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Study of Biological Properties of Chitin-binding Lectins from Potato Species (Solanum tuberosum) Cultivated in Bangladesh

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Study of Biological Properties of Chitin-binding Lectins from Potato Species (*Solanum tuberosum*) Cultivated in Bangladesh

M. Phil. Thesis

A

Dissertation

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August, 2012

EXAMINER'S COPY

Dedicated

To My Family

DECLARATION

I do hereby declare that the materials embodied in this thesis entitled "Study of Biological Properties of Chitin-binding Lectins from Potato Species (*Solanum tuberosum*) Cultivated in Bangladesh" prepared for submission to the department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh for the degree of Master of Philosophy in Biochemistry and Molecular Biology, are the original research works of mine and have not been previously submitted for the award of any degree or diploma anywhere.

(Imtiaj Hasan)

Signature of the M. Phil. Fellow

CERTIFICATE

This is to certify that the thesis entitled "Study of Biological Properties of Chitin-binding Lectins from Potato Species (*Solanum tuberosum*) Cultivated in Bangladesh" has been prepared by Imtiaj Hasan under my guidance and supervision for submission to the department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh for the degree of Master of Philosophy in Biochemistry and Molecular Biology. It is also certified that the materials included in this thesis are the original works of the researcher and have not been previously submitted for the award of any degree or diploma anywhere.

I have gone through the draft of the thesis and found it acceptable for submission.

(Dr. Syed Rashel Kabir)

Signature of the supervisor

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August, 2012 **Imtiaj Hasan**

ABSTRACT

Chitin-binding lectins were isolated from the tubers of two local varieties of potatoes (*Solanum tuberosum*) - 'Deshi' and 'Sheelbeelati' cultivated in Bangladesh. The approximate molecular mass of the Deshi Potato lectin mixtures were 22±1 kDa and 24±1 kDa whereas for Sheelbilatee potato lectins, those were 21±1 kDa and 23±1 kDa determined by SDS-PAGE. Both the lectin mixtures showed strong agglutination activity to mice blood as the minimum agglutination concentration was 4 µg/ml and possessed strong antibacterial activity against *Escherichia coli (0157:H18)*, *Listeria monocytogenes*, *Salmonella enteritidis* and *Shigella boydii* at the dose of 5µg/ml. In a bacterial agglutination study it was found that only *Salmonella enteritidis* was very sensitive to the presence of Sheelbilatee lectins whereas Deshi potato lectins could not agglutinate any bacteria. The lectins exerted marked growth inhibition against the same set of pathogenic bacteria at different concentrations and the Sheelbilatee potato lectins were found more active than the Deshi potato lectins against every bacteria in almost every concentration. In an antifungal assay, both the lectin mixtures had notable antifungal activity against the tested fungal species *Rhizopus sp.*, *Penicillium sp.* and *Aspergillus niger.* A quite high LC_{50} value of 75 µg/ml for Sheelbeelati potato lectins indicates cellular cytotoxicity whereas the LC_{50} value for Deshi potato lectin was 90 µg/ml. Both the protein mixtures inhibited the growth of Ehrlich's Ascites carcinoma (EAC) cells *in vivo* at

the dose of 1.38 mg/Kg/day by 83.04% and 79.84%. A mechanism study based on the measurement of β-lactamase efficiency of the protein mixtures against two bacterial species showed that both Sheelbilatee and Deshi potato lectins were weakly positive against *E. coli* but failed to show any positivity against *Klebsiella pneumoniae.* Another mechanism study showed that both the protein mixtures have marked inhibitory effect on the biofilm production of *Pseudomonas aeruginosa* in a concentration-dependent manner. In a structural stability study, the measurement of fluorescence spectra indicated the possible structural similarities in the two lectin mixtures. The number of Tryptophan residues may be higher in the Deshi potato lectins than the Sheelbilatee lectins and these lectins may be calcium-independent in nature.

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INTRODUCTION

INTRODUCTION

The potato is a starchy, tuberous crop from the perennial *Solanum tuberosum* of the *Solanaceae* family (also known as the nightshades). The word may refer to the plant itself as well as the edible tuber. Potato (*Solanum tuberosum*), is the only major tuber crop that is grown in temperate regions. It is also the most important tuber crop in terms of production, accounting for about 45% of the total world production of all tuber crops (Shewry, P.R., 2003). In the region of the Andes, there are some other closely related cultivated potato species. Potatoes were introduced outside the Andes region four centuries ago, and have become an integral part of much of the world's cuisine. It is the world's fourth-largest food crop, following rice, wheat and maize. Long-term storage of potatoes requires specialized care in cold warehouses.

Wild potato species occur throughout the Americas, from the United States to Uruguay. The potato was originally believed to have been domesticated independently in multiple locations, but later genetic testing of the wide variety of cultivars and wild species proved a single origin for potatoes in the area of present-day southern Peru and extreme northwestern Bolivia, where they were domesticated 7,000 – 10,000 years ago. Following centuries of selective breeding, there are now over a thousand different types of potatoes.

However, the local importance of potato is extremely variable and rapidly changing. It remains an essential crop in Europe (especially eastern and central Europe); where per capita production is still the highest in the world,

but the most rapid expansion over the past few decades has occurred in southern and eastern Asia. China is now the world's largest potato-producing country, and nearly a third of the world's potatoes are harvested in China and India.

While there are close to 4000 different varieties of potato, it has been bred into many standard or well-known varieties, each of which has particular agricultural or culinary attributes. In general, varieties are categorized into a few main groups, such as russets, reds, whites, yellows (also called Yukons) and purples — based on common characteristics. Around 80 varieties are commercially available in the UK. For culinary purposes, varieties are often described in terms of their waxiness. Floury or mealy (baking) potatoes have more starch (20 – 22%) than waxy (boiling) potatoes (16 – 18%). The distinction may also arise from variation in the comparative ratio of two potato starch compounds: amylose and amylopectin.

