of ENL under nonreduced (A) and reduced (B) conditions.

By gel chromatography using Sephadex G-150, the lectin eluted as a single peak, indicates the homogenity of the lectin. The elution volume of the lectin was 78 ml (Figure- 3.3). On comparison of the elution volume of the lectin with that of standards, it was estimated that the lectin had a molecular weight of 60 kDa, which was same as the molecular weight obtained in SDS-PAGE.

3.3.4. Thermal and pH stability

ENL was significantly stable for 10-15 days at room temperature. Figure 3.4 shows the temperature stability curve of the lectin. It showed that the lectin was slable at 65 °C for 15 min with no change in the haemagglutination titre. But as the temperature was increased, the titre decreased. In the case of 30 min. incubation, the lectin was stable at 60 °C beyond which the haemagglutination titre decreased. It was interesting to note that haemagglutination activity of the lectin was not completely lost even at 95 °C for 30 min. However, at this temperature, most of the lectin precipitated. It was also noticed that in the presence of 0.2 M galactose, the thermal stability of the lectin was increased significantly ie., the lectin was stable at 70 °C for 30 min. Such an enhancement in thermal stability was not observed when nonspecific sugars were added to the lectin solution.

Demetalized lectin showed low temperature stability than the native lectin. Demetalized lectin was stable only up to 55 °C and the haemagglutinating activity was completely lost at 85 °C (Figure-3.4).

pH stability studies showed that the lectin exhibited haemagglutination activity even at pH 3 and 10. No haemagglutination was observed at pH 11 or above. The haemagglutination titre at extremes of pH was low (ie., 4 and 16), when compared with optimal pH titre, where the titre was 512 (Figure-3.5). Demetalized lectin showed the same pH stability pattern. After metaperiodate treatment of lectin, haemagglutination titre was not reduced even after 24 h incubation.

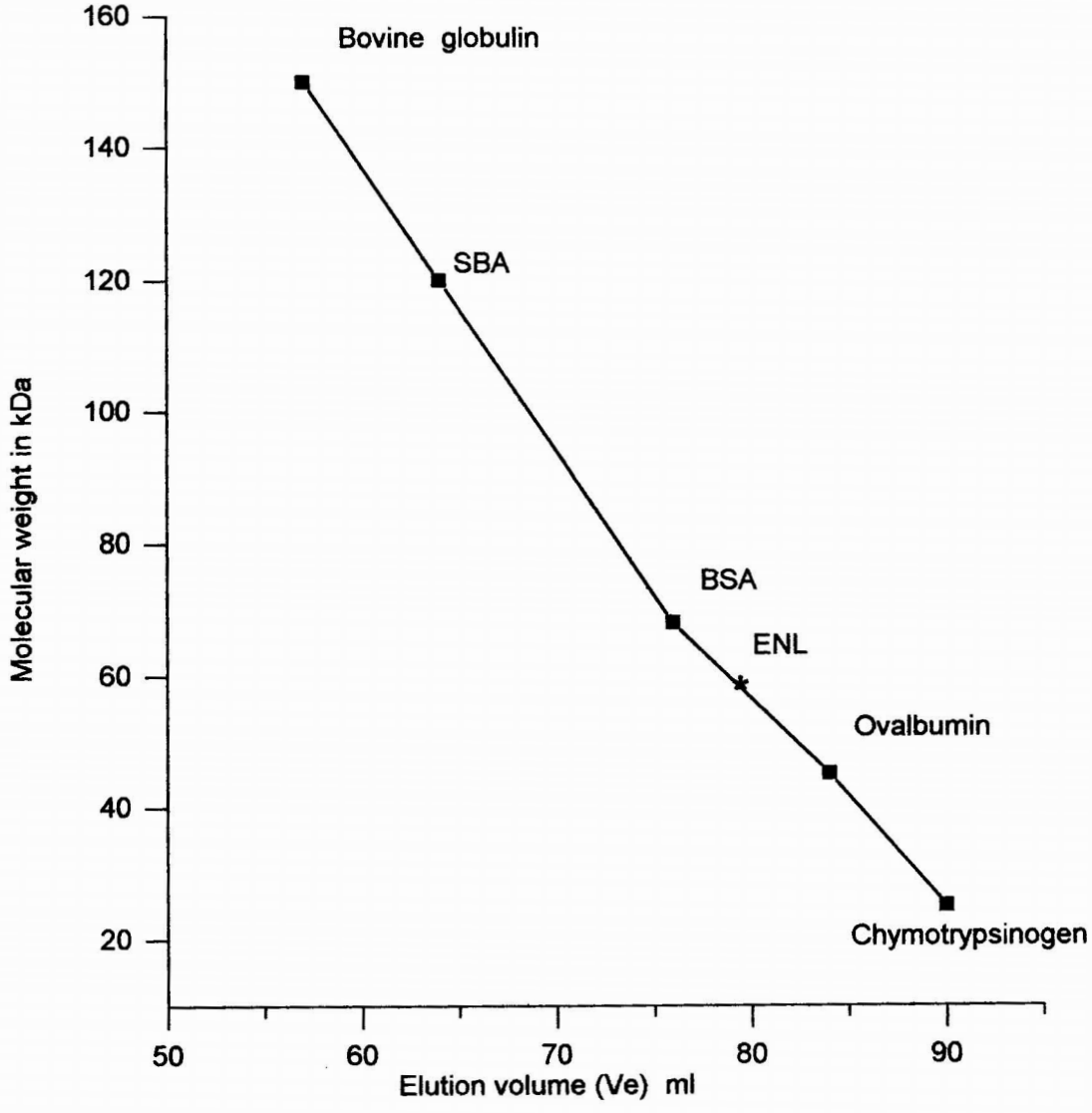


Figure 3.3 Determination of molecular weight of ENL using Sephadex G-150 (19 X 50 cm) gel filtration chromatography.

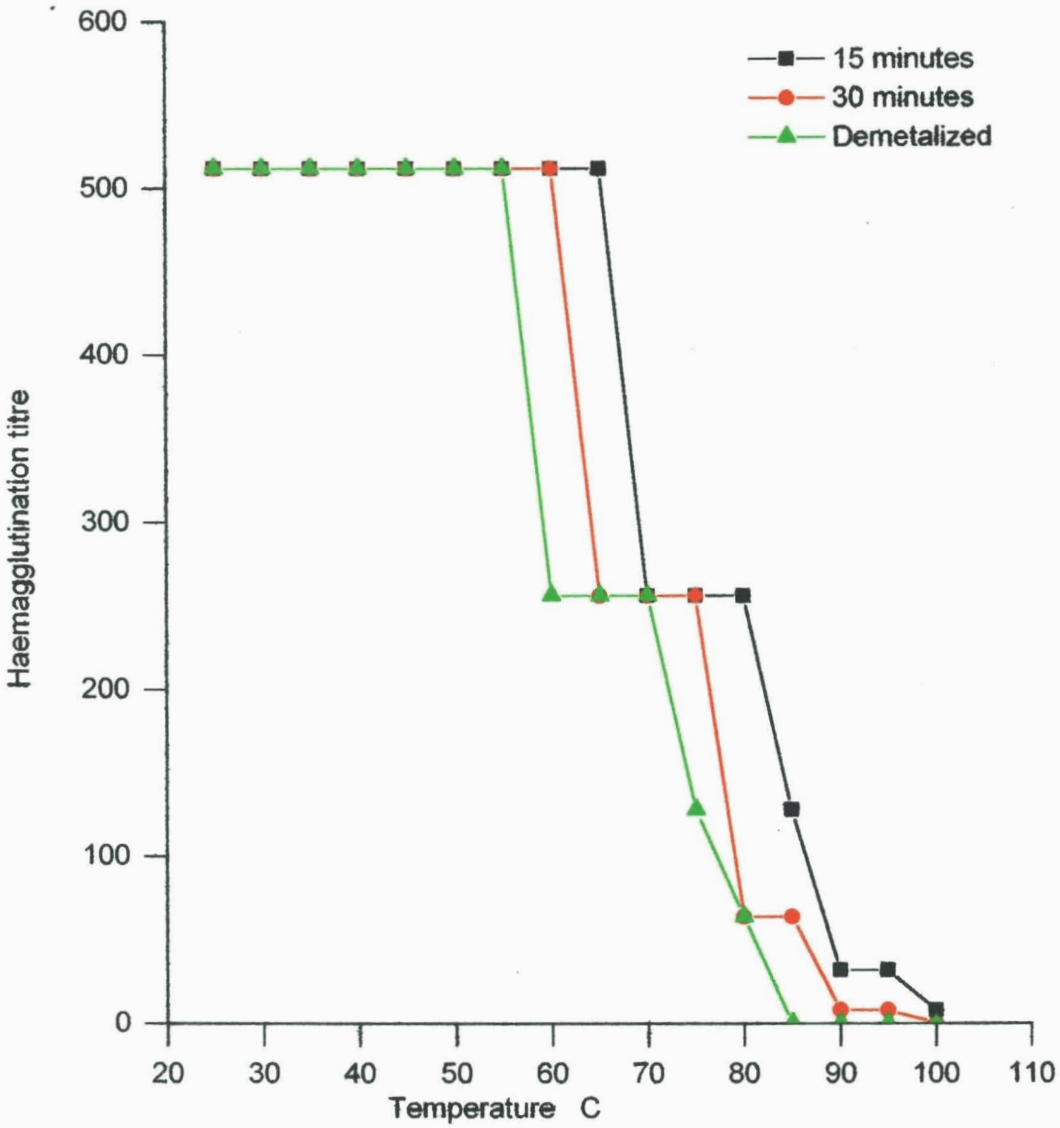


Figure-3.4 Temperature sensitivity of ENL.

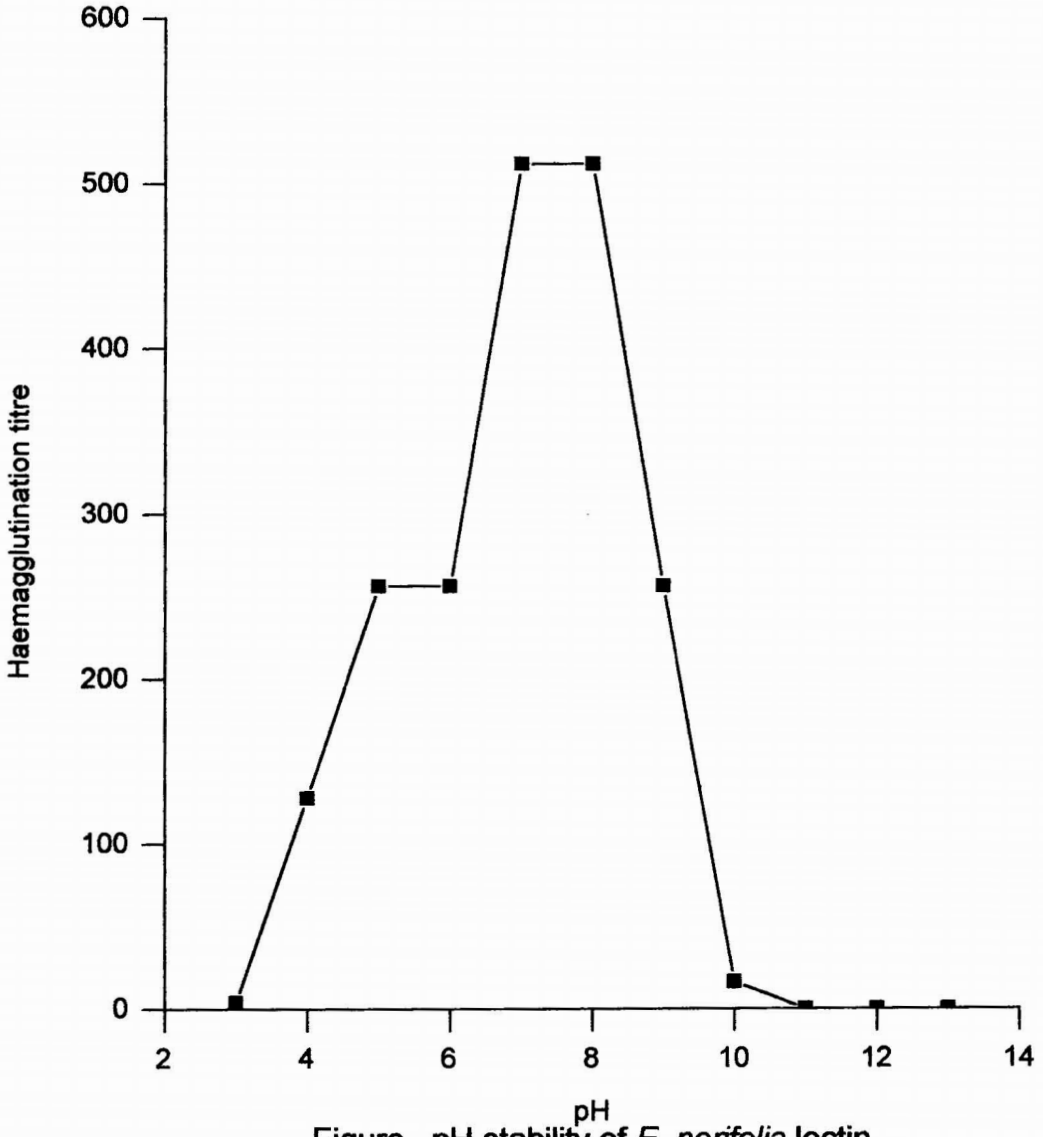


Figure pH stability of *E. nerifolia* lectin.

3.3.5. Metal content

The results of metal ion determination by atomic absorption analysis indicated that ENL is a metalloprotein containing 2.9 - 3.2 g atoms of Ca^{2+}, 1.8 - 2.2 g atoms of Mg^{2+} and 0.8 - 1.4 g atoms of Mn^{2+} per mole of lectin. ENL does not contain Fe^{2+}, Cu^{2+} and Zn^{2+} ions. Demetalization of ENL resulted in the significant removal of metals from the lectin but complete removal of Ca^{2+} and Mg^{2+} was not attained. ENL after demetalization showed same haemagglutination titre as that of native lectin.

3.3.6. Immunological studies

Ouchterlony double diffusion of ENL showed precipitation arc when tested against ENL antiserum (Plate 3.3A). No reaction was observed with preimmune serum. Precipitation arc was not observed when the ENL was preincubated with its specific sugar GalNAc.

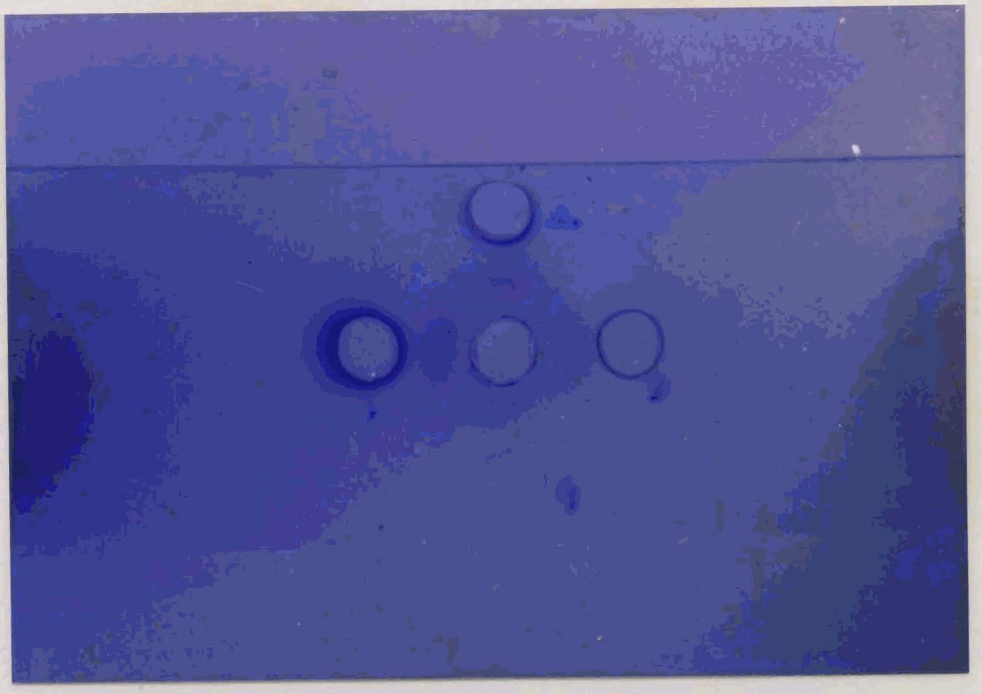
3.3.7. Crossreaction of ENL with other lectins

Ouchterlony double diffusion studies showed that ENL does not cross react with any constituent of rat, rabbit and human serum. But precipitation arc was observed when the lectin was allowed to diffuse against *Canavalia virosa* lectin (CVL) and Con A (Plate-3.3B). Precipitation arc formation was not observed when ENL was preincubated with its specific sugar GalNAc. Other lectins tested did not cross react with ENL.

3.3.8. Purification of CVL using ENL- Sepharose 4B column

Ouchterlony double diffusion gave precipitation band between CVL and ENL, which showed the possibility of using ENL-Sepharose column for the

A)



B)

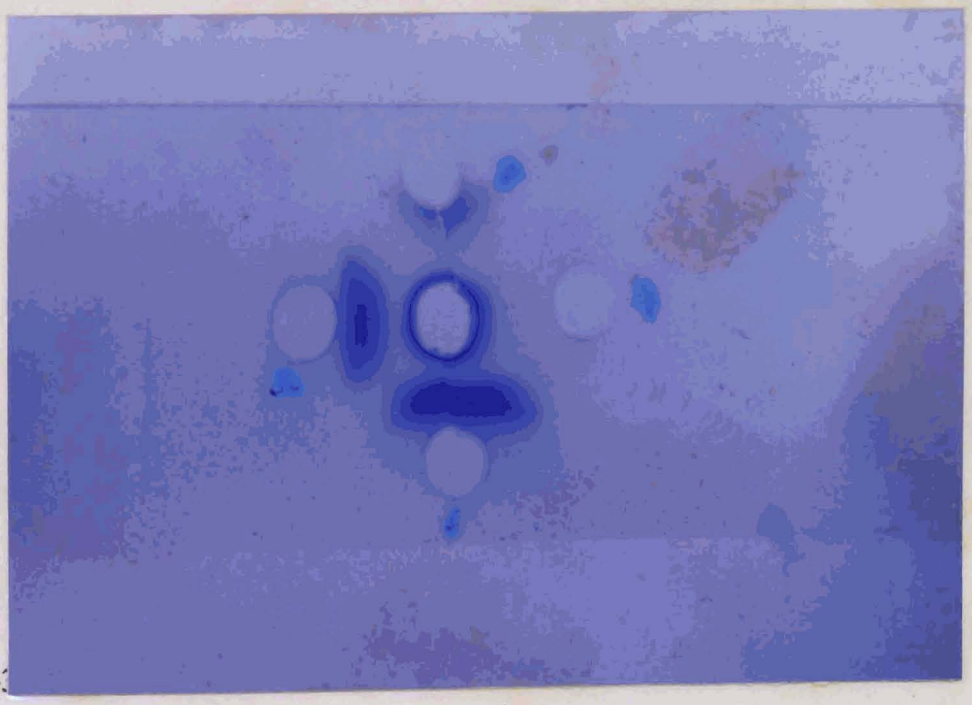


Plate-1

Ouchterlony double diffusion technique showing:-

A) Cross reaction of ENL with anti ENL mice serum. Central ring contained ENL and surrounding wells contained antiserum at different concentrations. Well -1 contained undiluted mice antiserum.

B) Cross reaction of ENL with Con A (well-1) and CVL (well-2 and 3). PNA (well-4) doesnot show cross reaction with ENL

purification of CVL. Figure-3.6 shows the elution profile of CVL. 3.9 mg of the lectin was purified in a single run. The purification fold was 205, which was higher than the purification fold using Sephadex as affinity matrix which is conventionally used.

3.3.9. UV absorption spectra

Figure-3.7 shows the UV absorption spectra of native ENL in the presence and in the absence of 0.2 M galactose. The UV-spectra of the lectin showed a maximum absorbance at 278-281 nm. The presence of galactose caused an increase in absorbance of lectin with maximum perturbation occuring at the maxima. No change in absorbance spectra was observed in the presence of 0.2 M GlcNAc, which is a non inhibitory sugar.

3.3.10. Ion dependence in ENL binding

Treatment of RBC with 2% glutaraldehyde permitted their exposure to hypotonic solutions. Figure-3.8 and 3.9 shows the dependence on salt concentrations for the agglutination of erythrocytes by ENL, Con A and PNA. In all cases, a decrease in the extent of haemagglutination was observed with the decrease in salt concentrations and no significant haemagglutination occured in deionized water. This behaviour was not specific to the salt employed ie., both NaCl and $CaCl_2$. The concentration of NaCl to produce half maximal (50%) haemagglutination with ENL, Con A and PNA was 44 mM, 38 mM and 37 mM respectively and to produce 100% haemagglutination, the concentration was 80 mM in all the three lectins.

In the case of $CaCl_2$, ENL, Con A and PNA, haemagglutination attained half maximal (50%) at concentrations of 5.7 mM, 3.2 mM and 2.4 mM respectively and 100% haemagglutination at concentration of 6 mM in the case of PNA and 10 mM in the case of ENL and Con A. (Figure-3.9).

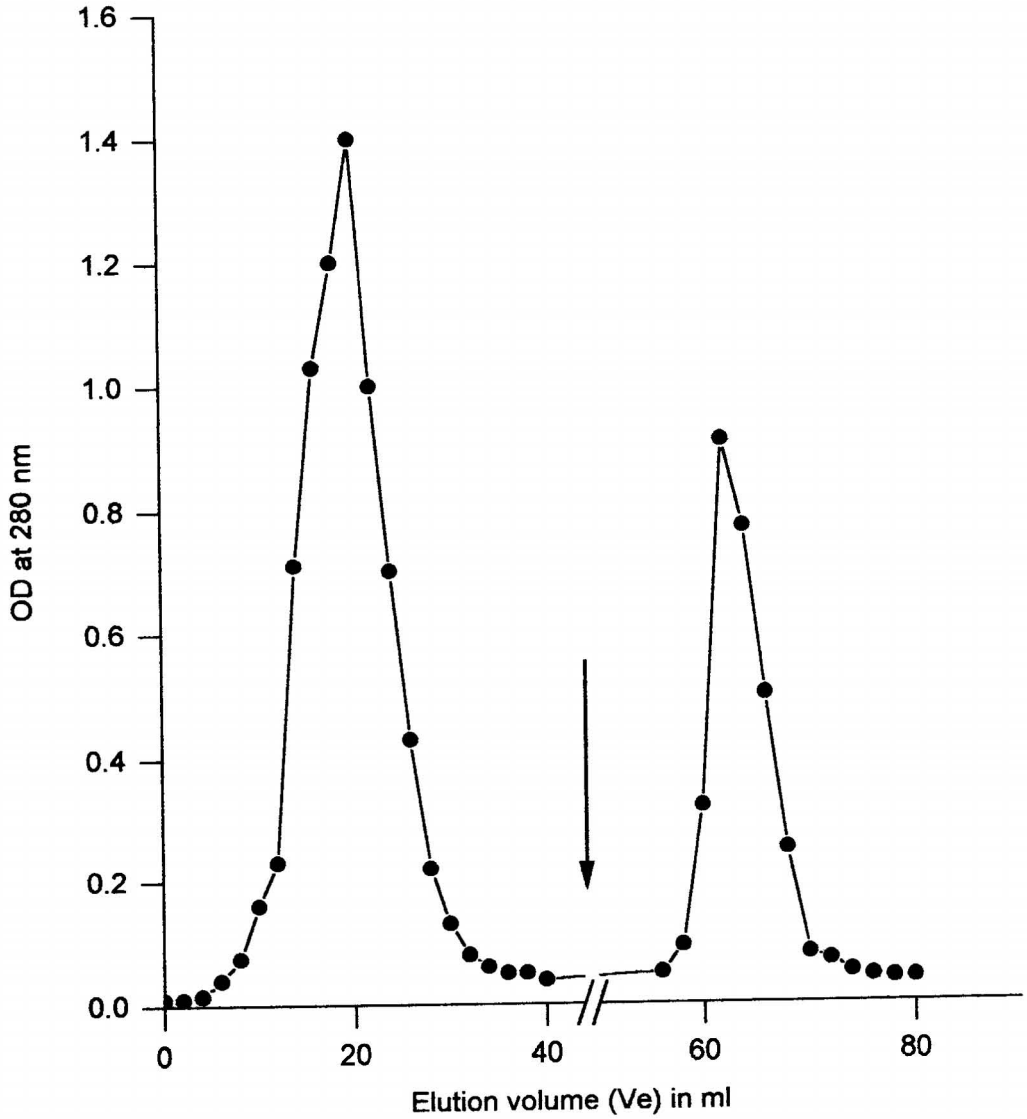


Figure-3.6 Elution profile of CVL on Sepharose 4B-ENL column (0.5 X 10 cm). 2 ml fractions were collected. The arrow indicates the application of 0.2 M galactose.

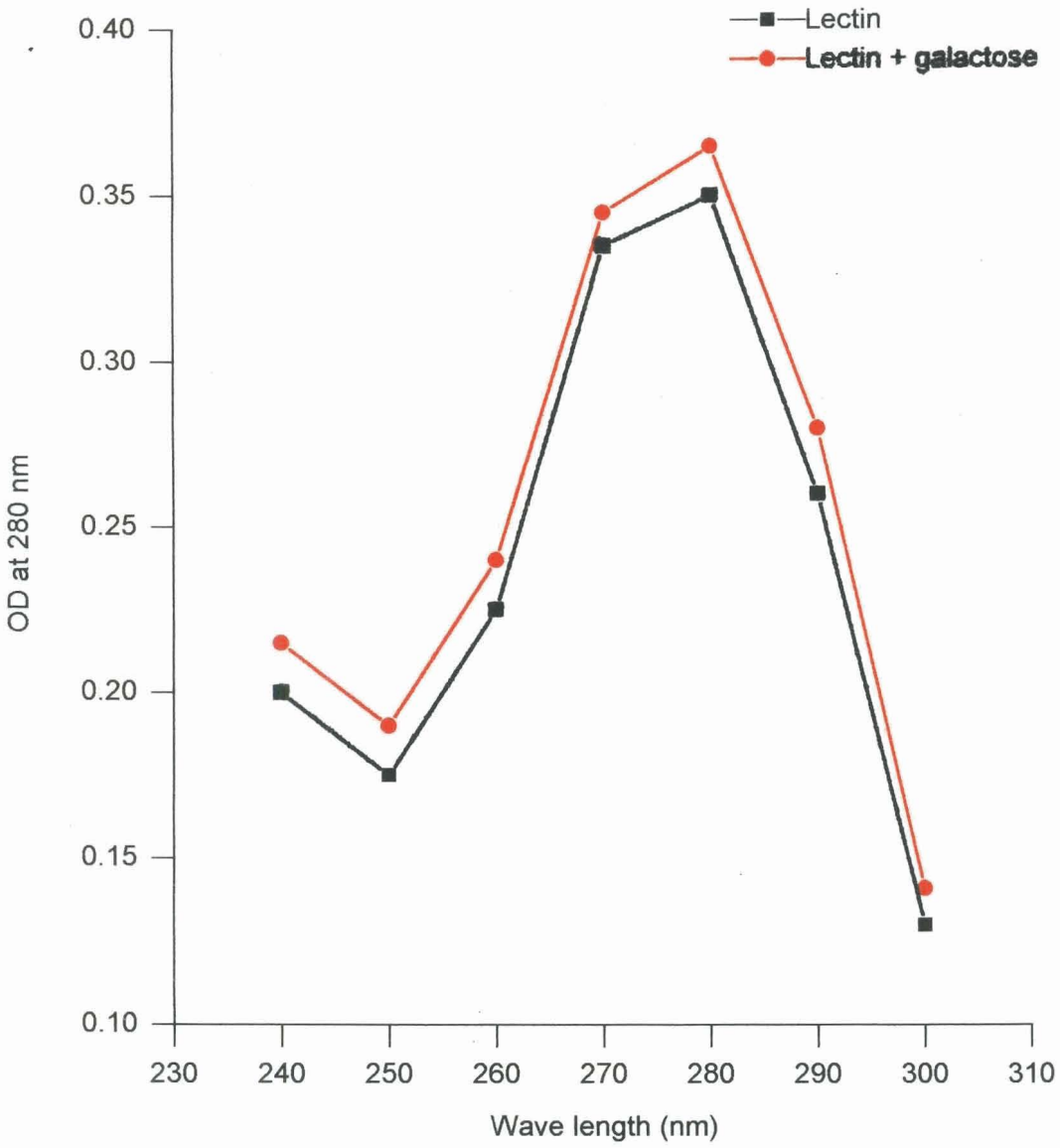


Figure-3.7 UV spectra of ENL (400 ug/ml) in the presence and absence of galactose

The chemical modification of the erythrocytes with ethylenediamine allowed PNA, Con A and ENL to attain half the maximal haemagglutination (50%) at a significantly lower concentration of NaCl, ie., 16 mM, 17 mM and 16 mM respectively and 100% at concentration 40 mM for NaCl in all the three lectins. Figure-3.10, 3.11 and 3.12.

3.3.11. Lectin toxicity

The body weight of the ENL treated animals of group II and III from day one through day nine fluctuated. But finally there was a decline in the body weight by 1.92% and 4.2% in group II and III respectively. The control group exhibited a steady increase in body weight of 5%. Supernatant of faecal extracts of group II and III were found to have haemagglutinating activity but the titre was reduced significantly.

Histologically the villi height of group II and III was considerably reduced and appeared to be abnormal when compared to the control group. (Plate 3.4).

3.3.12. Jerne's plaque assay

Evaluation of humoral immune response was done by Jerne's plaque assay. Spleen cells from SRBC immunized control mice, found to give maximum number of plaques on day four, 327 ± 7.9 (Figure- 3.13). ENL administered group showed a significant increase in the number of plaques from third day onwards which peaked on the fifth day (655.33 ± 10.3) and there after the activity declined steeply. The plaque forming activity of levamisole almost showed a similar pattern. The results of ENL treated and control showed a significant difference at 5% level in one way ANOVA.

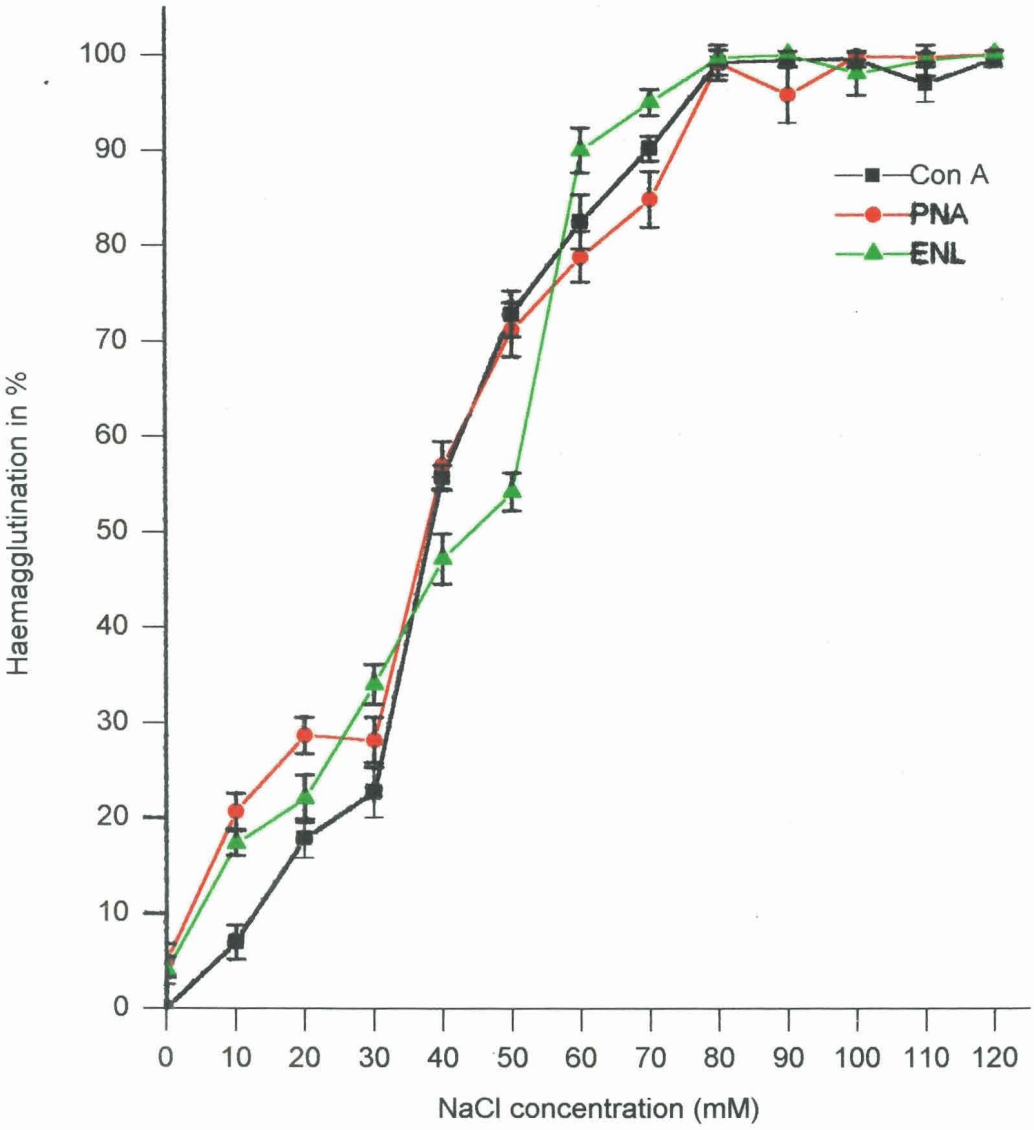


Figure-3.8 Dependence of NaCl concentration on haemagglutination of trypsin treated glutaraldehyde fixed human 'A' group erythrocytes.

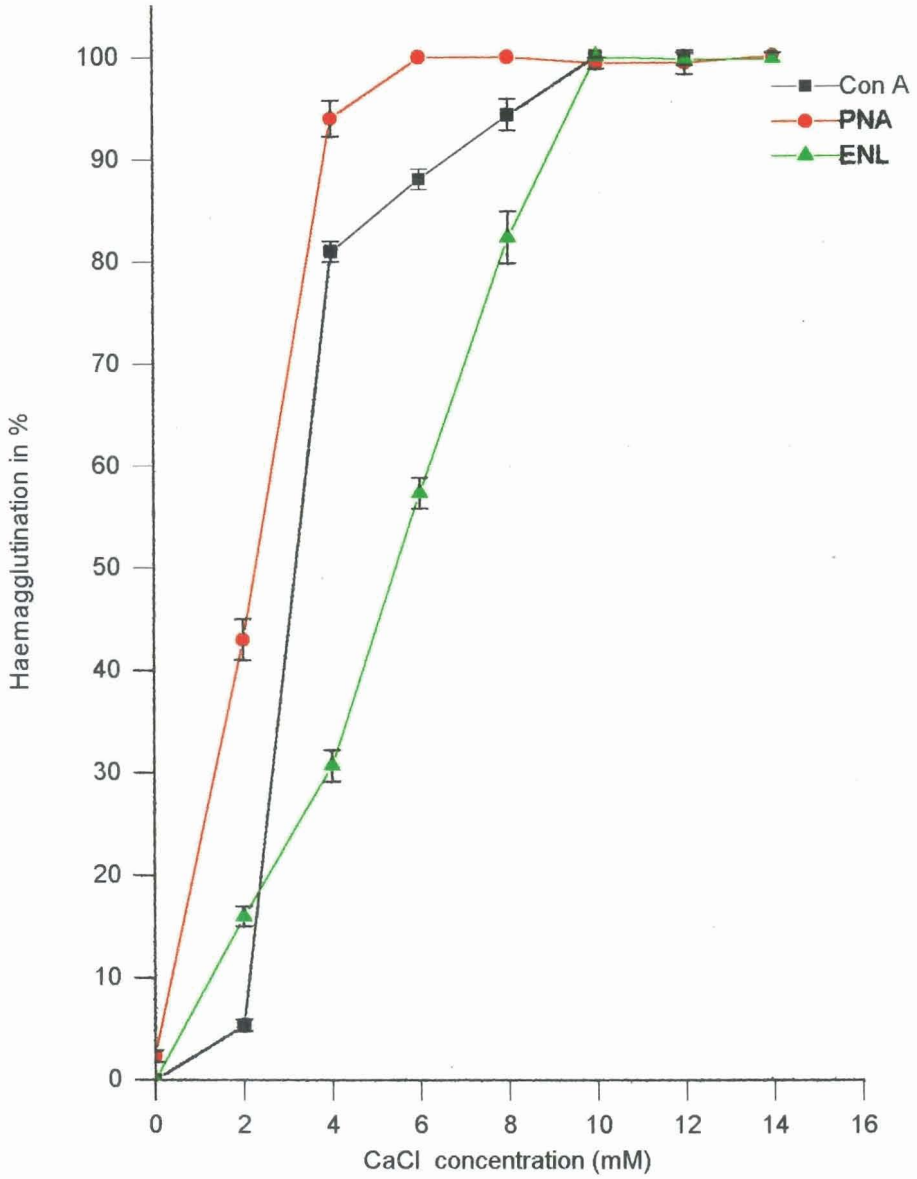


Figure-3.9 Dependence of CaCl concentration on haemagglutination of trypsin treated glutaraldehyde fixed human ' A' group erythrocytes.

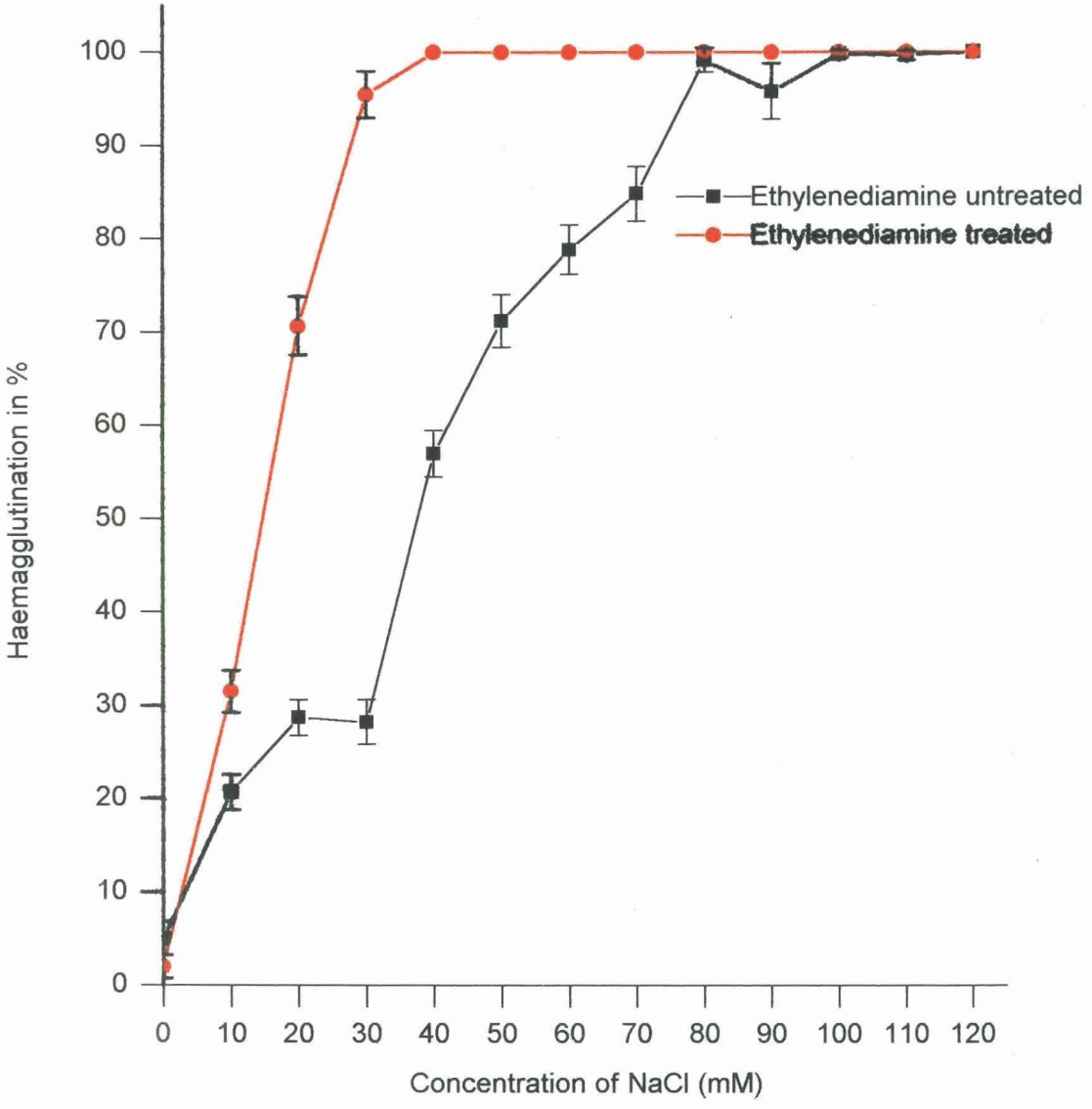


Figure-3.10 Dependence of NaCl concentration on haemagglutination of trypsin-, glutaraldehyde-, glycine- treated and trypsin-, glutaraldehyde-, glycine- ethylenediamine treated human 'A' blood group erythrocytes by PNA.