In Bangladesh, there are about 27 local varieties of potatoes cultivated in different parts of the country. They have familiar local names. It is estimated that local varieties were cultivated in about 11, 3540 acres of land, producing 3, 09,800 m tons of tubers during 1997-98.

Potato varieties in Bangladesh, as across the territory of colonial India, have been derived from two subspecies, *Solanum tuberosum;* subspecies *tuberosum* and subspecies *andigena* (Shekhawat *et. al.* 1992). As of independence (i.e. of India and Pakistan in 1947), most varieties were of recent European import, and few were suitable to the short-day conditions of the Indo-Gangetic Plains.

In the last few decades, several dozens of high yielding varieties (HYV) of potato were brought to Bangladesh and tried experimentally under local conditions before being recommended for general cultivation. During the 1970s, about 16 varieties were initially selected, but subsequently 10 varieties were dropped. Through constant evaluation of the traits, varietal performance, and considerations of other characteristics, about 10 varieties of HYV have been released for cultivation in the country. However, huge amount of potato seeds are imported every year by the Bangladesh Agricultural Development Corporation (BADC) for distribution among farmers. Bangladesh Agricultural Research Institute (BARI) has also established a farm at Debiganj in Panchagar district for production of HYV seed potatoes. Among the high yielding popular varieties the following are notable: (a) *Cardinal*- probably most popular among the foreign varieties with oblong, reddish tubers, shallow eyes, and smooth skin. The variety has been introduced from Holland and has yield potential of 20 - 25 metric tons per hectare. (b) *Diament* - another Holland variety with oval to oblong, pale yellow tubers, skin smooth, and eyes shallow. It is quite disease resistant. Per hectare yield ranges from 18 - 24 metric tons. (c) *Kufri Shindhury* - tubers reddish, round, and eyes deep with rough skin. This variety was introduced from India and is comparatively less susceptible to pests and diseases. It has a yield potential of 18 to 22 metric tons per hectare. Other notable exotic varieties are Patronis, Alpha, Archa, Multa, Ukama, Hira, Maurin, Origo, Alisa etc.

The most common types of Potatoes in Bangladesh are sometimes designated as *deshi*, a term often treated as synonymous with "local

varieties" or "indigenous," generally referring to varieties introduced prior to independence in 1947 which are favored within a particular region. Most are relatively low yielding and have somewhat longer vegetative cycles than more recently introduced varieties, but are retained by farmers for other factors, such as storage properties and cooking and culinary qualities (Scott, G., 1985; Mahfuz-Ul-Hoque, M., 1985). These are now cultivated all over the country.

The other familiar local varieties are (a) *Sheel Bilatee*- mostly cultivated in Rangpur. The tuber is oblong, reddish. Each tuber weighs about 30 g. (b) *Lal Sheel*- primarily cultivated in Bogra with tubers rounded, reddish, each having a weight of about 55 g. This variety is also known as *Lal Madda* and *Bograi*. (c) *Lal Pakri* - cultivated widely in Dinajpur, Bogra and Sirajganj districts with tubers reddish and round, each weighing about 30 g. (d) *Du Hajari* - mostly cultivated in the Chittagong area. Tubers appear round and pale, each weighing about 25 g. Among other indigenous varieties *Jhau Bilatee*, *Suryamukhi*, *Indurkani*, *Shadaguti*, *Challisha*, *Jam Alu* etc. are notable.

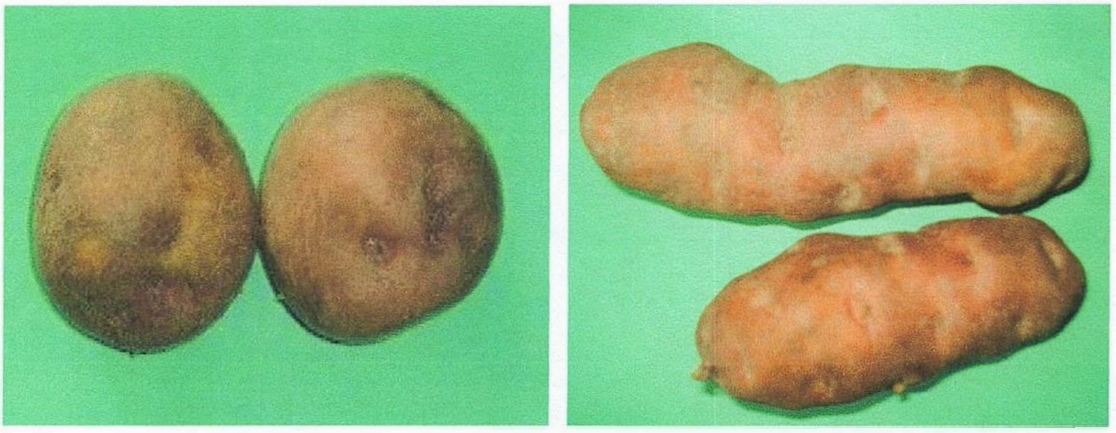


Fig - 1: Deshi (on the left) and Sheelbilatee (on the right)

Two of the most common indigenous varieties, *Deshi* and *Sheel Bilatee* (Figure -1) are used in this study.

1.1 Plant as a Source of Protein

Protein is essential for the growth, maintenance and function of major parts of human body. Therefore, every person requires protein, regardless of diet or lifestyle choice. Vegetarians can still get necessary amounts of protein from a variety of nutritious plant foods.