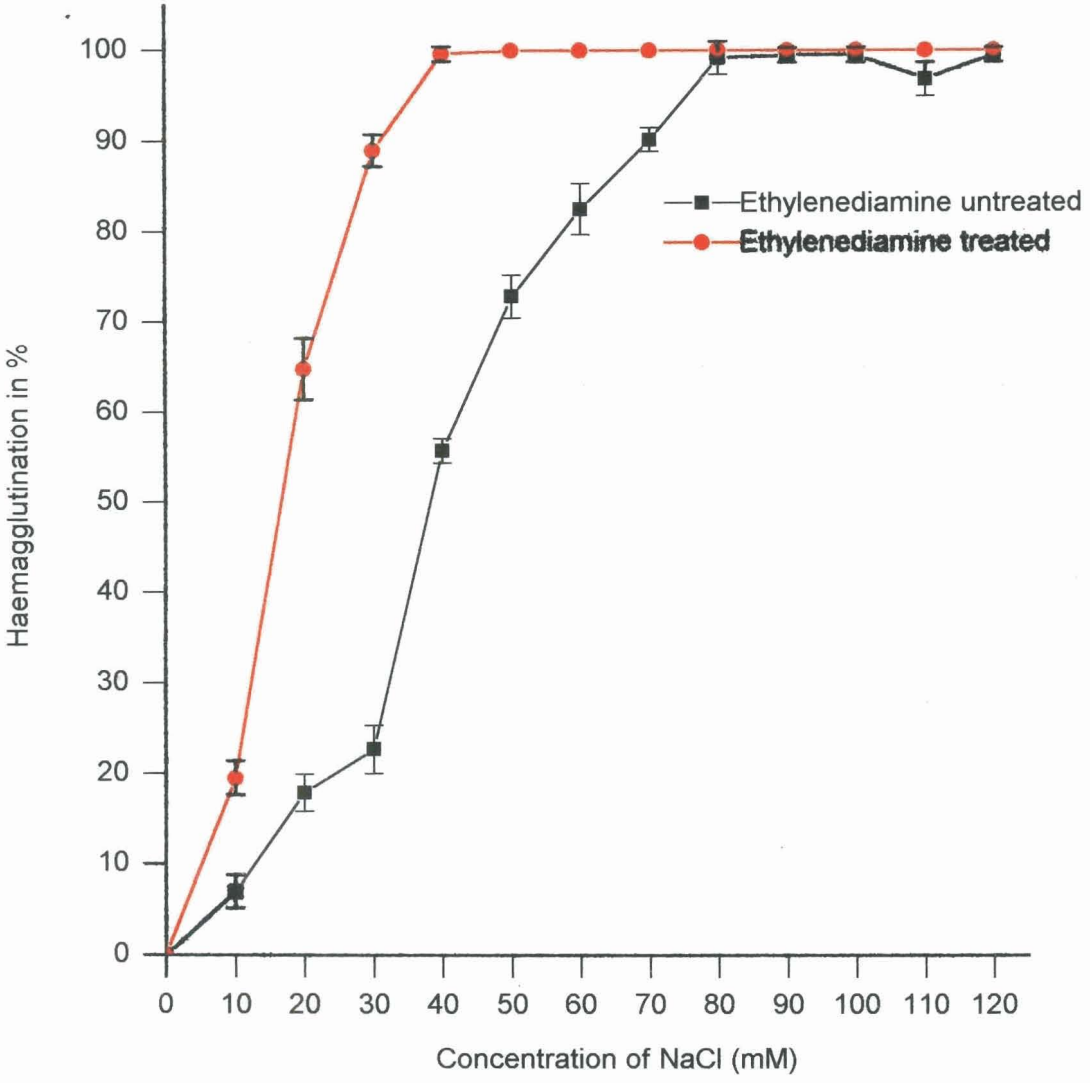


Figure-3.11 Dependence of NaCl concentration on haemagglutination of trypsin-, glutaraldehyde-, glycine- treated and trypsin-, glutaraldehyde-, glycine- ethylenediamine treated human 'A' blood group erythrocytes by Con A.

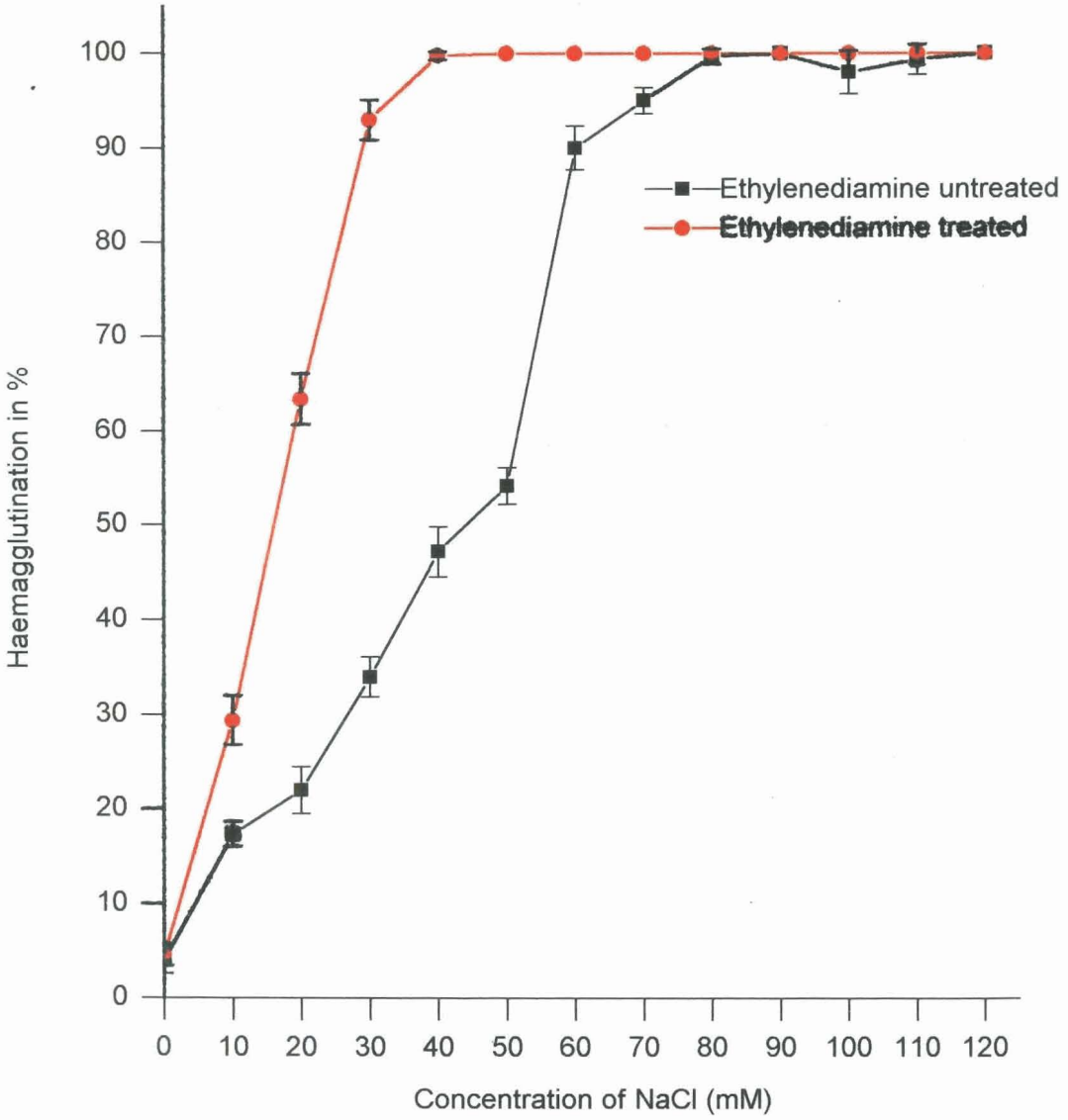
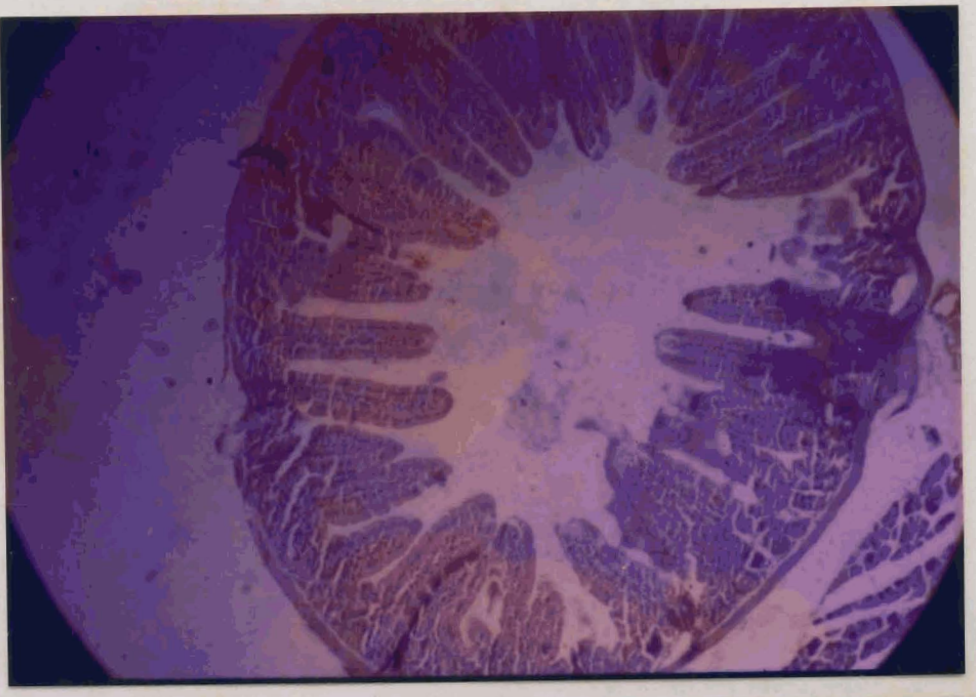


Figure-3.12 Dependence of NaCl concentration on haemagglutination of trypsin-, glutaraldehyde-, glycine- treated and trypsin-, glutaraldehyde-, glycine- ethylenediamine treated human 'A' blood group erythrocytes by ENL.

A)



B)

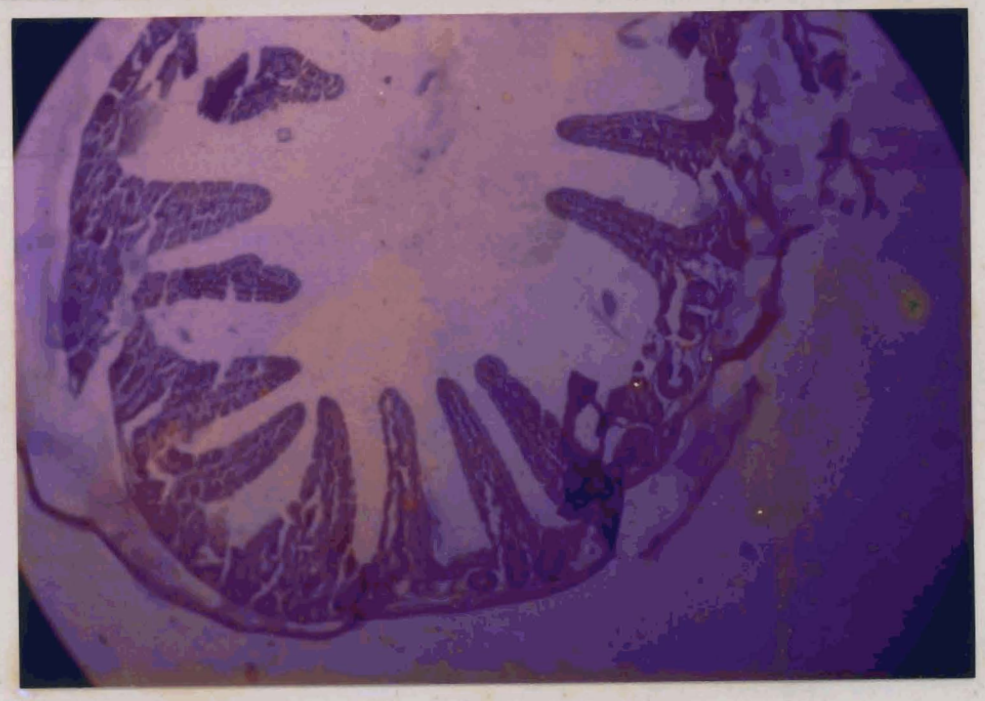


Plate 3.4

Cross section of the small intestine.

A) Control Balb/c mice.

B) ENL treated Balb/c mice (2 mg/100 gm body weight).

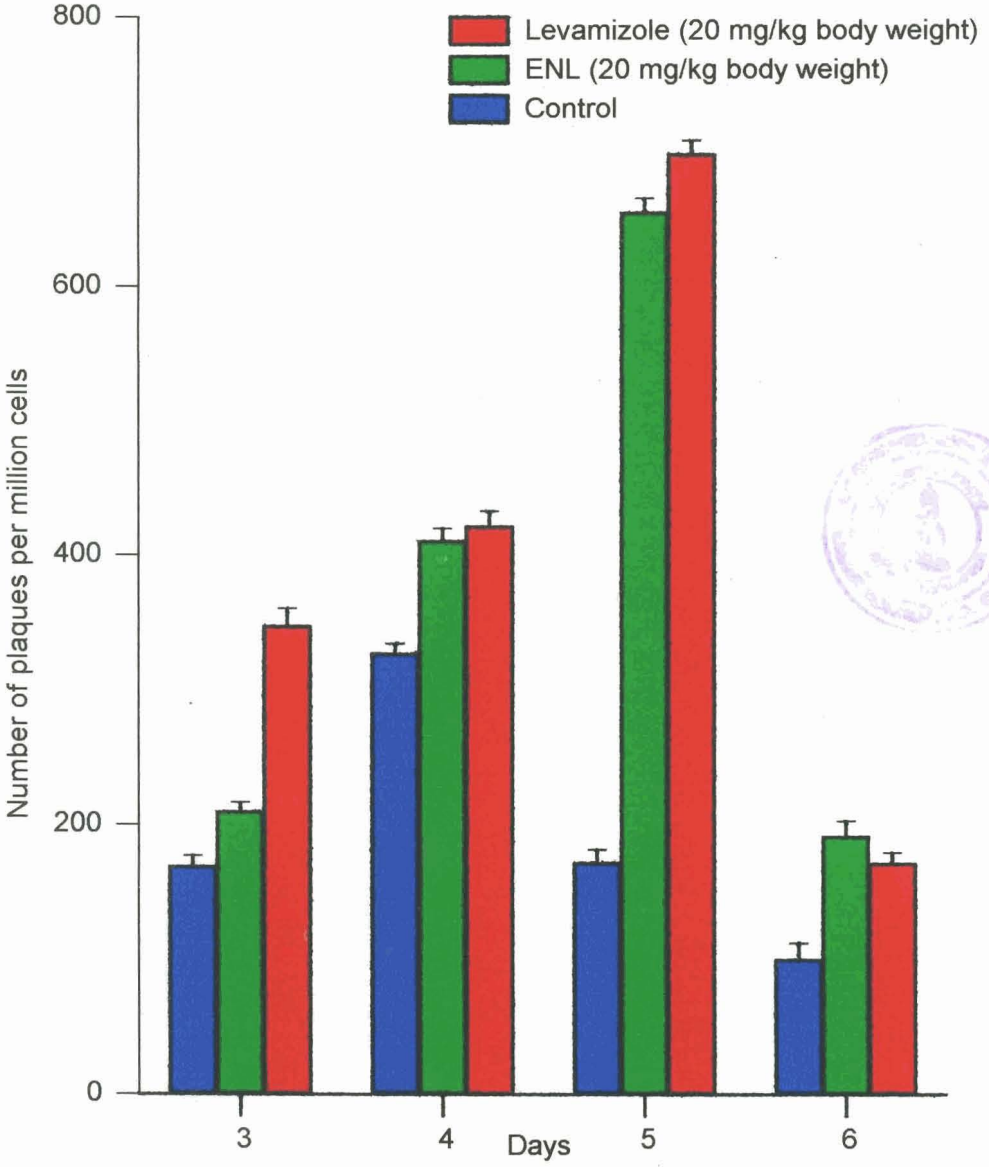


Figure-3.13 Effect of ENL on the antibody producing cells.

3.3.13. Mitogenic activity

ENL was found to have mitogenic activity similar to that of Con A. The dose response relationship was almost the same as that of Con A. (Figure-3.14). ENL and Con A showed maximum mitotic activity at 16 μg/ml and 12 μg/ml respectively. At higher concentrations, both the lectins showed decreased mitogenic activity.

3.3.14. Stimulation of pollen germination

The germination medium that contained 0.5 mg/ml and 1 mg/ml of ENL showed 17 ± 2.48 % and 20 ± 3.1% germination respectively in the first hour of incubation, whereas, Con A (positive control), showed 25 ± 3.6 % germination (Figure-3.15). No germination was noticed at that time in the medium devoid of lectin. In the 3rd h 72 ± 2.4% and 80 ± 4% germination was noticed in the media that contained 0.5 mg/ml and 1mg/ml of ENL respectively and positive control showed 86 ± 3.1 % germination. The control medium showed only 42 ± 2.4% germination in the 3rd h. At the 6th h the percentage of pollen germination in the presence of ENL was 85 ± 3.4 % at both the concentrations. Positive control showed 94 ± 2.8 % germination and the control showed 76 ± 2.9% germination. It was noticed that preincubation of ENL with 0.2 M galactose reduced the stimulatory effect significantly i.e., in the 1st h 8 ± 4.5% of pollens only germinated. In the 3rd and 6th h, the percentage of germinated pollens were 50 ± 3.1 % and 80 ± 2.2% respectively. The results showed a significant difference at 5% level by one way ANOVA.

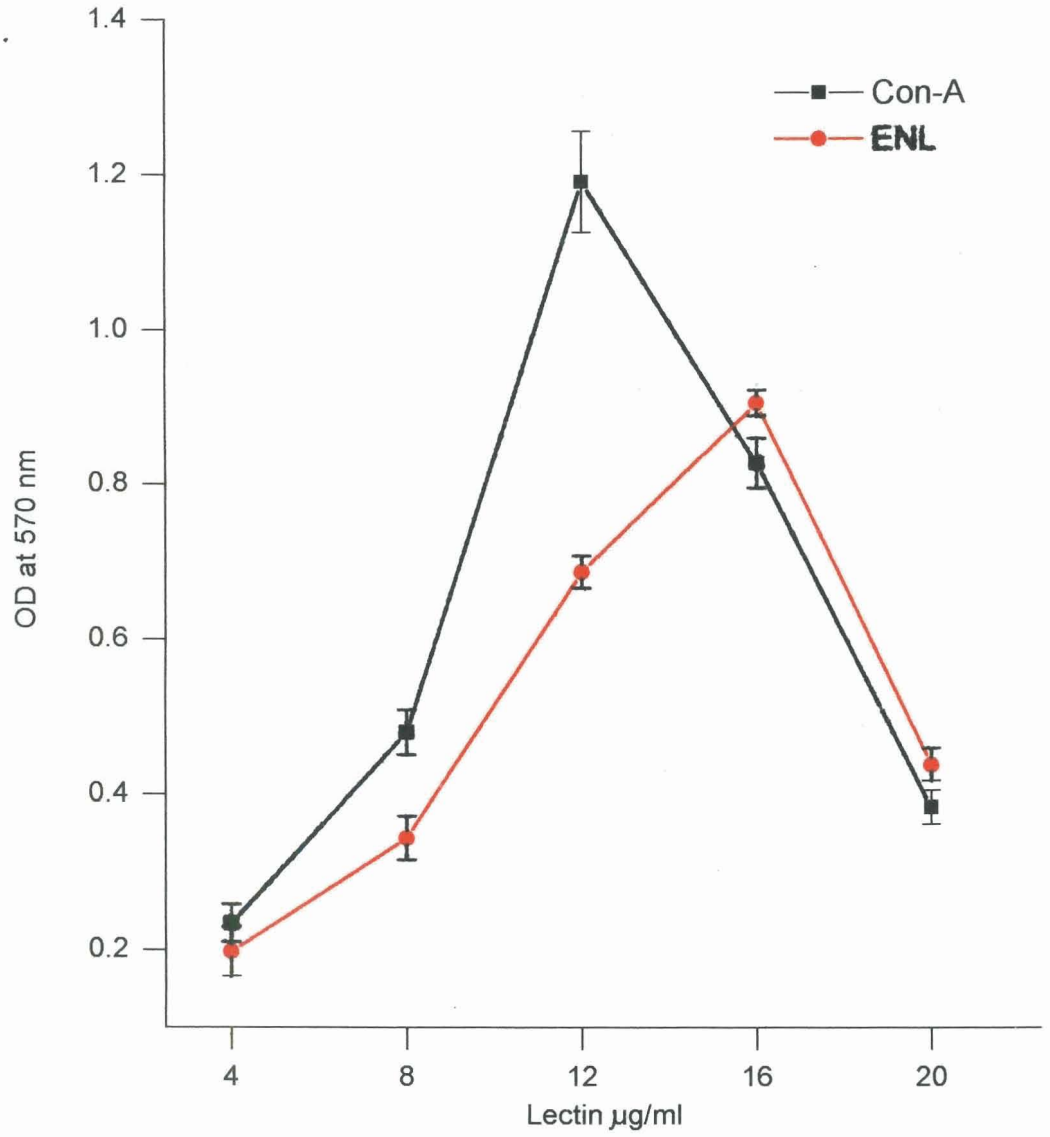


Figure 3.14 Mitogenic activity of ENL and Con-A.

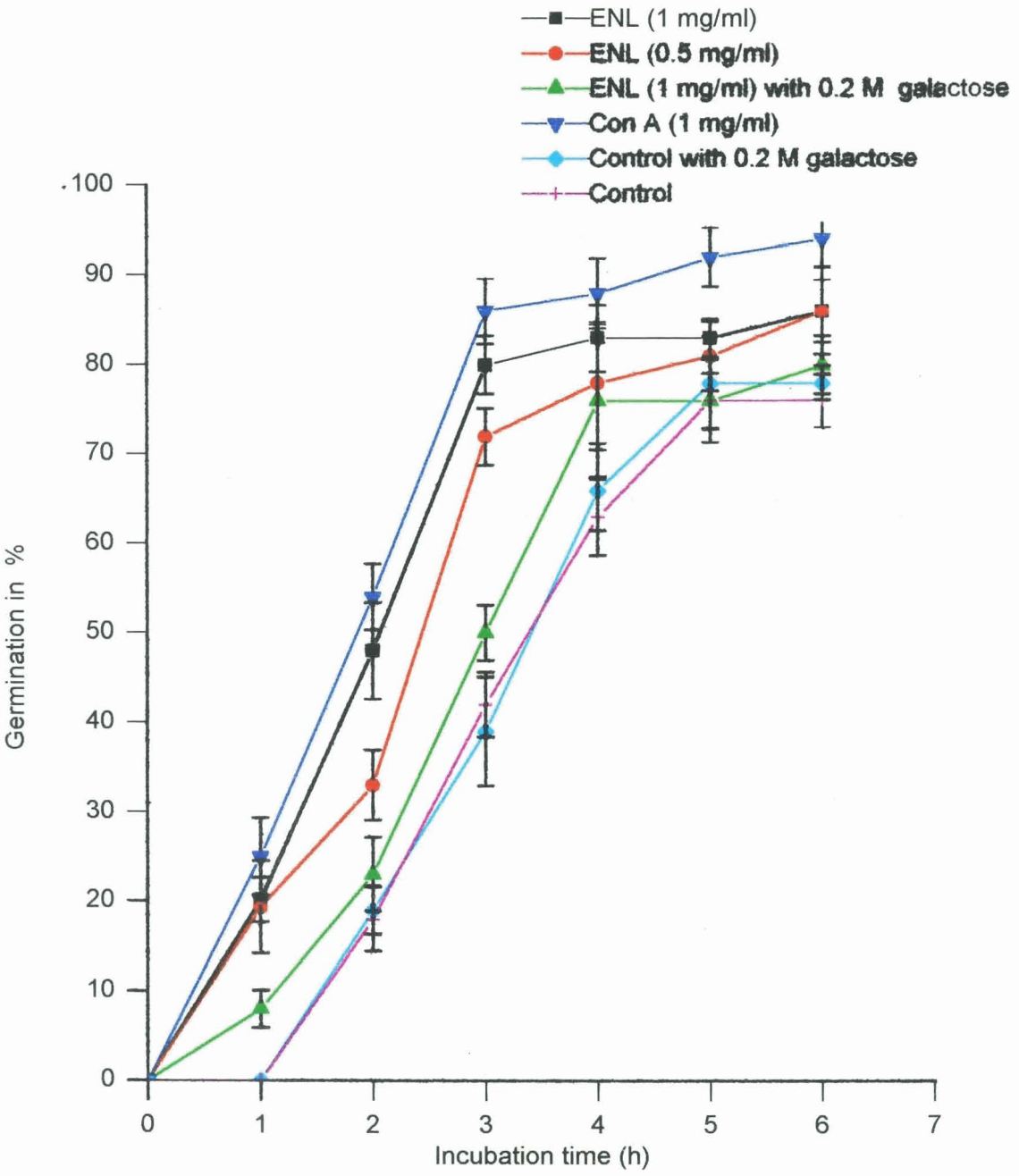


Figure-3.15 Effect on pollen germination by ENL and Con A.

3.4. DISCUSSION

Partial purification of lectin using $(NH_4)_2SO_4$ precipitation is a useful step in the purification of lectin. Many workers had employed precipitation of proteins by using $(NH_4)_2SO_4$ as an initial step in the purification of lectins. Prognet and Bourrithon (1976); Lee *et al.*, (1977); Wallie and Areeruk (1992), employed this technique for the partial purification of lectins from *Vicia graminea, B. simplicifolia* and *Parkia speciosa* respectively. In this study 60% $(NH_4)_2$ SO_4 fraction showed the highest haemagglutination titre and purification fold. So this fraction was employed in the affinity purification of the lectin.

Haemagglutination of ENL showed that the lectin is not specific to any blood group. This finding suggests that the lectin was a nonspecific agglutinin of the three different antigenic receptors of the RBC membrane, or it can be said that the lectin recognizes membrane receptory common to the three erythrocyte types. However, haemagglutination titre with 'A' and 'B' blood group cells was higher than 'O'blood group cells. This difference in titre may be due to the difference in the nonreducing ends of blood group substance. 'A' blood group substance have GalNAc side group and 'B' group have galactose (which are specific to the lectin), linked to the basic 'O' group substance, where there is no side group (Robert and Murray, 1999).

As against this nonspecificity of the lectin, many lectins have been shown to be specific agglutinins, like the anti-A from *Dolichos biflorus*, *Phaseolus limensis* and *Crotalaria lanceolata*, the anti-H from *Lotus tetragonolobus* and anti-M from *Vicia graminea* (Lis and Sharon, 1972). These lectins are valuable for blood typing, especially for distinguishing between different subgroups within the A B O system of blood grouping (Palatink *et al.*, 1980). Thus it is evident that ENL can not be used in blood typing as in the case of these specific lectins. Like ENL, many lectins have been reported to be nonspecific. Entlicher *et al.* (1970); Bhattacharyya

et al. (1981) and Magdolna *et al.* (1992), reported a nonspecific lectin from *Pisum sativum, Erythrina suberosa* and *Sechium edule* respectively.

Haemagglutination titre of all the fractions showed high titre against trypsinized erythrocytes and neuraminidase treated erythrocytes as compared with enzyme untreated erythrocytes. This increase in haemagglutination titre was due to the action of these enzymes on erythrocytes by exposing more lectin binding sites on these cells. Trypsin is a proteolytic enzyme and neuraminidase removes sialic acids from complex carbohydrates. So, these enzymes expose masked lectin binding sites on erythrocytes. Increase in haemagglutination titer by enzyme treatment was also noticed by Datta and Basu (1981), in *Erythrina varigata* lectin and in *Crotalaria striata* lectin (Sandhu et al., 1986).

Formalization of erythrocytes did not alter the haemagglutination titre of ENL, thus formalinized erythrocytes can be used for haemagglutination titre determination studies. Haemagglutination by lectin could be inhibited or prevented by the addition of certain simple sugars. The inhibitory effect of sugars is attributed to their ability to compete for the binding sites on the lectin molecule, thereby interfering with the attachment of the lectin binding sites on the surface of the erythrocytes.

Haemagglutination inhibition studies showed that, of the sugars tested, GalNAc was the most potent inhibitory sugar followed by galactose, lactose and galactosamine. Other sugars tested were found to be incapable of inhibiting haemagglutination. In galactose and galactosamine, the only difference is in their carbon –2 (C-2), of the sugar. In galactosamine, the hydroxyl group at C-2 in galactose is substituted by an amino group. This substitution caused a decrease in its inhibitory capacity to about four times. But, when the hydroxyl group at C-2 of galactose in substituted by $-NH.CO.CH_3$ (ie., GalNAc), the inhibitory capacity of the sugar was increased by two times.

Hammarstorm *et al.* (1977), reported that considerable number of lectins display preferential affnity to GalNAc but also react with galactose. Conversely, there is a group of lectins that display a primary specificity for galactose and cross react with varying degree to GalNAc. For example, lectin from *B. simplicifolia* (Hayes and Goldstein, 1974) and *Ricinus communis* (Nicolson *et al.*, 1974), are specific to GalNAc, also binds to galactose, where as some others like PNA interact with galactose only (Pereira and Kabat 1974).

Makela in 1957, reported that 4-hydroxy group is critically involved in lectin binding, so that lectin which bind mannose and glucose do not interact with galactose and *vice-versa*. This observation supports the finding that, binding of ENL is not inhibited by glucose and mannose but were inhibited by galactose, which differ from glucose only at the C-4 atom.

Haemagglutination inhibition studies showed that a column immobilized with any inhibitory sugar can be used in the affinity purification of ENL. Thus Sepharose- 4B, an ω-hexanoic acid derivative of agarose was used for this purpose. ENL adsorbed on the Sepharose - 4B column was eluted by 0.1 M galactose which showed that the ENL binding to Sepharose - 4B was reversible. Irimura and Osawa (1972), used Sepharose as an affinity adsorbent for the purification of *Abrus precatorius*, *Ricinus communis* and *Bauhenia purpurea* lectins which are specific to GalNAc and galactose. Bhattacharyya *et al.* (1981) used acid treated Sepharose-6B for the purification *Erythrina suberosa* lectin.

Estimation of total carbohydrates has shown that the lectin is a glycoprotein. PAS staining of the electrophoresed lectin confirmed the glycoprotein nature of ENL. Many lectins are known to contain carbohydrates. Kurokawa *et al.*, (1976) reported that *Wistaria floribunda* lectin is a glycoprotein. In 1987, Chowdury *et al.*, reported the presence of 11.7% neutral carbohydrate in *Artocarpus lakoocha* lectin. But the carbohydrate analysis of *Artocarpus*

integrifolia revealed the absence of covalently linked sugars (Vijayakumar and Forrester, 1986).

The homogenity of affinity purified lectin was checked by PAGE. In PAGE, ENL migrated as a single band, which reflects its purity. On SDS-PAGE, in the absence of β-mercaptoethanol, ENL appeared as a single band having a molecular weight of 60 kDa. After reduction of ENL with β-mercaptoethanol, ENL migrated as a single band having a molecular weight of 30 kDa. This showed that the native ENL was composed of two identical subunits, which are linked by disulphide bond/s. The linking of subunits was not by nonconvalent bond and/or electrostatic attraction, since the monomers were not dissociated in the presence of SDS. If the monomers were linked by noncovalent and/or electrostatic attraction, the monomers will be dissociated in the presence of SDS, but in ENL, dissocation of submit was not observed in SDS-PAGE. On the other hand, reduction of ENL with β-mercaptoethanol, reduced disulphide bond/s, which link the monomers. So, when the lectin was reduced with β-mercaptoethanol, the subunits got dissociated, having a molecular weight of 30 kDa, which is half the molecular weight of the native lectin. Thus ENL was composed of two identical subunits of molecular weight of 30 kDa, linked together by disulphide bond/s. It was also noticed that the subunits failed to produce haemagglutination.

Many workers have used gel chromatography and SDS-PAGE for the determination of molecular weight of lectins. Magdolna *et al.* (1992) used SDS-PAGE and Sephadex G-100 gel chromatography for the determination of molecular weight of *Sechium edule* lectin. They reported that this lectin has a molecular weight of 44 kDa by PAGE, but on SDS-PAGE under reduced condition, the lectin showed a single band of 22 kDa. Thus they suggested that the subunits were noncovalently linked. Lotan *et al.* in 1975c, observed similar results in *Arachis hypogaea* lectin.

The elution profile of ENL by Sephadex G-150 column showed that the lectin was eluted as a single peak, which confirms the purity of the affinity purified lectin. The elution volume of ENL also confirmed that the molecular weight of lectin was 60 kDa.

Like many other lectins, ENL was also thermostable to some extent. It was stable up to 65 °C for 15 min and 60 °C for 30 min. The thermal stability data showed that the stability of the lectin was temperature and time dependent. Ahmed and Chatterjee (1989), reported that jacalin was stable at 50 ° C for 30 min and the heat stability is temperature and time dependent.

Demetalized lectin showed decreased thermal stability. So this suggests the role of metal ions in thermal stability of ENL. It was also noticed that thermal stability of ENL was increased in the presence of 0.2 M galactose. The reason behind this may be the sugar present in the binding site of the lectin which might be protecting the site from thermal denaturation. This type of enhancement in thermal stability by specific sugar was reported in *Erythrina subumbrans* and in *Erythrina indica* by Bhattacharyya *et al.* (1981). Nonspecific sugars failed to enhance thermal stability of ENL.

pH stability of ENL showed that the lectin was significantly stable from pH 5-9. However, the optimal pH for lectin binding was between pH 7-8. Demetalization of the lectin does not affect the pH stability of lectin. So and Goldstein (1967) also reported that Con A gave maximum haemagglutination titre between pH 7-8.

Metal analysis of ENL by atomic absorption spectrocopy showed that the lectin contained Ca^{2+}, Mg^{2+} and Mn^{2+} cations. Demetalization of ENL removed these cations in appreciable amount, but complete removal was not achieved. It was noted that demetalization of ENL does not affect its agglutinating capacity, but it resulted in decreased thermal stability. This suggests that metal ions are not

essential for agglutination. Ahmed and Chatterjee (1989) reported that treatment of jacalin by acetic acid and EDTA, neither affected the haemagglutinating activity of the lectin nor did incorporation of Ca^{2+} and/or other bivalent ions in saline showed any enhancement in the haemagglutination titre. Doyle *et al.*, 1976 reported that metals confer a high degree of structural stability to Con A, protecting the lectin against heat inactivation and hydrolysis by proteolytic enzymes.

In Ouchterlony double diffusion, presence of precipitation arc between serum of immunised mice and lectin showed that antibody was produced against the lectin. No precipitation arc was observed with preimmune serum. The inhibition of precipitation arc formation by the lectin specific sugar GalNAc confirms that the precipitation arc was formed by the reaction between the antibody and the lectin. Lotan *et al.* (1975c) and Hosselet (1983) produced autibody against *Arachis hypogaea* and *Pisum sativum* lectins respectively.

Double diffusion also indicated that ENL failed to react with any of the normal serum constituents. But Maria and Antonio (1985) reported that jacalin reacts with IgA, which is specific for GalNAc. ENL also has the same specificity but it failed to react with any of the normal serum constituents.

ENL reacted with Con A and CVL when allowed to diffuse against it. But other lectins tested, failed to give any precipitation arc. It was also noticed that the precipitation arc was dissolved slowly by the addition of GalNAc indicating the specificity of lectin cross reaction. Moreover, nonreactivity of ENL, which was preincubated with GalNAc confirms the binding of ENL with Con A and *Canavalia virosa* lectin. Cross reaction between lectins was reported in many cases. Galbraith and Goldstein (1970), observed similar cross reaction between Lima bean lectin and Con A. Like this, Goldstein *et al.* (1969), reported the cross reaction between soybean and wax bean lectins. The reaction between ENL and CVL indicates that a column immobilized with ENL can be used as an affinity adsorbent for the isolation of CVL.

ENL gave precipitin arcs with Con A and CVL in double diffusion experiments. Similar reactions between Con A, lima bean and soy bean lectin were observed by Galbraith and Goldslein (1970). The specific binding of the CVL to ENL is further corroborated by the results of affinity chromatographic isolation of this lectin on ENL-sepharose colum. The bound CVL was eluted by 0.2 M galactose. The lectin purified on ENL-Sephorose column showed a high purification fold.

UV absorbance spectra showed that the lectin had a maximum absorbance between 278-281 nm. In 1986 Bhattacharyya *et al.* studied the UV- spectra of *Erythrina* species and reported that *Erythrina indica, Erythrina arborescens* and *Erythrina lithosperma* lectin showed maximum UV absorbance peak between 275-280 nm which is similar to the UV absorbance peak of ENL. They also noticed that the environments of the aromatic amino acid residues are similar in these lectins and that there was a change in the environment of tryptophan residue(s), upon sugar binding.

On addition of inhibitory sugar to lectin, the UV absorbance showed a hike especially in the 278-281 nm region. This shows that during the binding of sugar to lectin, there occurred structural rearrangement leading to the exposure of tryptophan residues, which might have increased the UV absorbance. Nonspecific sugars like, GlcNAc failed to cause such a structural rearrangement, which resulted in nonenhancement of the absorption spectra. In addition to this, perturbations of the UV- spectra of ENL in the presence of inhibitory sugar, indicates the involment of tryptophan residues in sugar binding.