Although animal kingdom is the main source of protein but plant kingdom also possesses a lot of protein sources. Green leaves, barks, roots, stems etc. contain small amount of protein; while seeds are the main sources of protein in plants. Aromatic grain and nut pilafs, grilled tofu and vegetable kebabs, whole-grain pancakes, and black bean enchiladas are just a few examples. Pulses are 20 to 25% protein by weight, which is double the protein content of wheat and three times that of rice (http://en.wikipedia.org/wiki/Pulse_%28legume%29). Oil seeds also contain a lot of proteins; cottonseed kernels contain 32.6 g of protein/100 g whereas Linseeds, Peanuts, Rapeseeds, Sesame seeds and Sunflower seeds contain 19.5 g of protein/100 g, 25.6 g of protein/100 g, 22.0 g of protein/100 g, 18.2 g of protein/100 g and 19.8 g of protein/100 g respectively. [Source: Food Standards Agency and Institute of Food Research (2002) and US Department of Agriculture, Agricultural Research Service (2003), http://www.hillfarmoils.com/oilseedreport.pdf]. In addition, wheat, barley, rice bran, maize etc. also contained significant amount of protein. Some plant seed proteins are toxic and some are nontoxic. Here are some examples of

vegetarian foods with high amounts of plant protein (http://www.happycow.net/vegetarian_protein.html).

Protein in legumes: Garbanzo beans, Kidney beans, Lentils, Lima beans, Navy beans, Soybeans, Split peas.

Protein in grains: Barley, Brown rice, Buckwheat, Millet, Oatmeal, Quinoa, Rye, Wheat germ, Wheat, hard red, Wild rice.

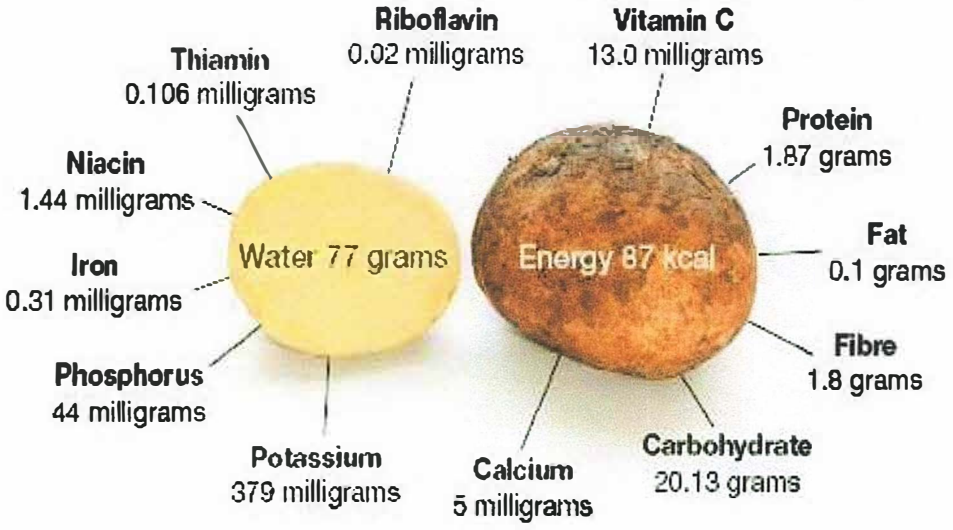
Vegetable protein: Artichokes, Beets, Broccoli, Brussels sprouts, Cabbage, Cauliflower, Cucumbers, Eggplant, Green peas, Green pepper, Kale, Lettuce, Mushrooms, Mustard green, Onions, Potatoes, Spinach, Tomatoes, Turnip greens, Watercress, Yams, Zucchini.

Protein in fruits: Apple, Banana, Cantaloupe, Grape, Grapefruit, Honeydew melon, Orange, Papaya, Peach, Pear, Pineapple, Strawberry, Tangerine, Watermelon.

Protein in nuts and seeds: Almonds, Cashews, Filberts, Hemp Seeds, Peanuts, Pumpkin seeds, Sesame seeds, Sunflower seeds, Walnuts.

1.2 Protein in Potatoes

Whether baked or raw, a medium potato contains approximately 4 g of protein. The protein content can vary slightly depending on the potato variety. For example, a medium white potato contains only 3.5 g protein, whereas a medium russet potato contains 4.5 g protein.



(Per 100 g, after boiling in skin and peeling before consumption)
Source: United States Department of Agriculture, National Nutrient Database

Fig - 2: Protein content of potatoes in comparison to the other nutrients

1.3 Proteins and Glycoproteins

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

The glycoproteins that contain carbohydrate group attached covalently to the polypeptide chains represent a large group of wide distribution with considerable biological significance. The percentage by weight of carbohydrate group in different glycoproteins may vary from less than 1% in ovalbumin to 80% in the mucoprotein. Many different types of monosaccharide derivatives have been found in glycoprotein. The linear or branched side chain of glycoproteins may contain from two to dozens of monosaccharide residues usually of two or more kinds. Some glycoproteins also contain oligosaccharide units. The sugar residues are generally mannose, galactose, lactose, xylose, glucose, raffinose, arabinose, glucosamine etc (Lis, H. and Sharon, N., 1981).

1.4 Lectins and Chitin-binding Proteins

Structural domains that recognize and bind specific carbohydrates without altering the recognized sugars are widely distributed in all sorts of living organisms ranging from simple viruses to the most complex higher animals. Proteins possessing such carbohydrate-binding domains are usually called lectins (Candy, L. *et al.*, 2003). According to another definition, Lectins are the heterogeneous group of proteins of non-immune origin that bind reversibly to mono and oligosaccharides with high specificity and are devoid of any catalytic activity (Lis, H., Sharon, N., 1998). Most of our daily foodstuffs (such as tomato, wheat, potato, fruits, fish etc.) contain lectins. Lectins have sugar specificity such as Galactose-specific lectin, Raffinose-specific lectin etc.