The studies on dependence of ions in lectin binding showed that a decrease in the extent of haemagglutination was observed with the decrease in salt concentration and almost no haemagglutination occurred in deionized water. Erythocytes were fixed with 2% glutaraldehyde to permit their exposure to

hyposmotic solution. The glutaraldehyde fixed, trypsin treated erythrocytes displayed a higher density of negative charges than the unfixed erythrocytes, since the fixed cells are exposed to glycine following the glutaraldehyde treatment. The latter fixative reagent, while cross-linking the amino groups on the cell surface, leaves some unreacted aldehyde residues which were then neutralized by Schiff's base coupling with the amino group of glycine, there by producing a significant increase in the number of carboxyl groups on the cell surface.

Since none of the lectins studied here is known to have a monovalent ion requirement for its activity, it is reasonable to ascribe the absence of agglutination in deionized water to an electrostatic repulsion either between the negatively charged lectin or the negatively charged surface of the erythrocytes or between the erythrocytes themselves.

To investigate these possibilities, we examined whether ENL, PNA and Con A were able to bind with erythrocytes in deionized water. The observation of such a binding would suggest that the absence of haemagglutination in deoinized water may be due to the electrostatic repulsion between the erythrocytes.

Accordingly, each lectin was mixed with a suspension of erythrocytes at a concentration allowing the complete agglutination of the cells and the clearing of the solution from free erythrocytes and free lectin. The experiments were performed in parallel using a salt free solution (deionized water which prevents agglutination) and a solution containing 150 mM NaCl (which allows agglutination). Once the erythrocytes in the latter solution were agglutinated by the lectin, the solutions were centrifuged and the respective supernatants were added to freshly pelleted erythrocytes. Using this protocol, it was observed that erythrocytes in salt free supernatant when supplemented with 150 mM NaCl showed haemagglutination, whereas, no agglutination was observed in the other case.

These results showed that no interactions took place in deionized water between the erythrocytes and the test lectins, the latter remaining entirely in solution, where as in the presence of salts, the lectins adhere snugly to the cells. So, it can be concluded that in deionized water, the electrostatic charges on the erythrocytes and the lectins repel each other so as to prevent binding, where as, salts, by providing counter ions to the electrostatic charges, neutralize them and allow the binding of the lectins to the erythrocyte cell surface.

To provide further evidence for the electrostatic repulsion between lectins and erythrocytes, the surface of the latter was chemically modified in order to decrease the number of negative charges. For this purpose, the erythrocytes were treated with ethylenediamine in the presence of N, N'-dicyclohexylcarbodiimide to allow coupling of the diamine to the carboxyl groups present on the cell surface and thereby obtaining a partial substitution of these negative charges with the positive charges originating from the substituting amino groups. This chemical modification allowed the tested lectins to agglutinate erythrocytes at a significantly lower NaCl concentration than that needed for the haemagglutination of the unmodified erythrocytes.

The dependence of ions for lectin binding was also reported by Teichberg *et al.*, (1988), in *Bandireae simplicifolia, Dolichos biflorus* and *Ricinus communis* lectins.

The impairment of growth in experimental animals such as rats, mice etc. by lectins has been known for a long time (Honavar *et al.*, 1982). Lectins have been shown to bind with the intestinal membrane glycoproteins and alter the membrane integrity, transport of ions across the membrane and membrane enzymes (Boldt and Banwell, 1985). The results showed that ENL is found to be toxic to mice. The body weight of the test animals was reduced. At the intestinal level, deodenal and jejunal villi were shortened in ENL ingested mice. Similar

observation has been made with other lectins like Con A (Lorenzsonn and Olsen, 1982), SBA (Jindal *et al.*, 1984) and *phaseolus vulgaris* (Rouanet *et al.*, 1985).

A decrease in the body weight and the alteration of the villi structures in ENL ingested mice may be due to a significant decrease in the activities of several nutritional enzymes like sucrase, maltase, alkaline phosphatase, γ -glutamyl tranferase etc. Some of these enzymes are predominantly localized in the brush border membrane of the intestinal epithelial cells. The involvement of these enzymes and their activities had been determined in rats by Higuchi *et al.*, (1984). The decrease in the villus height, number and the abnormality in the microvilli structure by ENL suggests the firm binding of the lectin to the epithelial cells, which may cause an increased cell loss from the villi.

The saccharide determinants present on cell surface glycoconjugates serve as important binding sites for specific lectins, exerting modulatory influences within the immune system Gabius (1987). The results of Jerne's plaque assay revealed a significant increase in immune response in ENL treated mice. Thus the above result provide some evidence for immunomodulatory potency of ENL.

Many lectins have been reported to possess mitogenic activity. PHA, the lectin from red kidney bean *Phaseolus vulgaris* was the first described mitogenic lectin (Nowell, 1960). Con A is also a well known mitogen for murine and human T-lymphocytes (Weckster *et al.*, 1968). It was observed that ENL could also be used as a mitogen. The mitogenic mechanism of ENL was not clear but it would appear to be a direct action on lymphocytes. Possibly it alters the cell membrane to permit entry of some factors from the culture medium, which in turn initiates mitotic process.

Pollen germination involves the conversion of the quiescent vegetative cells into a metabolicaly active and rapidly growing pollen tube. The results

presented here shows that the ENL stimulate pollen germination of *Coleus species* by reducing the length of lag period before the pollen tube germination.

The stimulation of pollen germination by lectin, which binds to different cells like RBC, lymphocytes and spermatozoa suggests that primary control of the germination process may occur at the plasma membrane. Pollen germination is affected by many sugars and ion especially Ca^{++}, which are taken up from the external medium (Nicolson *et al.*, 1974). So, an alternation of the permeability properties of the plasma membrane by bound lectin could lead to a greater uptake of water or nutrients for germination. The stimulation of pollen germination by Con A and PHA is manifested by reduction in the length of the lag period, which is similar to the stimulatory effect of the stigmatic exudate on pollen germination (Loewus, 1973).

CHAPTER 4

Callus Culture of *E. neriifolia*

4.1. INTRODUCTION

Lectins are widely distributed throughout the plant kingdom, which is seen in a variety of tissues like seeds, roots, leaves and saps (Etzler, 1985). Del Campillo *et al.*, (1981) isolated lectin from plant tissue cultures, where they have been found on the cell surface, in the walls and in the cytoplasm of callus cells (James *et al.*, 1985). At present, more information is available on the biochemical and molecular properties and the distribution of lectins in plants. A little knowledge is available about the function and the factors which regulate the lectin production. So the callus culture study of *E neriifolia*, aims to elucidate the possible mode of regulation of the biosynthesis of lectin and also to produce lectin by callus culture technique.

4.2 MATERIALS AND METHODS

Murashige and Skoog (MS), medium was used as the basal medium. The callus cultures were maintained in agar medium. The MS medium was supplemented with different plant growth hormones such as indole acetic acid (IAA), 2 mg/l or 2,4-dichlorophenoxy acetic acid (2,4-D), 2 mg/l.

Leaves and shoot apex were washed thoroughly with tap water and with 5% v/v extran. Then the specimens were washed thrice with water. The leaves were then cut to 4-5 cm in length and were washed with double distilled water to remove the detergents completely. The leaves and shoot apex was treated with 0.1% mercuric chloride for 7 minutes. The specimens were washed with sterile double distilled water thrice and cut to 1 cm pieces and inoculated to MS medium, and incubated at 25 $\pm$ 2 °C. The subcultures were done from the primary callus.

4.2.1 Tissue extraction and lectin assay

2 g of callus was extracted in 10 ml of PBS and lectin titre was estimated as described in section 3.2.6. The lectin content was determined in three consecutive subcultures.

4.3 RESULTS

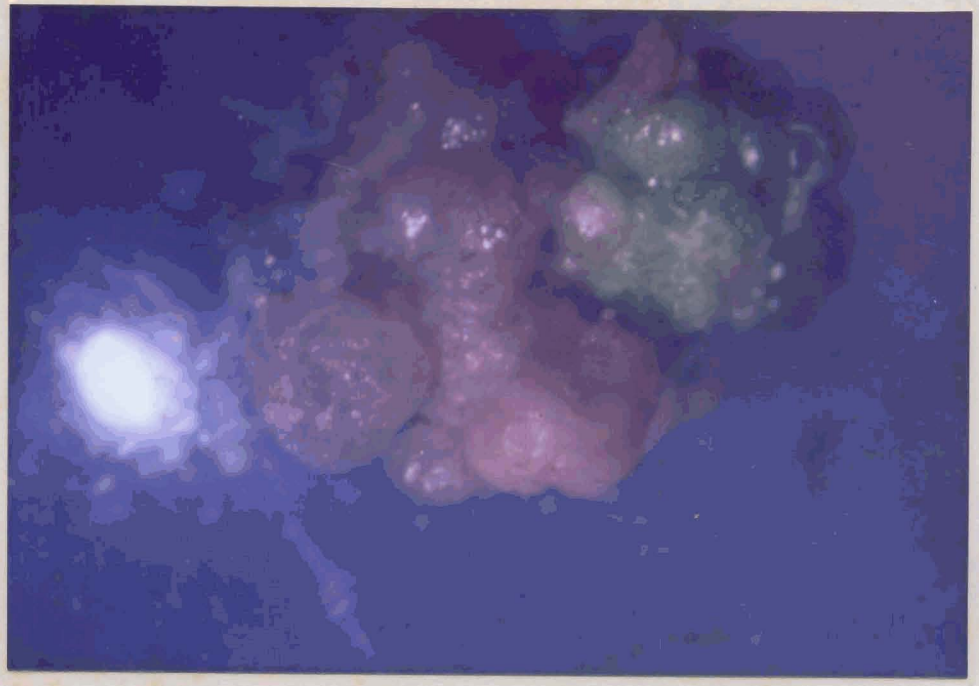
The callus was developed both form the shoot apex and leaves. The initiation of callus was found to be better in MS media fortified with 2,4-D (2 mg/l). Therefore, this medium was used for subsequent cultures. The callus started developing between 1-2 weeks. After 4-5 weeks, the callus was subcultured. The callus developed is shown in plate 4.1.

The haemagglutination titre of the callus extract was 1:32, which was far lower than that of leaf extracts (1:256). No lectin was detected in the medium. The callus was then transferred to fresh medium. After four weeks of incubation, sufficient quantity of callus was produced. But no lectin was detected in the subcultured callus. The successive subcultures gave the same results.

4.4 DISCUSSION

The regulation and synthesis of plant lectins can be demonstrated by tissue culture technique. There have been a few reports in the literature of efforts to produce plant lectins by tissue culture technique. In 1981, Del Campillo *et al.*, reported that soybean callus cultures contained SBA like material on the surface of the callus. *Psophocarpus tetragonolobus* callus cultures also found to contain lectin (Meimeth *et al.*, 1982). The localization of lectins in callus cultures was done by radioimmunoassay, which enables subcellular localization of lectin. This was introduced by James *et al.*, (1985).

A)



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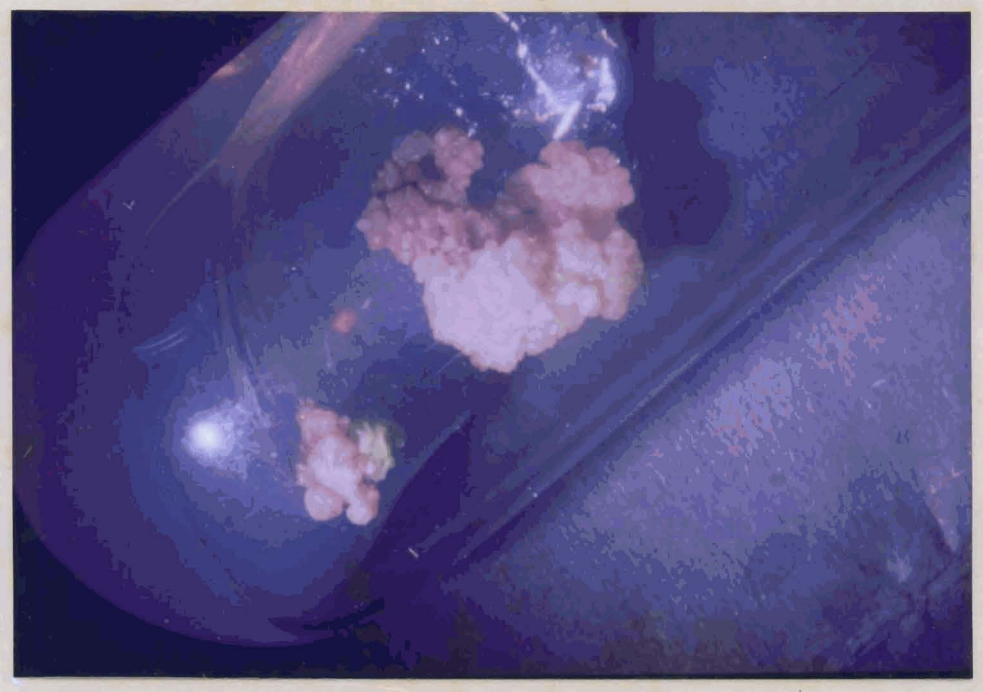


Plate-4.1

A) Callus developed from leaf of *E.neriifolia.*

B) Callus developed from shoot apex of *E.neriifolia.*

The primary callus cultures of *E. neriifolia* showed the presence of lectin, but the titre was low. The initiation of callus was observed in MS medium supplemented with 2,4-D. Subsequent subcultures of the callus failed to show any lectins, which was demonstrated by nonagglutination of callus extracts. In 1967, Jones *et al.*, detected no lectin in tissue derived from *Maclura pomifera*. Same results were also reported in potato callus cultures by Owens and Northcole (1981). MS medium supplemented with different hormonal combinations also failed to produce any lectin in subcultured callus. Borrebaeck and Linsefors (1985) and Raikhel *et al.*, (1986) have observed that the production of lectin in callus culture was hormone dependent. The callus derived from immature wheat embryo, grown in the presence of 10 μM of absisic acid exhibited increased levels of lectin content compared to callus grown on other media combinations.

The subcultured callus did not show any lectin activity. This failure may therefore indicate that the callus did not contain the appropriate enzymes or they are at lower concentration to produce lectins. The primary callus showed the presence of lectin, since the callus cells utilized the limited supply of enzymes from the explant for the production of lectin.

In order to study whether the lectin production was due to the lack of enzymes, we cultured the callus devoid of lectin content on MS media, supplemented with the leaf extract of the same plant at various concentrations i.e., 250 μl/l, 500 μl/l 750 μl/l and 1000 μl/l, which showed that all these combinations failed to produce any callus. This suggests that the leaf extract contained some active principle which inhibit callus growth.

CHAPTER 5

Lectin Histochemistry

5.1. INTRODUCTION

Carbohydrate chain of glycoconjugates are the sugar constituents of cellular membranes, which probably confer physiological information relevant to cell interaction, growth regulation and cell differentiation. Detection of cell differentiation and related changes in the expression of the sugar component of glycoconjugates has already found common application in pathology (Caselitz 1987; Damjanov 1987). Neoplastic transformation is associated with important changes in the epithelial glycosubstances. Biochemical, histochemical and immunohistochemical studies demonstrate the differences between the glycoconjugates of the normal mucosa and those of carcinomas (Gold and Schochat 1977). The cellular membrane carbohydrates play an important role in functions related to the neoplastic transformation of cells as do cellular antigenicity, contact regulation of cell growth and survival and metastatizing ability of the neoplastic cells (Hakomori, 1981). Plant lectins have proven to be valuable for defining changes in the structure and composition of cellular glycoconjugates upon neoplastic changes (Alroy *et al.*, 1984).

Breast carcinoma is the common malignant tumor (23.4% of total cancer cases) and the leading causse of cancer death in women. The principal risk factors are menstrual and reproductive history and family history. Women who have their first child before the age of eighteen have only one third the risk compared with those whose first child is delayed until age thirty.

The two key determination to make in the morphologic study of breast carcinoma are a) whether the tumor is confined to the glandular component of the organ or whether it has invaded the stroma and b) whether it is of duct or tubular type (Rosai, 1990). The first criterion, whose prognostic significance far out weighs that of the second. The term ductal carcinoma may be taken to imply that the tumor is arising from a duct or at least invading duct.

Gastric adenocarcinoma is of major importance world wide as a case of death from malignant disease. In Kerala, stomach cancer comes to about 5% of the total cancer cases reported (Gangadharan *et al.*, 1992). Men are often infected by this disease than women. Microscopic examination of gastric carcinoma shows marked variation in their structures. WHO, classification divides adenocarcinoma of the stomach into pappilary, tubular mucinous and signet ring cell types. The typing of any particular tumor is based on its predominant component. Signet ring cell carcinoma are composed of mainly isolated tumor cells with large amounts of intracellular mucin and often associated with considerable fibrosis. Gastric carcinoma are highly infiltrative tumors and in ressection specimen, majority have extended into suberosa.

In Kerala, of the cancer patients, 1.5% have colon cancer (Gangadharan *et al.*, 1992). Adenocarcinoma is the commonest malignant tumor of colon and rectum. Other much less frequently encountered neoplasms include undifferentiated adenosquamous, squamous and endocrine cell carcinomas. There are marked variations in the incidence of colorectal cancer throughout the world. There is a good correlation between the consumption of meat and the incidence of colorectal cancer in various populations. Colorectal cancer becomes more frequent with increase of age. About 85% of adenocarcinoma of the colorectum are composed of relatively well differentiated tubules secreting small quantities of mucin.

In this chapter we discuss the diagnostically favourable feasibility of detecting the presence and difference in expression of endogenous receptors for cellular glycoconjugates using newly isolated ENL. ENL after conjugating with horse radish peroxidase (HRP) and a panel of other HRP conjugated lectins such as HPA and PNA, are tested against routinely processed tissue section of gastric cancer, colon cancer and breast cancer.

5.2. MATERIALS AND METHODS

5.2.1. Specimen collection

Surgical specimens of normal and cancerous tissues were collected from Aswini Diagnostic Centre, Calicut and Medical College, Calicut during the year 1997-2000. These included normal and malignant lesion of breast, colon and stomach. The cases included 16 breast, 20 stomach and 18 colon. Of the 16 breast cases, 7 cases were normal and 9 cases were infiltrating duct carcinoma breast. Of the 20 stomach cases 6 were normal, 6 were mucin secreting adenocarcinomas and 8 cases were signet ring cell carcinomas. In the case of colon, 8 cases were normal and 10 cases were adenocarcinoma colon. All the specimens were fixed routinely in 10% formalin and embedded in paraffin. Serial paraffin sections of 5-7 μ thick were prepared on slides and one was stained with haematoxylin-eosin stain and others were used for lectin binding studies. All the sections were examined by two experienced pathologists and only the cases with clear histological diagnosis were included for this study.

5.2.2. Processing of specimens

The preserved specimens in formalin were passed though the following series.

10% formalin	30 minutes
Acetone I	30 minutes
Acetone II	30 minutes
Acetone III	45 minutes
Chloroform + Acetone (1:1)	30 minutes
Chloroform I	30 minutes
Chloroform II	30 minutes
Chloroform III	15 minutes
Molten wax with ceresin	30 minutes at 56 °C

Molten wax with ceresin	incubated overnight at 85 °C
Molten wax with ceresin	30 minutes

a) Embedding

The tissues were transferred to a mould filled with paraffin wax. The wax was allowed to solidify slowly without trapping air bubbles. The L-blocks were removed and the block was then labelled.

b) Trimming

The wax block was attached to a wooden block. The block was trimmed to leave 2-3 mm of wax all over the tissue.

c) Section cutting

The block was fixed to the block holder of the microtome. Sections were cut at about 5-7 μ thickness. The sections were gently laid on a water bath kept at 45 °C.

d) Egg albumin glue

Equal parts of egg white and glycerine was mixed well and filtered through a coarse filter paper. A pinch of thymol was added as a preservative.

The sections were taken on to the slides applied with glue and left in an incubator at 50 °C for 1 h.

5.2.3. Haematoxylin – eosin staining

1) The incubated section was cooled and placed in xylene twice for 15-30 minutes to remove wax.

2) Hydrated through alcohol series.

Absolute alcohol	8-10 dips.
90% alcohol	5-10 dips.

70% alcohol 3 minutes.

50% alcohol 3 minutes.

3) Washed in running tap water for 5 minutes.

4) Stained with haematoxylin for 3-5 minutes.

5) Washed in water for bluening.

6) Stained with 1% aqueous eosin stain for 1 minute.

7) Dehydrated through alcohol series.

50% alcohol	8-10 dips.
70% alcohol	8-10 dips.
90% alcohol	5-10 dips.
Absolute alcohol	2 minutes (2 changes).

8) Cleared using xylene 15 minutes (2 changes)

9) Mounted under a cover slip using DPX

5.2.4. Lectins used in tissue binding studies

We have studied the binding pattern of ENL in normal and cancerous tissues by conjugating the lectin with HRP. Along with this, HRP conjugated Con A and Helix pomatia lectin was also included in this study.

Details of lectins used in histochemistry.

Lectin	Source	Inhibitory sugar
Euphorbia neriifolia lectin	*Euphorbia neriifolia*	GalNAc
Concanavalin A	*Canavalia ensiformis*	Methyl-α-D-mannopyranoside
Helix pomatia agglutinin	*Helix pomatia*	GalNAc

5.2.5. Preparation of lectin peroxidase conjugate

The conjugation of lectins with HRP was done in three steps *viz.*, activation of HRP, conjugation and purification of the conjugate. All the procedures were done at room temperature.

I Activation of HRP

1) Dissolved 10 mg of Sigma type VI HRP in 2 ml of 0.3 M bicarbonate solution.
2) Added 20 µl of 1% FDNB in ethyl alcohol. Mixed gently for 1 h.
3) Added 1 ml of 0.06 M Sodium periodate.
4) Added 2 drops ethylene glycol and mixed gently for 1 h.
5) Desalted the peroxidase on Sephadex G-25 column (0.5 X 15 cm), equilibrated with 0.2 M bicarbonate buffer. pH 9.5.

II Conjugation

1) Dissolved 10 mg of the lectin in 0.2 M bicarbonate buffer (pH 9.5) and added sufficient amount of specific sugar to give a 50 mM solution. Left for 1 h.
2) Added 10 mg of activated HRP. Mixed gently for 3 h.
3) Added 1 mg of sodium borohydride. Left for 1 h.
4) Reduce the pH to 6.9 using 1 N HCl. Left overnight.

III Purification

The conjugate was separated from free lectins and free peroxidase by gel chromatography on Sephadex G-200 (1.5 X 40 cm). 2 ml fractions were collected and the absorbance of the purified conjugate was measured at 280 nm and 403 nm in a UV-spectrophotometer.

5.2.6. Staining of tissue sections with lectin- HRP conjugates

1) The slides were incubated for 30 min at 56 °C for deparaffinization.
2) Left in xylene for 10 min. with two changes.

3) Left in absolute alcohol for 10 min with two changes.
4) Left in 0.3% H_2O_2 in methanol for 20 min to eliminate endogenous peroxidase activity.
5) Hydrated through alcohol series.
6) Washed in distilled water twice for 2 min.
7) Washed in distilled water twice for 1 min.
8) Washed in PBS thrice for 2 min.
9) The sections were incubated in HRP conjugated lectins for 30 min.
10) Washed in PBS thrice for 2 min.
11) Stained with Diamino benzidine dihydrochloride (DAB), (50 mg/ml in 0.1% H_2O_2), for 20 min.
12) Washed in distilled water thrice for 2 min.
13) Counter stained with Haematoxylin for 1-2 min.
14) Washed in tap water, dehydrated through alcohol series and cleared in xylene for 10 min and mounted in DPX.

The observations were mainly done on the following features under a light microscope.

1) The number of cells which showed binding and evaluated as percentage.

2) The patterns of cellular binding such as: a) membrane binding. b) focal cytoplasmic binding. c) diffused cytoplasmic binding. d) uniform binding and e) irregular binding.

Intensity of staining was graded from + to +++ (- for no binding, + for weak binding, ++ for moderate binding and +++ for intense binding). The diagnostic accuracy of the lectin on tissue sections were compared with haematoxylin - eosin stained sections.

5.3. RESULTS

Slides were examined by two pathologists and the number of reactive cells were estimated subjectively. The cells which bound by the HRP lectin conjugate gave a brown colour at the site of binding when stained with DAB. For controls, the conjugates were blocked by the respective specific sugar, before the incubation of slides with the conjugate.

5.3.1. Binding pattern in breast

Of the 9 carcinoma cases studied, ENL, Con A and HPA showed staining in 7 cases (78%), 5 cases (56%) and 6 cases (67%), respectively. Whereas, normal tissues showed staining with ENL Con A and HPA in 4 cases (57%), 3 cases (43%) and 4 cases (57%), respectively (Table–5.1). In most cases the cytoplasmic staining was seen to be more intense than membrane binding. Stromal cells were stained by all the three lectins used.

The binding pattern of the ENL and HPA were almost similar. But the binding pattern of Con A was different from the other two. In carcinomatous cells, the intercellular space stained uniformly with HPA and ENL, but with Con A, the staining was irregular (Plate5.1and 5.2).

5.3.2 Binding pattern in stomach

Of the 6 cases of mucin secreting adenocarcinoma stomach studied, ENL, Con A and HPA showed staining in 4 (67%), 2 (33%) and 5 (83%) cases respectively. In signet ring cell carcinoma, of the 8 cases 5 (62%), 4 (50%) and 6 (75%) cases showed staining with ENL, Con A and HPA respectively (Table - 5.3). Where as in normal cases both ENL and Con A showed staining in 3 (50%) cases and HPA showed staining on 2 (33%) cases. Cytoplasmic staining of cells

Table 5.1. Lectin staining in normal and infiltrating duct carcinoma breast.

Specimen	ENL		Con A		HPA	
	No binding	Binding	No binding	Binding	No binding	Binding
Normal breast (n=7)	3 (43 %)	4 (57 %)	4 (57 %)	3 (43 %)	3 (43 %)	4 (57 %)
Infiltrating duct carcinoma stomach (n=9)	2 (22 %)	7 (78 %)	4 (44 %)	5 (56 %)	3 (33 %)	6 (67 %)

Values in parenthesis shows the number of cases studied.

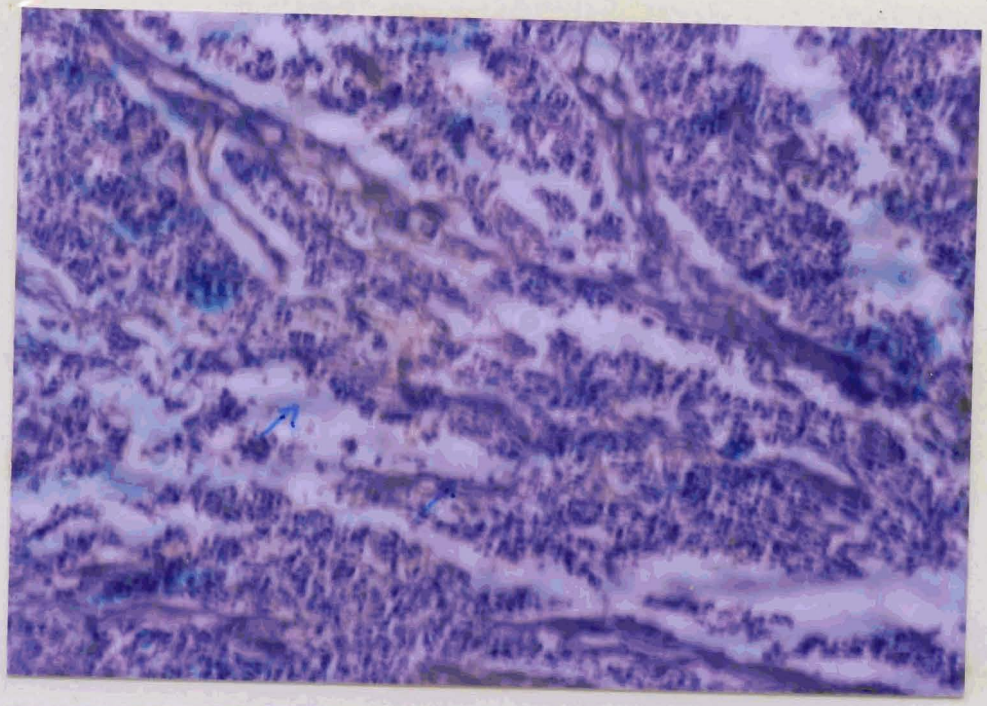
Table 5.2. Binding pattern of ENL, Con A and HPA in normal and infiltrating duct carcinoma breast.

Binding site	Normal (n=7)			Infiltrating duct carcinoma (n=9)		
	ENL	Con A	HPA	ENL	Con A	HPA
Cytoplasm	Uniform +2/7 -5/7	Uniform ++1/7 +1/7 -5/7	Uniform +3/7 -4/7	Diffused ++3/9 +4/9 -2/9	Uniform +2/9 -7/9	Uniform ++2/9 +4/9 -3/9
Intercellular space	Uniform +3/7 -4/7	Uniform +3/7 -4/7	Uniform +3/7 -4/7	Uniform +5/9 -4/9	Irregular +3/9 -6/9	Uniform ++3/9 +2/9 -4/9
Cells at basement membrane	Uniform +3/7 -4/7	Irregular +3/7 -4/7	Diffused +2/7 -5/7	Diffused +6/9 -3/9	Irregular +3/9 -6/9	Diffused +5/9 -4/9

\- no binding + weak binding ++ moderate binding

Values in parenthesis shows the number of cases studied.

A)



B)

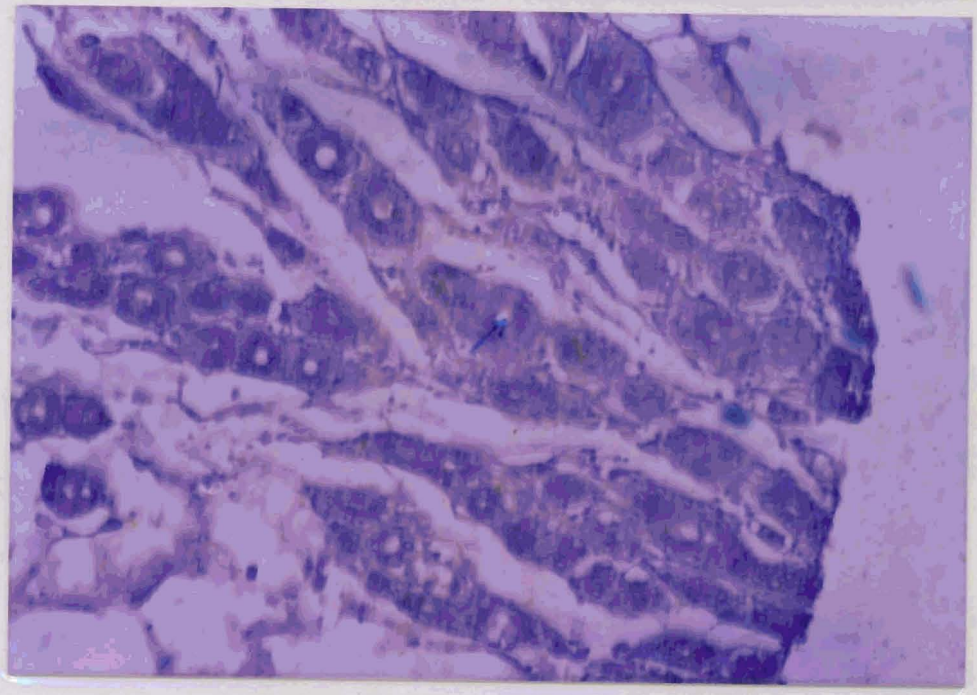


Plate-5.1

A) Infiltrating duct carcinoma breast stained with ENL. Arrows indicate the connective tissue were moderately stained with the lectin and the mucin pools not stained with the lectin (X 200).

B) Normal breast showing HPA in acina of ductules. Arrow indicates the site of lectin binding (X 200).

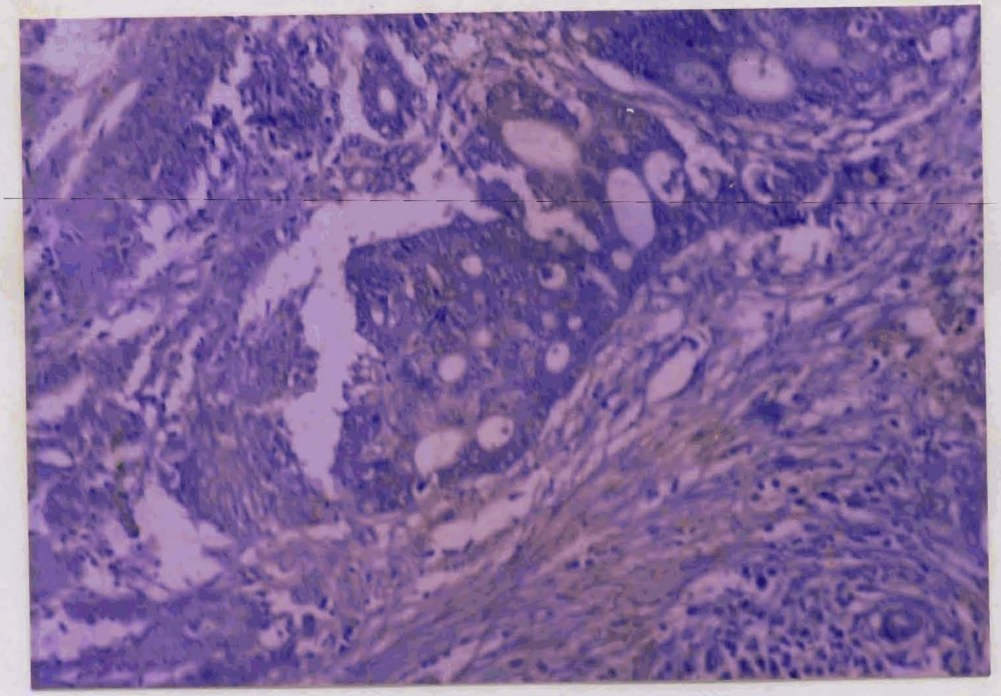


Plate-5.2

Infiltrating duct carcinoma breast stained with Con A. Arrow indicates diffused binding of the lectin (X 200).

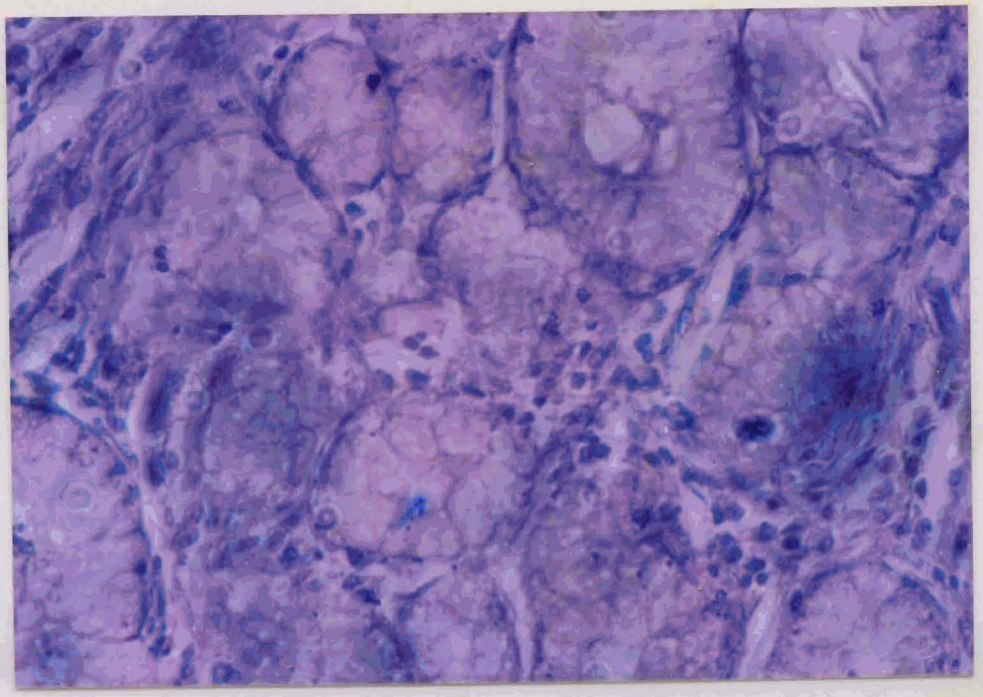
by all the three lectins in all cases (except in mucin secreting adenocarcinoma with Con A), was uniform in normal as well as in carcinoma specimens (Table 5.4 and Plate-5.3). In most cases the basement membrane was irregularly or diffusely stained and the intensity of staining was also low. In normal tissue, positive staining with ENL and HPA was recognized in goblet cells, mucus neck cells and pyloric glands. The mucin was intensely stained by ENL, in mucin secreting adenocarcinoma cases (Plate-5.3A, 5.4 and 5.5A).

The staining pattern of ENL showed that it has no specificity for gastric cancer tissue. However, a little higher positive rate (67%) was noticed in mucin secreting adenocarcinoma than in signet ring cell carcinoma stomach (62%). But in normal, only 50% of cases showed positivity. HPA also showed almost similar staining pattern (Plate-5.6). But in Con A 50% staining was observed both in normal and signet ring cell carcinoma cases. In mucin secreting adenocarcinoma stomach, Con A staining was observed only in 2 (33%) cases. In normal, Con A showed intense staining in stomach lining (Plate-5.3B).

5.3.3. Binding pattern in colon

The reactivity of each lectin in normal and adenocarcinoma colon were summerized in table 5.5. It showed that the binding percentage of ENL and HPA decreased considerably in adenocarcinoma colon than in normal colon (Plate-5.7 and 5.8). In the 8 normal cases, 7 cases (87%) and 6 cases (75%) showed staining by ENL and HPA respectively. The percentage of staining by ENL and Con A in normal cases were same. But in carcinoma cases the percentage of ENL and HPA binding was reduced to 20% and 30% respectively, while that of Con A remained almost same i.e., 80%. All the three lectins stained goblet cell and weak positivity was seen in the glycocalyx area of columnar cells (Plate-5.7). Con A stained goblet cell and glycocalyx area irregularly. Occasionally, goblet cell with completely unstained mucin were intermingled with nongoblet cells showing cytoplasmic staining was also noticed in normal cases (5.7).