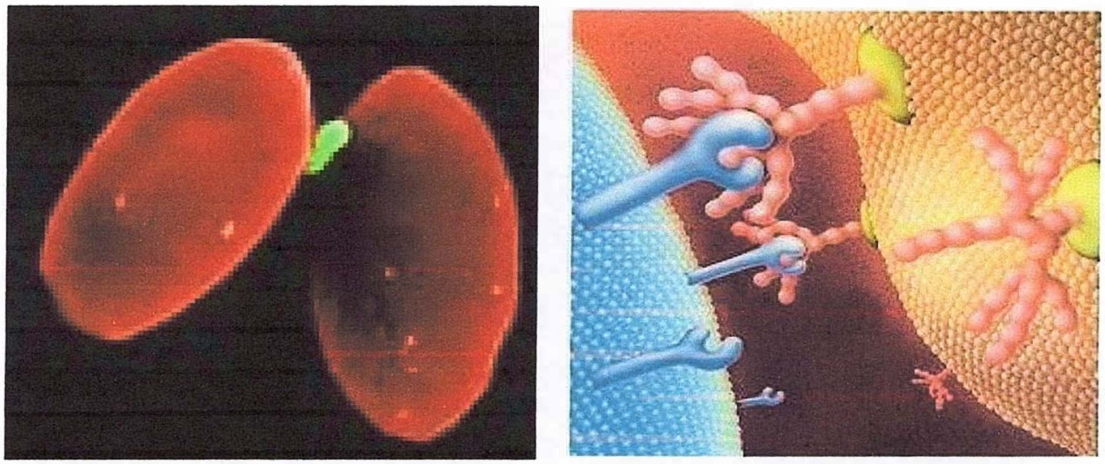


Fig - 3: The mechanism of lectin action

Lectins are currently attracting much interest, primarily because they serve as invaluable tools in diverse areas of biomedical research. Because of their unique carbohydrate binding properties, lectins are useful for the separation and characterization of glycoproteins and glycopeptides, in studies of glycolipids, following changes that occur on cell surfaces during physiological and pathological processes, for cancer treatment due to its anticancer activity, for histochemical studies of cells and tissues, for tracing neuronal pathways, typing blood cells and bacteria, for fractionation of lymphocytes and of bone marrow cells for bone transplantation. They are also used to stimulate lymphocytes to assess the immune state of patients and for chromosome analysis in human cytogenetics, as well as for the production of cytokines. In addition, lectins are excellent models to examine the molecular basis of specific reactions that occur between proteins and other types of molecules, both of low or high molecular weight, such as the binding of antigens to antibodies, of substrates to enzymes, of drugs to proteins and of hormones and growth factors to cells. Lectins can also be used in protein chemistry for

column packing in affinity chromatography e.g. Concanavalin A lectin and wheatgerm agglutinin. Lectins also have biomedical importance as they can be used for ulcer treatment.

1.5 Biological Properties of Lectins

Role in defense mechanisms: An essential feature of any active defense mechanism of a biological system is the ability to specifically recognize the offensive agent and responding to its presence. The specificities of lectins and the properties conferred on them by the multivalency of their combining sites led early investigators to note the similarities of lectins to antibodies and to propose that perhaps lectins may function as plant antibodies (Punin, W. J., 1952; Boyd W. C., 1963). Although lectins may well play a role in the defense system of plants, there is no evidence of their presence in plants immune system that can be directly comparable with the animal immune system. Many studies have demonstrated the interaction of plant lectins with various microorganisms (Pistole, T. G., 1981). Suggestive evidence for such a defense role of lectins in plants has come from studies of their interactions with fungi, using fluorescein isothiocyanate conjugates of wheat germ agglutinin (Mirelman *et al.,* 1975). It was found that this lectin, which has specificity for chitin oligomers (Allen, A. K, Neuberger, A. 1973: Lotan R, Sharon N., 1973), binds to the hyphal tips and septa of *Trichoderma viridae*, a fungus with chitin-glucan hyphal cell walls; the binding was inhibited by chitotriose. The lectin inhibited the growth and spore germination of the fungus and the investigators found evidence that it interferes with chitin synthesis. Further studies showed that the wheatgerm lectin can bind to the hyphal tips and septa and young spores of a number of species chitin

containing fungi (Barkai-Golan, R. *et al*., 1978) and has a strong affinity for the zygospores, which are hyphal branches involved in sexual reproduction (Jones, B. E., Gooday, G. W., 1977)

On the basis of above studies, it was proposed that lectins might protect plants against fungal pathogens during the imbibitions, germination, and early growth of the seedlings (Barkai-Golan, R. *et al*., 1978).

Role in pathogenesis: Lectins may aid in the invasion of plants by having some roles in pathogenesis. The infection of sugarcane by the fungus *Helminthosporium sacchari* is an example of this type of pathogenesis.

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

For agglutination to occur the bound lectin must form multiple cross bridges between the opposing cells. The agglutination does not depend on the amount of the lectins. Sometimes a considerable amount of any lectin cannot bind to the cells and cause agglutination. This is because agglutination is affected by many factors such as the molecular properties of the lectin (e.g. numbers of saccharide-binding sites, molecular sizes), cell-surface properties (for example, number and accessibility of receptor sites, membrane fluidity), and metabolic state of the cells (Nicolson, G. L., 1976). In addition, agglutination is affected by some external conditions such as temperature,

concentration of the cells, proper mix-up and so on. Agglutination can be inhibited by various sugars on that the lectins are dependent.

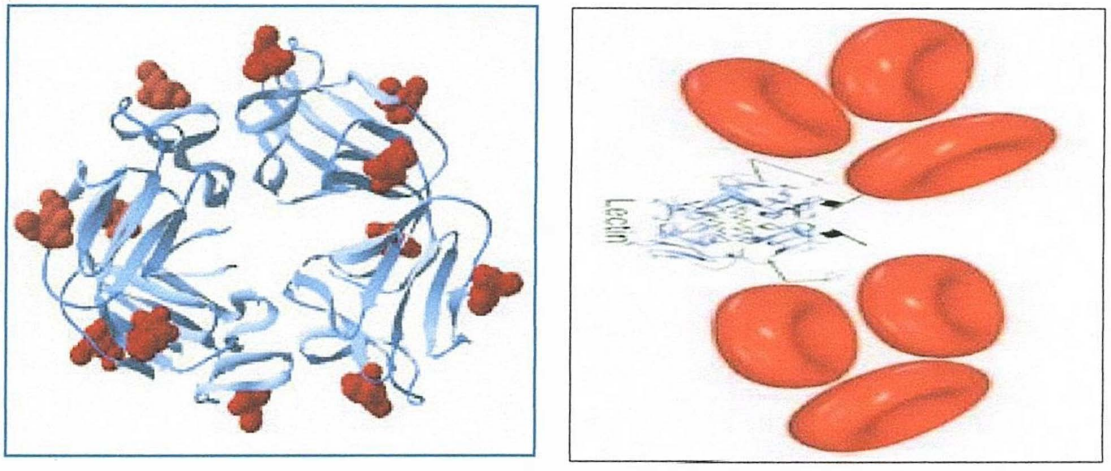


Fig - 4: Agglutination of RBC by lectins

Mitogenic stimulation of lymphocytes: One of the most dramatic effects of the interaction of lectins with cells is mitogenic stimulation, i.e. the triggering of quiescent, nondividing lymphocytes into a state of growth and proliferation.

Induction of suppressor cells: Treatment of human and murine lymphocytes with various mitogens, particularly Concanavalin-A can induce the generation of potent suppressor cells capable of inhibition activities of T and B cells *in vitro*.

Lectin-dependent cytotoxicity of lymphocytes and macrophages: The interaction and killing of target cells by cytotoxic T Lymphocytes requires the specific recognition by the effector cells of major MHC-antigens of the target cells. In contrast, in the presence of concanavalin – A or other

mitogenic lectins, a wide verity of antigenically unrelated target cells is lysed by the cytotoxic T- Lymphocytes, a phenomenon known as lectin-dependent cytotoxicity (Asherson *et al.*, 1973).

Both antigen specific and lectin-dependent cytolysis can be dissected into three distinct stages; binding and/or recognition, lethal hit and programming for lysis, and killer- cell-independent lysis. (Berke, G., 1983) The lack of immune specificity in the lectin-dependent reaction has been attributed to the ability of the lectin to bind to both the effector and target cells.

Similarly anti-tumor antibodies that can induce macorophage-mediated tumor lysis, lectins such as those from wheat germ (Kurisu *et al.*, 1980), *Griffonia simplicifolia* (Maddox *et al.,* 1982) and the dissect *sarcophaga peregrina* (Ohkuma *et al.,* 1985; Yamazaki *et al.,* 1983) possess the ability to mediate carbohydrate specific binding of mouse macrophages and tumor cells and to induce the killing of the tumor cells by the macorophages.

Lectin-mediated killing of both syngenic and allogenic tumor cells was observed in case of wheat germ agglutinin, but not by soybean agglutinins or concanavalin-A. The lectin can initiate the lysis of erythrocytes by human blood leukocytes (MacDermott *et al.,* 1976; Wei, W. Z., Lindquist R. R., 1981). With guinea Pig macrophages, however, both concanavalin-A and wheat germ agglutinin (but not Soybean agglutinin) induced cytolysis of homologous erythrocytes (Keisari, Y. 1982; Keisari, Y. and Pick, E. 1981).

Lectin-mediated phagocytosis of target cells: In addition to lectin-dependent killing of tumor cells by macrophages, lectins can mediate the

binding and occasionally, the phagocytosis of other types of cell (Sharon, N. 1984). Thus, The binding of concanavalin-A to the surface of mouse macrophages mediated the attachment of bacteria such as *Bacillus subtilis* to the macrophages, although no phagocytosis of the bacteria was observed (Allen, J. M., Cook, G. M. W, 1970) wheat germ agglutinin markedly enhanced the binding and phagocytosis of bacteria such as *Staphylococcus aureus H, Staphylococcus albus* or *Micrococcus luteus* by mouse peritoneal macrophages (Gallily, R. *et al.,* 1984).

Insulin immitic activity: Concanavalin-A, wheat germ agglutinin and several other Lectins (Cuatrecasas, P. 1973; Czech, M. P. *et al.,* 1974; Katzen, H. M. *et al.,* 1981) mimic the effects of insulin on adipocytes, such as stimulation of lipogenesis and of glucose transport and oxidation and inhibition of lipolysis, other target tissues are similarly stimulated. It was concluded that due to its glycoprotein-like nature, lectins like concanvalin-A or wheat germ agglutinin compete with the insulin receptors (Rouiller, D. G. *et al.,* 1986).

Lectin toxicity: Several lectins e.g. concanavalin A, wheat germ agglutinin, PHA, and the Lectin from *Robini pseudoacacia* are toxic to the mammalian cells both *in vitro* and *in vivo*.

1.6 Application of Lectins

Lectins are powerful tools for the study of carbohydrates and their derivatives, both in solution and on cell surfaces. They are being widely used for preparative and analytical purposes in biochemistry, cell biology,

immunology, and related areas, particularly with glycoconjugates. The list of application of lectins is getting bigger and still the potentiality of these proteins is far from being completely explored.

Isolation and Structural Studies of Glycoconjugates

Because of the analogy of lectin-saccharide interactions with the same between antibody and antigen, the lectins are applied either in solution or, more commonly, in immobilized form, for the detection and purification of a variety of carbohydrate containing compounds. It is a natural extension of the use of antibodies for the isolation of corresponding antigens. Moreover, lectins are often stable in the presence of low concentrations of certain detergents, permitting their use for the purification of membrane glycoproteins.