A)



B)

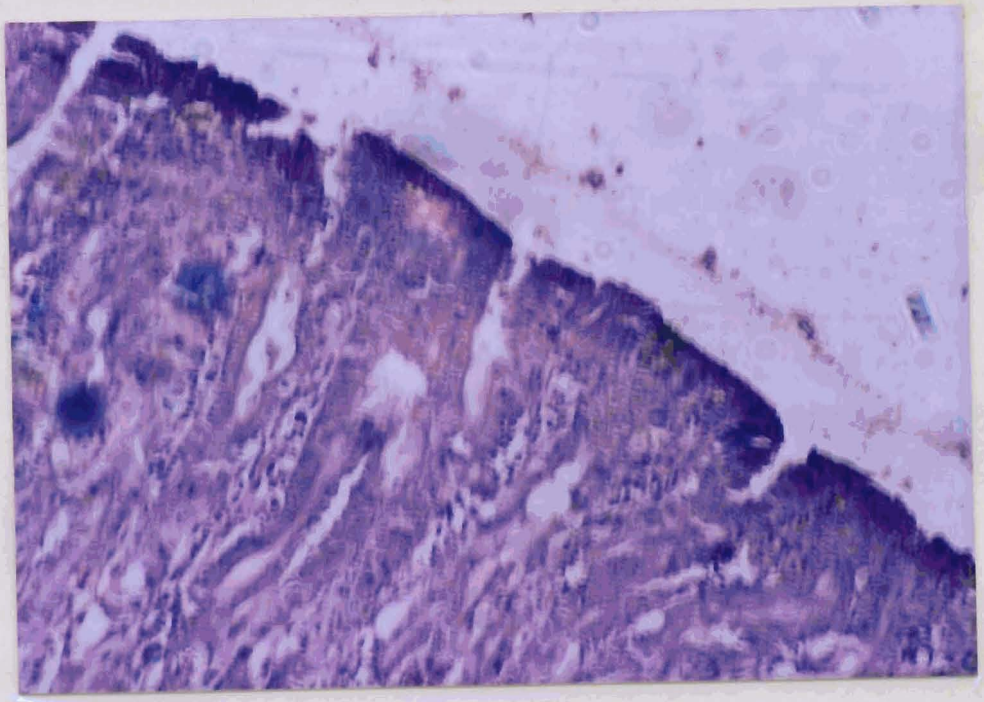
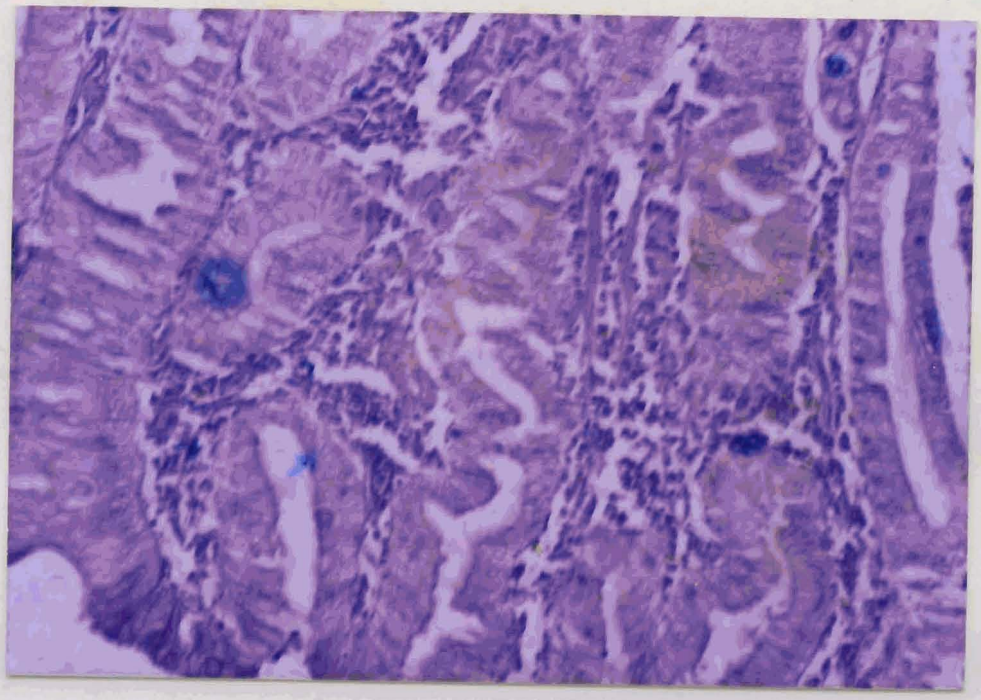


Plate-5.3

A) Normal stomach stained with ENL. Arrows indicate goblet cells and intercellular areas are weakly stained (X 200).

B) Normal stomach stained with Con A. Gastric lining show intense staining (X 200).

A)



B)

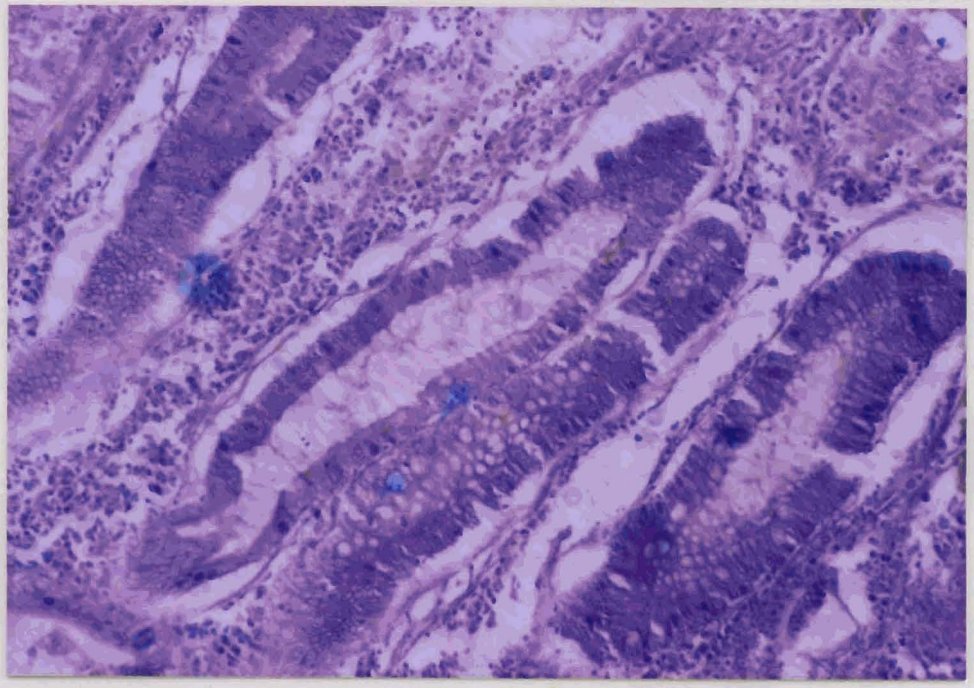
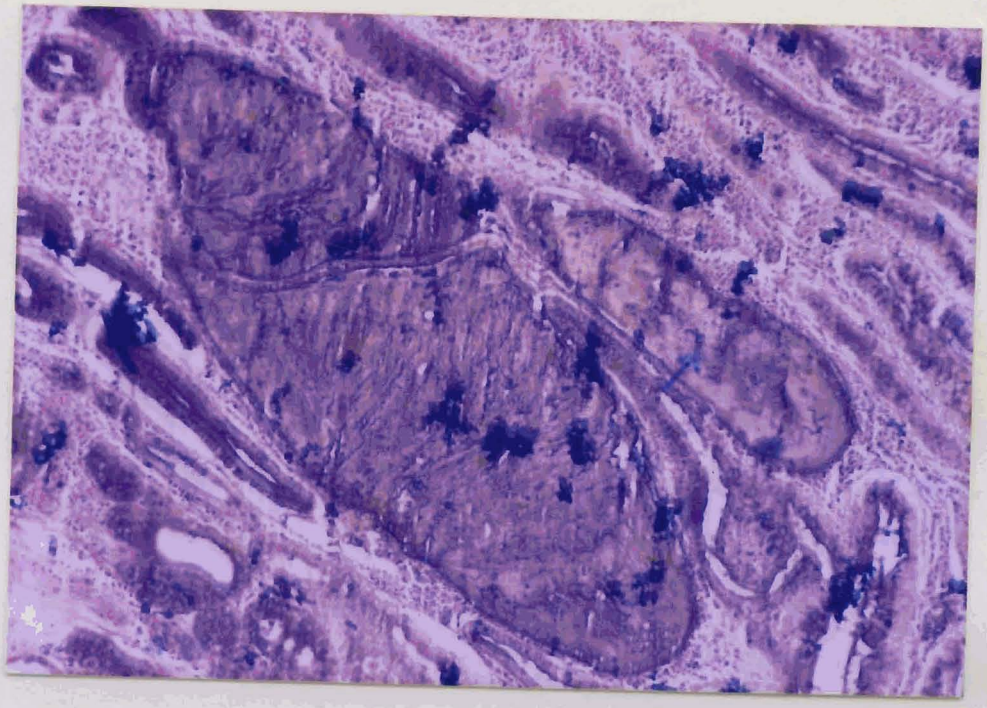


Plate-5.4

A) Normal stomach stained with ENL. Arrow indicates moderate binding in glandular cells (X 200).

B) Normal stomach (showing metaplasia) stained with ENL. Arrow indicates intense binding of lectin on goblet cells. Cells at basement membrane are less/not stained with the lectin (X 200).

A)



B)

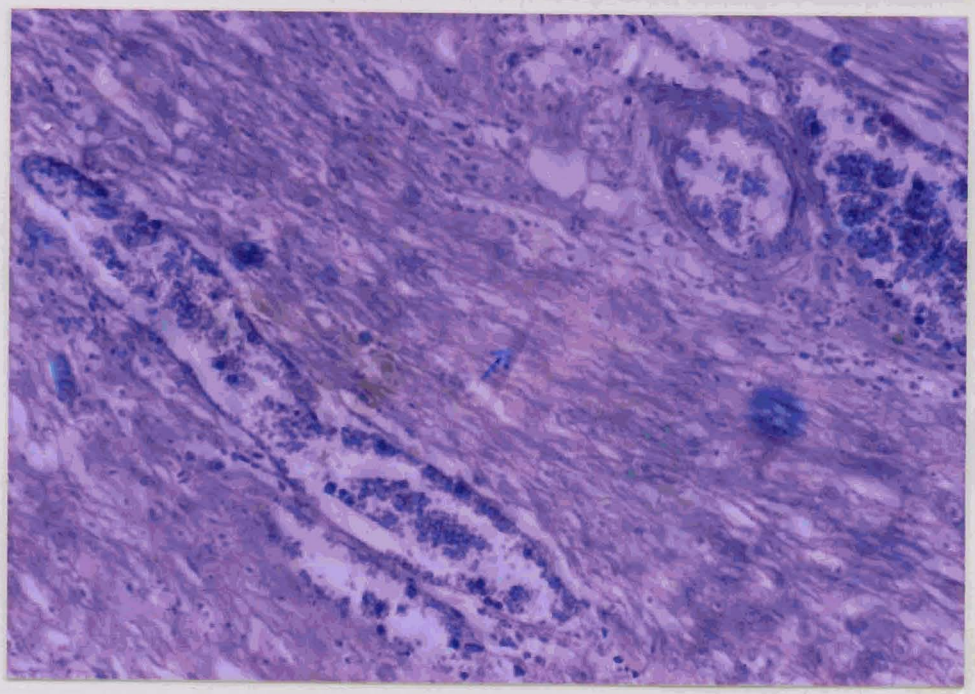
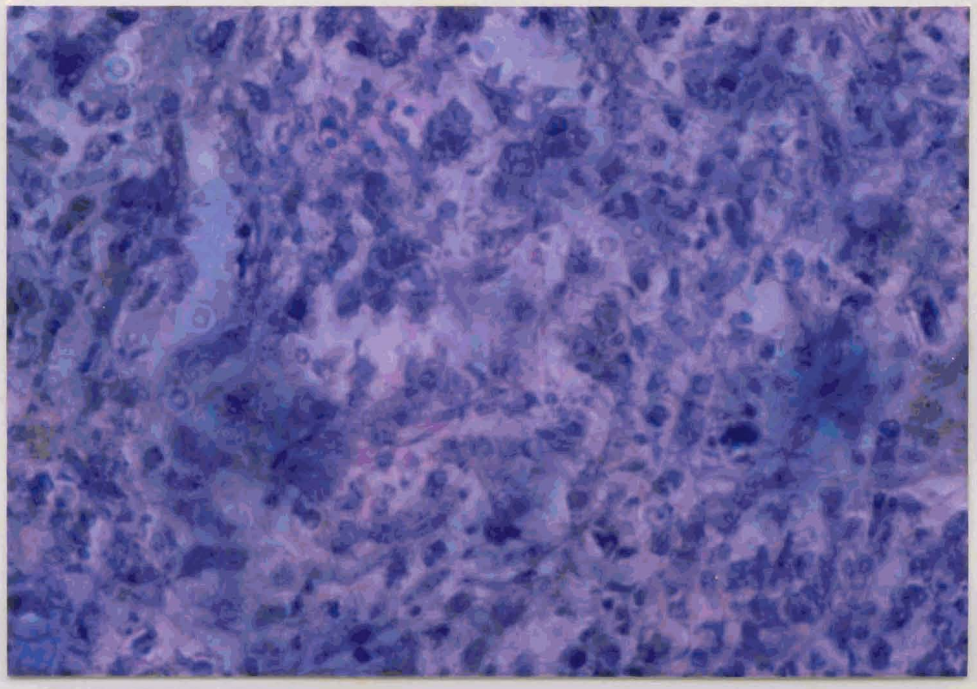


Plate-5.5

A) Mucin secreting adenocarcinoma stomach stained with ENL. Arrow indicates intense staining of mucin with the lectin (X 200).

B) Ulcerated, diffusely infiltrating signet ring cell carcinoma stomach stained with ENL. Arrow indicates moderate staining of connective tissue. Tumor cells were irregularly stained with the lectin (X 200).

A)



B)

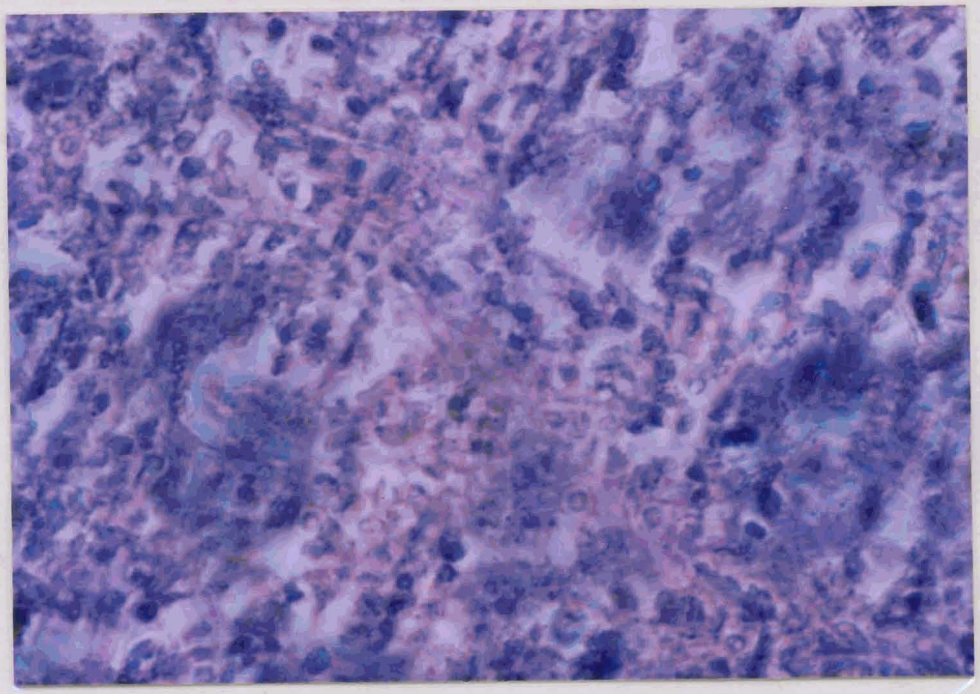


Plate-5.6

A) Signet ring cell carcinoma stomach stained with ENL. Signet ring cell shows diffused cytoplasmic staining with the lectin (X 200).

B) Signet ring cell carcinoma stomach stained with HPA. Signet ring cell shows diffused cytoplasmic staining with the lectin (X 200).

Table 5.3. Lectin staining in normal, mucin secreting adenocarcinoma and signet ring cell carcinoma stomach.

Specimen	ENL		Con A		HPA	
	No binding	Binding	No binding	Binding	No binding	Binding
Normal stomach (n=6)	3 (50 %)	3 (50 %)	3 (50 %)	3 (50 %)	4 (67 %)	2(33 %)
Mucin secreting adenocarcinoma (n=6)	2 (33 %)	4 (67 %)	4 (67 %)	2 (33 %)	1 (17 %)	5 (83 %)
Signet ring cell carcinoma (n=8)	3(38 %)	5 (62 %)	4(50 %)	4 (50 %)	2 (25 %)	6(75 %)

Values in parenthesis shows the total number of cases studied

Table 5.4. Binding pattern of ENL, Con A and HPA in normal, mucin secreting adenocarcinoma and signet ring cell carcinoma stomach.

Binding site	Normal (n=6)			Mucin secreting adenocarcinoma (n=6)			Signet ring cell carcinoma (n=8)		
	ENL	Con A	HPA	ENL	Con A	HPA	ENL	Con A	HPA
Cytoplasm	Uniform ++1/6 +2/6 -3/6	Uniform +++3/6 -3/6	Uniform ++2/6 +1/6 -4/6	Uniform +++1/6 ++3/6 -2/6	Diffuse +2/6 -4/6	Uniform +++1/6 ++4/6 -1/6	Uniform +++1/8 ++1/8 +3/8 -3/8	Uniform ++1/8 +3/8 -4/8	Uniform +++3/8 +2/8 -3/8
Intercellular space	Uniform ++2/6 -4/6	Uniform +++1/6 +2/6 -3/6	Uniform +2/6 -4/6	Diffuse ++2/6 +2/6 -2/6	Uniform +2/6 -4/6	Uniform +3/6 -3/6	Diffuse ++3/8 +2/8 -3/8	Diffuse +4/8 -4/8	Uniform +++1/8 ++3/8 +2/8 -2/8
Cells at basement membrane	Diffuse +2/6 -4/6	Irregular +1/6 -5/6	Irregular +1/6 -5/6	Uniform +2/6 -4/6	Diffuse +1/6 -5/6	Uniform ++2/6 +1/6 -3/6	Uniform +4/8 -4/8	Irregular +3/8 -5/8	Diffuse +4/8 -4/8

- no binding ++ moderate binding + weak binding +++ intense binding

values in parenthesis show the total number of cases studied.

Table 5.5. Lectin staining in normal and adenocarcinoma colon.

Specimen	ENL		Con A		HPA	
	No binding	Binding	No binding	Binding	No binding	Binding
Normal colon (n=8)	1(13 %)	7(87 %)	1(13 %)	7(87 %)	2(25 %)	6(75 %)
Adenocarcinoma colon (n=10)	8(80 %)	2(20 %)	2(20 %)	8(80 %)	7(70 %)	3(30 %)

Values in parenthesis shows the number of total cases studied

Table 5.6. Binding pattern of ENL, Con A and HPA in normal and adenocarcinoma colon.

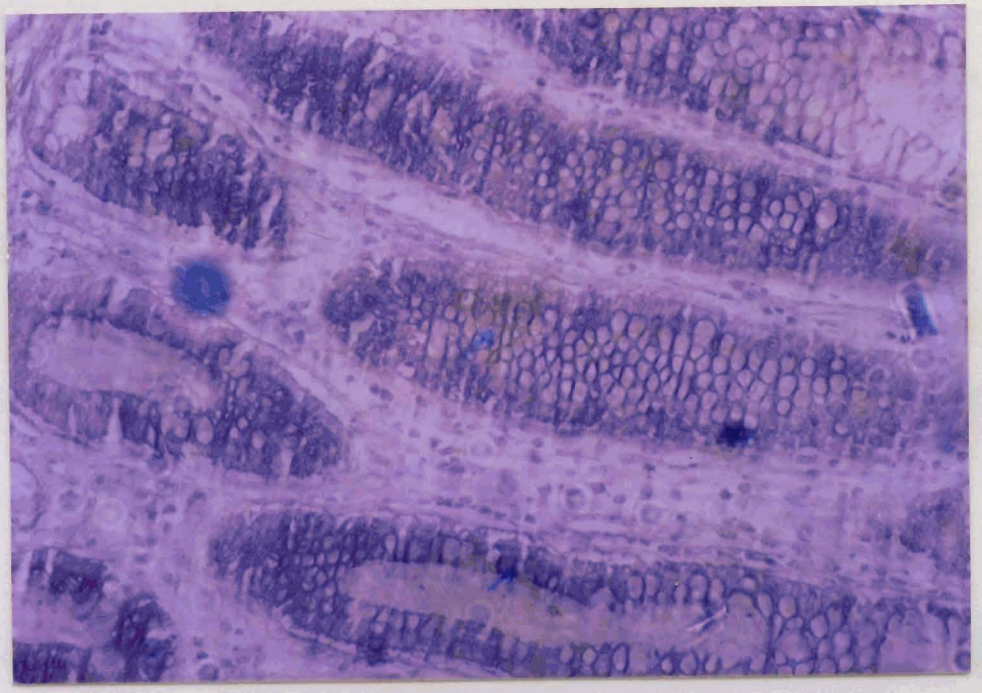
Binding site	Normal (n=8)			Adenocarcinoma (n=10)		
	ENL	Con A	HPA	ENL	Con A	HPA
Cytoplasm	Uniform +++2/8 +3/8 -3/8	Uniform ++5/8 +2/8 -1/8	Uniform +++3/8 +3/8 -2/8	Diffuse ++2/10 -8/10	Irregular +++2/10 ++5/10 -3/10	Diffuse ++2/10 +1/10 -7/10
Intercellular Space	Uniform ++3/8 +3/8 -2/8	Uniform ++5/8 +2/8 -1/8	Uniform +++1/8 ++3/8 +1/8 -3/8	Uniform ++1/10 +1/10 -8/10	Uniform +++2/10 ++1/10 +3/10 -4/10	Uniform ++1/10 +2/10 -7/10
Cells at basememt membrane	Uniform +6/8 -2/8	Uniform ++1/8 +5/8 -2/8	Diffuse +4/8 -4/8	Irregular +1/10 -9/10	Uniform ++4/10 +2/10 -4/10	Diffuse +3/10 -7/10

- no binding ++ moderate binding

\+ weak binding +++ intense binding

Values in parenthesis shows the number of total cases studied.