Detection and Identification

The specific interaction of a biopolymer with a lectin may be taken as evidence that the polymer contains carbohydrates. For example, the first indication that yeast invertase in a glycoprotein was obtained with concanavalin-A (Sumner, J. B., Howell, S. F., 1936). More recently, lectin chromatography has been widely used to demonstrate the glycoprotein nature of membrane receptors for hormones, growth factors, neurotransmitters, immunoglobulins, and related compounds (Gioannini, T. *et al.,* 1982; Hedo J. A. *et al.,* 1981; Helm, R.M., Froese, A. 1981; Shirakawa, O. *et al.,* 1983).

Immune electrophoresis also has been adapted for using with the lectins, for example, to study the heterogeneity of glycoproteins with respect to carbohydrate content and structure (BØg –Hansen, T. C. *et al.,* 1975).

An assay system has recently been introduced in which an enzyme –lectin complex is utilized for the detection, and possibly quantization, of glycoproteins (McCoy, J. P. *et al.,* 1983). This system (ELLA, enzyme-linked lectin assay) is conceptually similar to ELISA.

Preparative Applications

For preparative purposes, affinity chromatography of glycoproteins on lectins is very useful. Membrane glycoproteins isolated with the aid of lectins are often referred to as lectins receptors.

Affinity chromatography using lectins with complex biological extracts e.g. from membranes or cell homogenates, usually results in the isolation of mixtures of glycoproteins and polysaccharides.

Fractions of glycoproteins and glycopeptides that differ only slightly in their carbohydrate composition or in the structure of their oligosaccharide units can be isolated using lectin columns. In this way, the isolation of the molecular variants of α-fetoprotein from calf-serum (Lai, P.C.W., and Lorscheider, F. L., 1978) and rat amniotic fluid, α_1-acid glycoprotein (Bayard, B. and Kerckaert, J. P., 1980), α-protease inhibitor from human serum (Bayard *et al.,* 1982) became possible. Application of lectins became useful in the isolation of tRNA species containing glycosylated bases

(Kolberg, J. *et al.,* 1983). tRNA ASP was readily isolated on a column of concanavalin A- Sepharose, and tRNA Try by the chromatography on a column of *Ricinus communis* agglutinin – Sepharose.

Structural Studies of Blood Group Substances

The contribution of lectins to the information of the chemical structure of the ABO blood group determinants in humans is well established (Watkins, W. M. *et al.,* 1981) Hapten inhibition studies with the blood type A – specific lectins of Lima bean (*Phaseolus limensis)* and of tufted vetch (*Vicia creacea)*, and the blood type O-specific lectins from *Lotus tetragonolobus* and from the Eel (*Anguilla anguilla)*, provided the first clue that N-acetyl galactosamine plays an important part in blood type A specificity and L-fucose in O(H) specificity.

Lotus tetragonolubus lectin for mono or di-L-fucosyl derivatives of Gal-β-4-GLCNAC was used for the structural elucidation of certain dis-substituted blood group oligosaccharides (Rovis, L. *et al.,* 1973).

Studies of Cellular & Sub-cellular Membranes

The presence of lectin receptors on cells is readily demonstrated with the aid of suitable lectin derivatives. A lot of techniques are developed by the immunologists for the study of cell-surface antigens (Lotan, R. 1979; Molday, R. S. 1981; Roth, J. 1980; Schrevel, J. *et al.,* 1981).

Radioactively-labeled lectins may be used to measure the number of lectin receptor sites of the cell surface and their homogeneity, as well as the degree of affinity of the lectin-receptor interactions.

Cell separation

The wide use of lectins to separate different surface carbohydrates is a recent development. Most of the part of this work has been done with mammalian cells, particularly with lymphocytes. In principle, any sub-population of cells, whether from animals, plants, or microorganisms may be sorted into subpopulations by interaction with lectins, provided the cells differ in their cell-surface sugars. Since the binding of lectins to cells can be reversed by the addition of an appropriate sugar without damaging the cells. Both the lectin-reactive and non-reactive cells are readily recovered, resulting in high yields of fully viably cells. The first application of lectins to cell separation was reported in 1949 by Li and Osgood, who developed a method for the separation of leucocytes from erythrocytes in human blood with the aid of PHA. The erythrocytes were selectively agglutinated by the lectins and were removed from the mixture by centrifugation.

Peanut agglutinin is the most popularly used for a variety of purposes in numerous laboratories, predominantly with murine and human lymphocytes, but also with lymphocytes of some other species.

Identification of microorganisms

Agglutination of a microorganism from a primary isolate with a particular lectin may represent a confirmatory identification of the organism making it possible to dispense with subsequently expensive and time-consuming culturing or serological testing, e.g. *Neisseria* and related bacteria by its agglutination with wheat germ agglutinin (Schaefer, R. L. *et. al.,* 1979). *Bacillus anthracis* and *Bacillus mycoides* were agglutinated with soybean agglutinin; of these two species, only the latter was agglutinated with the lectin from *Helix pomatia* (Cole M. D. 1994). These results suggested a rapid and convenient means for identifying *Bacillus arthracis* from a primary isolation media.

Coagulase-positive *Staphylococcus aureus* can be distinguished from coagulase-negative *Staphylococci* because only the latter strains are agglutinated by wheat germ agglutinin (Davidson, S. K. *et al.,* 1982).

Lectin as drug carriers

The ability of some lectins to interact preferentially with certain transformed cells has led to the attempts to use these compounds as carriers for chemotherapeutic agents. Examples of such conjugates are the chimeric toxins, consisting of concanavalin-A and the α chains of diphtheria toxin (Gilliland, D. G. *et al.,* 1978) or ricin (Yamaguchi, T. *et al.,* 1979), in which the lectin serves to direct the cytotoxic agent to appropriate target cells. Conjugates of concanavalin-A with antitumor drugs such as daunomycin (Kitao, T. and Hattori, K. 1977), chlorambucil (Lin, J. Y. *el al.,* 1981) and

Methotrexate (Lin, J. Y. *et al.,* 1981; Tsuruo, T. *et al.,* 1980), had a higher activity against various cultured tumor cell lines than the equivalent doses of free drugs and lectins.

Lectin resistant cells

The cytotoxic properties and sugar specificities of lectins make them ideal agents for the selection of cell variants with altered cell-surface carbohydrates. These variants provide a new tool for studying the genetics, biosynthesis, and functions of cell-surface glycoconjugates.

1.7 Clinical Uses of Lectins

Blood typing: One of the earliest and still widely used applications of lectins for distinguishing between the erythrocytes of different blood types. Several Lectins are specific for the blood types A, B, O (H), M, N (Bird, G. W. G. *et al.,* 1978, Judd, W. J. *et al.,* 1980). Some are also used in blood banks as an aid to blood typing.

Clinical states assessed by lectins

a) Immune dysfunction

b) Lymphoproliferative diseases

c) Chronic lymphatic leukemia

d) Acute myeloid leukemia

e) Hodgkin's disease

f) Sézary syndrome

g) Malignant lymphoma

h) Nonlymphoid malignancies

i) Immune disorders

j) Lupus erythematosus

k) AIDS

l) Rheumatoid arthritis

m) Stress

n) Hepatitis

o) Leprosy

p) Diabetes mellitus

q) Inflammatory bowel cell diseases

1.8 Dietary Lectins

Dietary lectins can cause diseases if the individual is lectin sensitive (because of genetic and immunogenic abnormalities and also by viral or bacterial infections). There are some properties of dietary lectins that are responsible for causing those diseases:

(a) Lectins are hardy proteins that do not break down easily. They are resistant to stomach acid and digestive enzymes.

(b) Lectins may bind to the gut wall and damage the gut lining and may pass through the gut into general circulation.

(c) Lectins can cause alterations in gut function that may be related to colitis, Crohn's Diseases, Celiac-Sprue, IBS (Irritable bowel syndrome) and gut permeability.

(d) Lectin damage to the gut wall may allow other non-lectin proteins to cross undigested into general circulation and cause allergic reactions, including anaphylaxis.

(e) Having gained access to general circulation, lectins may bind to surface cell membranes of organs and glands including the thyroid, pancreas, kidney and adrenals, in susceptible animals and humans.

(f) This binding may begin antigen-antibody reactions leading to autoimmune disorders and so-called degenerative diseases.

(g) Dairy lectins may be implicated in juvenile onset type 1diabetes. Wheat lectins may be implicated in juvenile nephropathy.

1.9 Plant Lectins

Plant lectins are classically considered a heterogenous group of proteins with strikingly different biochemical properties and biological activities. However, it becomes increasingly apparent that the great majority of all currently identified plant lectins can be classified into seven families of evolutionary and structurally related proteins, each of which is characterized

by its own typical sugar-binding motif (Van Damme, E. J. M. *et al.*, 1998). One of these families comprises the plant lectins that possess one or more chitin-binding domains equivalent to the so-called 'hevein domain'. This hevein domain was named after hevein, a small 43 amino acid residue chitin-binding protein found in the latex of the rubber tree (Walujono, K. *et al.*, 1975) Biochemical analyses indicated that the lectins found in potato (Allen and Neuberger, 1973; Allen *et al.*, 1978), tomato (Nachber, M.S. *et al.*, 1980), and thornapple (Ashford, D. *et al.*, 1982) distinguish themselves from all other plant lectins by two peculiarities. First, these Solanaceae lectins have an unusual amino acid composition characterized by a very high content (up to 30%) of hydroxyproline. Second, these lectins are more extensively glycosylated than any other plant lectin, the major carbohydrate compounds being arabinose and galactose.

1.10 Chitin-binding Lectins in Potatoes and Other Tubers

Lectins are widely distributed in the nature occurring in animals, micro-organisms and plants. In the plant kingdom, these proteins are abundant in seeds, roots, fruits, flowers, and leaves (Sharon, N. and Lis. H. 1990; Sharon, N. and Lis. H. 1995). According to the molecular weight and evolutionary relationships, plant lectins can be subdivided into seven different families characterized by its own typical sugar-binding motif which include the legume lectins, type-2 ribosome-inactivating proteins, monocot mannose binding lectins, jacalin-like lectins and chitin-binding lectins (Van Damme, E. J. M. *et al.* 1998). In the recent years, lectins have been proved to be attractive for research works due to their role in cell agglutination, toxicity, antifungal, antibacterial, antiviral, anti-proliferative and antitumor effects.

In general, plant tubers are rich in starch and indeed they are often considered solely as a source of carbohydrate for diets and industrial uses. However, they do contain protein which varies in amount from about 1-10% (dry weight). Plant tubers are known to contain defense-related proteins such as chitinases and lectins (Shewry, P. R., 2003). Chitinases are groups of proteins which catalyzes the hydrolysis of β-1,4-linked homopolymer of N-acetylglucosamine. Chitinases are widely distributed in a wide range of organisms, including plants, insects, fungi, bacteria and marine invertebrates (Flach, J. *et al.*, 1992). These enzymes vary widely in size (20 kDa to about 90 kDa). Bacterial chitinases have a molecular weight range of ~20-60 kDa, which is similar to that of plant chitinases (~25-40 kDa) and are smaller than insect chitinases (~40-85 kDa) (Bhattachrya, D. *et al.*, 2007). Chitinases have important physiological roles to hydrolyze polymers found in fungal cell walls and so it has been hypothesized that they are involved as part of the plant defense mechanism against fungal infection (Collinge, D. B. *et al.*, 1993). Furthermore, in filamentous fungi, chitinase is thought to be involved in cell wall metabolism for processes such as apical growth, branching, and autolysis of hyphae (Yanai, K. *et al.*1992). Chitinase has also shown inhibitory activity against a number of pathogenic fungi (Schlumbaum, A. *et al.* 1986; Sela-Buurlage, M. B. *et al.* 1993). In recent years, chitinase has been extensively studied through its isolation and purification from various organisms such as plants, insects, fungi, bacteria, and marine invertebrates (Flach, J. *et al.* 1992).