A)



B)

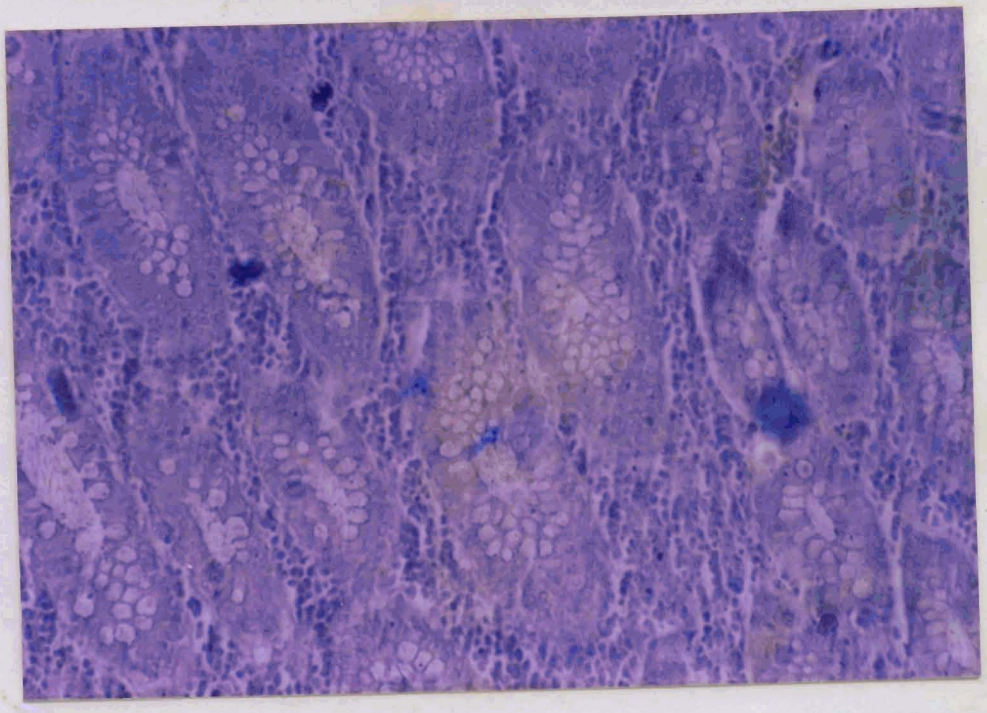
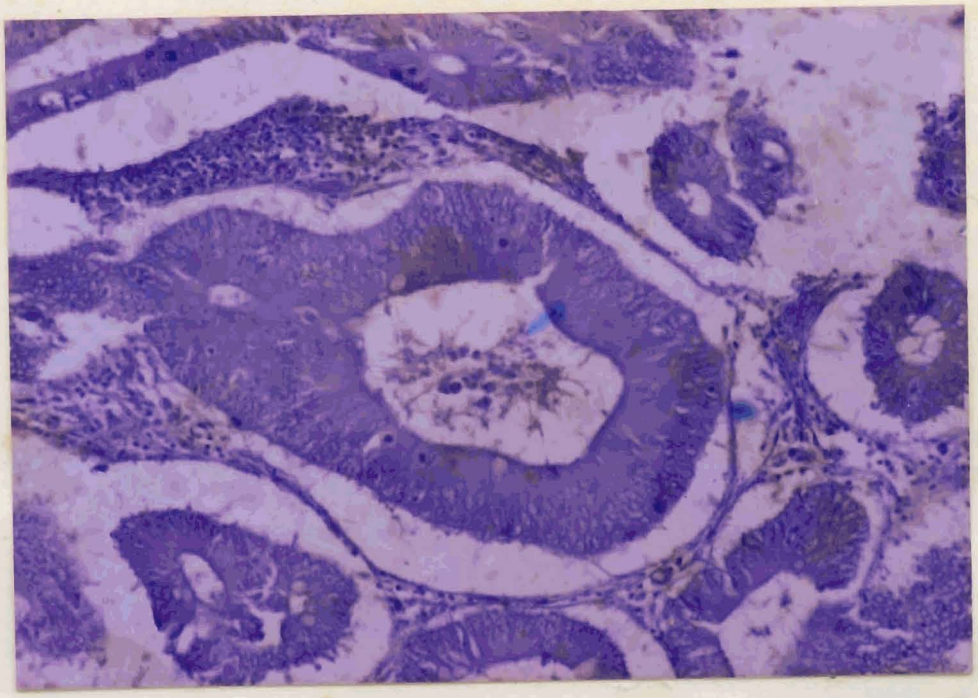


Plate-5.7

A) Normal colon stained with ENL. Arrows indicate intense staining of goblet cells and moderate staining of mucin with the lectin (X 200).

B) Normal colon stained with HPA. Arrows indicate moderate staining of goblet cells with the lectin. Cells at basement membrane were diffusly stained by the lectin (X 200).

A)



B)

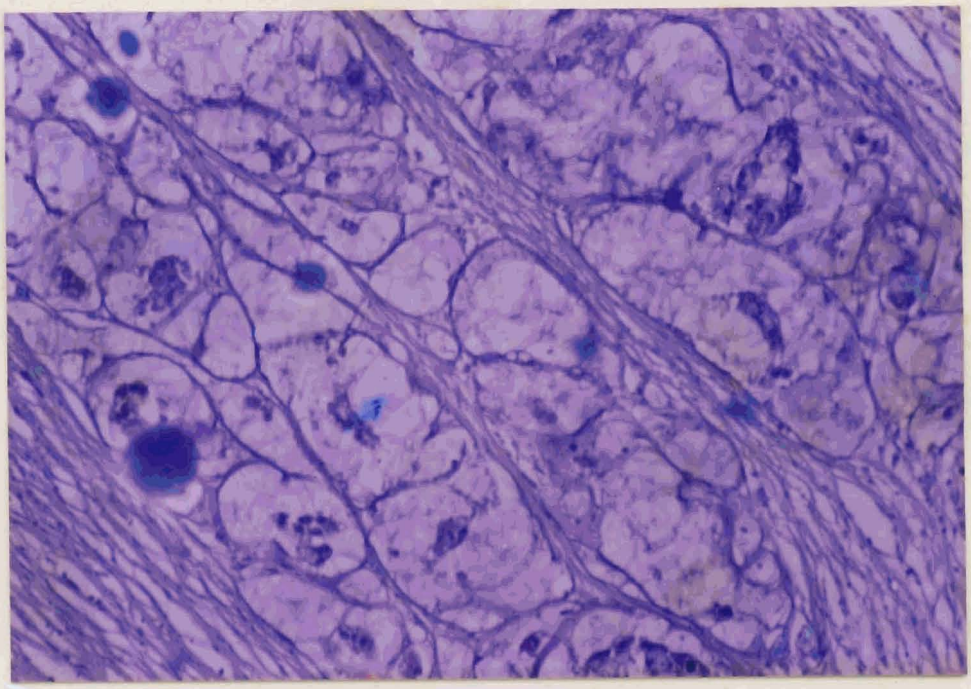


Plate-5.8

A) Adenocarcinoma colon stained with ENL. Arrow indicates only the luminal cells were stained with the lectin. Columnar cells were not stained with the lectin (X 200).

B) Adenocarcinoma colon stained with ENL. Arrow indicates mucins were not stained by the lectin (X 200).

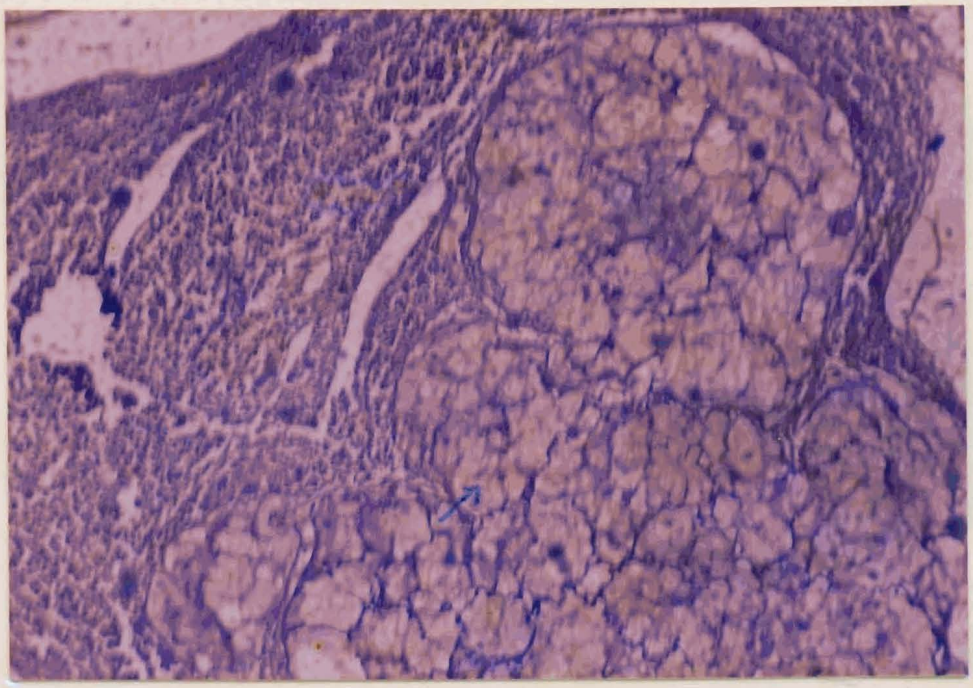


Plate-5.9

Adenocarcinoma colon stained with Con A. Arrow indicates weak binding of the lectin with mucin (X 200).

In normal cases ENL, Con A and HPA showed uniform cytoplasmic staining (some goblet cells did not show cytoplasmic staining with ENL and HPA) but in carcinoma cases, irregular and diffuse staining was noticed (Plate-5.8A and 5.9). Intercellular space was uniformly stained by all the three lectins, in both normal and adenocarcinoma colon. The basement membrane was uniformly stained by Con A in adenocarcinoma cases whereas, in ENL and HPA, it was diffusely stained (Table-5.6). Controls of all the three lectins – HRP conjugates did not show binding with the tissues.

5.4. DISCUSSION

Reisner *et al.*, (1976) used the lectin binding technique to study carbohydrate moities on cells and it has been well documented that there are alterations to carbohydrate and residues in glycoconjugates during cellular differentiation and malignant transformation. So, lectin peroxidase conjugate can be used as a useful tool to visualize the lectin receptors in routine pathological specimens (Davina *et al.*, 1985). We employed a panel of three HRP conjugated lectins *viz.*, ENL, Con A and HPA to detect the receptors for these lectins in breast, stomach and colon cancer specimens. The binding pattern of ENL was not studied earlier, whereas Con A and PHA was studied by many workers (Elias *et al.*, 1988; Yoshihiro *et al.*, 1991). These two lectins were included in this study to compare the binding pattern of these lectins with ENL.

In the present study, ENL biniding in normal tissue was evenly distributed along the intercellular regions and cytoplasm, Whereas, in malignant tissue a much more heterogenous staining pattern was observed. The difference in binding may be due to the alterations of the normal cells during the malignant transformations. Davina *et al.*, (1986) noted that the lectin binding method is based on the fact that the cells contain higher amounts of oligosaccharide residues on the surface and the number of cells with such binding sites differ significantly between normal and

cancerous tissues. A semiquantitative difference in the cytoplasmic intensity of the histochemical reaction could also be detected with these probes between different types of malignancies.

The binding of ENL in breast tissue was not specific to normal or cancerous tissues, but the cytoplasmic and basement membrane staining was uniform in normal cases but it was diffuse in cancerous breast. These histochemical finding are of limited diagnostic value. However, there was progressive increase in the percentage of ENL binding in tumor cases than in normal cases. Similar binding pattern was reported with DBA by Dansey *et al.*, (1988). A few studies have indicted that lectin binding can provide prognostic information on breast cancer (Fenlon *et al.*, 1987). For the last few years PNA has been under trial as a histochemical reagent to study malignant transformation. PNA reacts with Thomson-Friedenreich (TF), antigen (Stanley *et al.*, 1986) that was supposed to be present on human mammary tumors in a free state and to be absent or masked by sialic acid residues in benign for normal breast tissue (Howard and Taylor, 1979). But later studies have shown that PNA also reacts with benign lesions without neuraminidase treatment.

Tumorigenic transformation is almost invariably associated with altered glycosylation on surface glycoproteins (Smels and Van Beek 1984). These quantitative changes of cell surface glycoproteins are thought to be associated with altered cell adhesion and the development of invasive and metastatic properties (Steck and Nicolson, 1983; Altevogot *et al.*, 1984). Sialic acid on the cell surface protects the cell against proteolytic digestion, servers as receptors, attenuates immunogenicity by masking carbohydrates, plays a role in adhesion, tends to increase solubility and appers to correlate with metastatic potential (Yogeeswaran and Tao, 1980).

The results showed that ENL does not bind specifically either to normal or gastric carcinoma cells. Macartney (1986) also reported that the binding pattern of galactose and GalNAc specific lectins did not correlate with the stage or differentiation of gastric carcinoma. Both ENL and HPA gave the same result since both are specific for GalNAc. However, Yoshihiro *et al.*, (1991) reported that HPA positive tumors were characterized by tumor enlargement, penetration, infiltrative spread or lymphatic invasion and metastasis. These factors are significant prognostic elements in gastric cancer.

The percentage of ENL staining in normal and carcinoma stomach cases showed the staining percentage was high in both mucin secreting adenocarcinoma and signet ring cell carcinoma cases. Same was the case with HPA. Shue *et al.*, (1993) also reported that the percentage of VVA staining increased considerably in carcinoma stomach, which have the same sugar specificity as that of HPA and ENL (GalNAc).

The binding patten of ENL and HPA showed that cytoplasm and intercellular spaces were intensely stained in carcinoma stomach than in normal stomach. But in the case of Con A, no hike in binding intensity was observed. Mucin was intensely stained by both ENL and HPA but in the case of Con A diffuse staining was noticed. Raju and Lee (1988) also reported the same type of binding pattern with Con A and DBA. All the three lectins included in this study does not show much difference in binding pattern in mucin secreting adenocarcinoma and signet ring cell carcinoma stomach cases.

Lectin binding studies in adenocarcinoma colon showed that both ENL and HPA binding percentage is low in adenocarcinoma colon than in normal colon. Dall'Olio and Trer'e, (1993) reported that about 90% of human colon carcinomas express increased levels of sialyl transferase which adds sialic acids α-2-6 linkage to galactose/GalNAc residues on N-linked chains of glycoprotein masking galactose/GalNAc residues, which ultimately lowers the binding site of

galactose/ GalNAc specific lectin. The percentage of Con A binding in normal and adenocarcinoma colon was almost the same. Jorbi *et al.*, (1989) also reported the same in colon tissues.

The binding of ENL, Con A and HPA in normal colon was uniform in cytoplasm, intracelluar space and basement membrane. But in HPA the basement membrane was diffusely stained. In adenocarcinoma colon, both ENL and HPA showed diffuse cytoplasmic staining whereas, intercellular space showed uniform staining. Moreover, the intensity of staining was also reduced in adenocarcinoma colon as compared with normal colon. Bresailer *et al.*, (1985) also reported that DBA showed diffused cytoplasmic staining in adenocarcinoma colon. Lectin-HRP conjugates blocked by specific sugars, which failed to bind with tissues confirms the biniding specificity of the lectins.

In conclusion, the differential expression pattern of various neoglycoproteins in the carcinoma of breast, stomach and colon, implies that, the sugar receptor expression can be used as a discriminating marker in the differentiation of these cancers to some extent. The difference in the staining pattern of lectins suggest the coexpression of respective receptosrs in different types of carcinomas.

CHAPTER 6

Summary and Conclusions

6.1. SCREENING

The presence of lectins has been detected in large number of plant species in India, but very few lectins have been separated in pure form. So the present study demonstrates the wide occurrence of lectin in locally available plants. Of the 101 plant seeds screened, 30 plants showed presence of haemagglutination against the RBCs tested. In addition to the seeds tested, latex of two plants showed haemagglutinating activity, of which, the lectin from *E. neriifolia* was studied in detail. The lectins were not restricted in a particular family but it is distributed in different families and the sugar specificity of lectins was different. However some lectins showed common sugar specificity. Lectin from *E. neriifolia* was not studied earlier, so this lectin was purified and subjected to a detailed study.

6.2. PURIFICATION AND CHARACTERIZATION OF ENL

1. Partial purification of the lectin was done by using fractional precipitation of the extract by $(NH_4)_2SO_4$. The fraction precipitated by 60% $(NH_4)_2SO_4$ showed higher haemagglutination titre. Thus this fraction was employed in the affinity purification of lectin.

2. Haemmaglutination titre showed that 'A' and 'B' blood groups showed higher titre than 'O' blood group. The haemagglutination titre was increased considerably after treatment of the cells with neuraminidase and trypsin.

3. Sugar inhibition studies showed that the lectin is specific for GalNAc followed by galactose.

4. Lectin can be purified by using a column of Sepharose-4B.

5. The lectin is a glycoprotein and the molecular weight of the native lectin was 60 kDa. The native lectin is a dimer and monomers have a molecular weight of 30 kDa under reduced conditions.

6. Thermal and pH stability studies showed that the lectin is stable at 60 °C and pH 7-9. Demetalized lectin showed decreased thermal stability but does not have any effect on pH stability.

7. Atomic absorption spectroscopic studies showed that the lectin is a metalloprotein.

8. Antibodies were raised against the lectin and it cross reacted with the lectin.

9. The lectin also cross reacted with Con A and CVL.

10. CVL can be purified on a ENL-Sepharose column.

11. Lectin toxicity studies have shown that the lectin is toxic to mice and causes impairment in villi.

12. The lectin has immunopotentiating activity.

13. The lectin is mitogenic to mouse lymphocytes.

14. The lag period of the pollens of *Coleus species* was reduced by the lectin.

6.3. CALLUS CULTURE

Callus culture of *E. neriifolia* can be done on MS medium supplemented with 2,4-D. Primary callus culture of shoot apex and leaf showed the lectin activity, but the subsequent cultures failed to produce lectin. So the callus culture

technique is not an ideal method for lectin production. But it may be helpful in studying the mechanism of lectin synthesis and regulation etc.

6.4. HISTOCHEMISTRY

The binding studies of ENL on breast, colon and stomach showed that the percentage of lectin binding is high in infiltrating duct carcinoma breast than in normal cases. In the case of stomach,same is the condition but the percentage is slightly lower than breast. In colon, the binding pattern was reversed.

Here the percentage was lowered considerably during malignant transformation. HPA and ENL showed similar binding pattern. The binding pattern of Con A was different from the other two lectins. So, ENL binding studies can be used in the detection of malignant transformation in breast, stomach and colon to some extent.

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