Chitin-binding lectins are believed to be involved in plant defense system because many of them possess antifungal or insecticidal activity.

Till now different lectins are purified from various species of potatoes (Kielisewski, M. J., *et al*, 1994; Siddanakoppalu N. P., Yeldur P. V., 2006; Van Damme, E. J. M. *et al,* 2006; Feng, J. *et al,* 2003). Beside that chitin binding lectins were also purified from wheat agglutinin, stinging nettle rhizomes, Vibrio parahemolyticus etc. (Gildemeister, O., *et al* 1994; Broekaert, W. F. *et al*, 1997).

1.11 Tuber Lectins Isolated from Different Plant Sources

Till now most of the lectins are purified from plant sources and the main sources of lectins in plants are mature seeds, fruits, rhizome, and tuber. Research on lectins is currently attracting much interest to the scientist because of their unique biological properties. Although approximately thousand of lectins are known but the structure of most lectins are not fully elucidated even detailed physiological functions of plant lectins are still unknown.

The lectin have attracted great interest on according of their various biological activities, such as cell agglutination (Konkumnerd, W. *et al.,* 2010), antifungal (Sitohy, M. *et al.,* 2007), antiviral (Tian, Q. *et al.*, 2008) and antiproliferative activities (Liu B. *et al.,* 2010). Specially mannose and *N*-acetyl-D-glucosamine-binding lectins are considered biologically important defense proteins, because these sugars are widely distributed in micro-organisms, insects and animals (Kaur, A. *et al.,* 2005; Trindade, M. B. *et al.,* 2006). Although different sugar specific lectins were purified from the tubers of different plants e.g. a mannose binding homotetrameric lectin of approximately 50 kDa was purified from edible Arum maculatum tuber,

another novel mannose-binding tuber lectin with in vitro antiproliferative activity towards human cancer cell lines was. Its apparent molecular weight was 48 kDa. A tuber lectin of 13.4 kDa was isolated from the fresh tubers of a traditional Chinese A tuber lectin from Arisaema jacquemontii Blume belonging to family Araceae by employing a single step affinity chromatography using column of asialofetuin-linked amino activated silica. An anti-insect and anti-cancer lectin has been isolated from *Arisaema helleborifolium* Schott by affinity chromatography using asialofetuin-linked amino activated silica beads. The lectin was a homotetramer having subunit molecular mass 13.4 kDa while its native molecular mass was 52 kDa.

1.12 Potato Lectins

The lectin in potato (Solanum tuberosum) tubers was first described by Marcusson-Begun H., 1926 and has since been the subject of detailed studies (Marinkovitch, V. A., 1964; Allen, A. K, Neuberger, A., 1973; Allen, A. K. *et al*., 1978). Later two lectins differing in saccharide specificity had been detected in crude homogenates of potato fruits, one occurring in the seeds, the other in the pericarp and juice within the pericarp (Kilpatrick, D. C. *et al*., 1980).

Varieties of potatoes are cultivated worldwide and a number of chitin-binding proteins and lectins have been identified in potatoes (*Solanum tuberosum, L*) on the basis of their affinity, enzymatic properties or amino acid sequence (Allen, A. K, Neuberger, A., 1973; Millar, D. J., *et al*, 1992; Desai, N. N., Allen, A. K., 1979; Kilpatrick, D. C. *et al*., 1980; Matsumoto,

I. *et al*, 1980; McCurrach, P. M., Kilpatrick, D. C., 1986; Owens, R. J., Northcote, D. H., 1980; Pramod, S. N. and Venkatesh, V., 2006).

1.13 Research Plan

We have a huge variety of indigenous plants and animals that can serve as the source of lectins. The aim of this study is to isolate chitin binding lectins from locally cultivated species of potatoes that could have antimicrobial activity and to develop the microbicidal agents that can be used for the treatment of various infectious diseases. Then it can be implemented in the health sector of our country. A few lectins are already isolated and purified from various species of potatoes. In this research work, chitin-binding lectins will be isolated from the indigenous varieties (Deshi and Sheelbilatee) of our country and their biological activities will be studied. As the other members of the tuber lectin family have antibacterial, antifungal and antiproliferative activities, the lectins isolated from these local potato species are expected to have the same and will be investigated in this regard. Though it is not a comparative study, the differences and similarities in the biological activities of these chitin-binding lectins from two varieties will add a variation in this thesis work. So the aims of this research work are:

- to isolate chitin binding lectins from two locally cultivated species ('Sheelbilatee' and 'Deshi') of potatoes

- to determine their molecular weights

- to study the biological properties like the antimicrobial and antineoplastic activities

- to observe the mechanism of action of the lectins under study by:

  (a) the measurement of beta-lactamase efficiency by fluorescent end product emission

  (b) observing the inhibitory effect on the production of biofilm by *Pseudomonas aeruginosa*

- to study the protein conformational changes upon binding to different molecules by fluorescent spectroscopy

Chapter-Two

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Collection of Sheelbilatee and Deshi Potatoes

Sheelbilatee potato tubers were collected from Rangpur and Deshi potatoes were collected from the local market near Rajshahi city. After collection, the potato tubers were cleaned, washed and stored.

2.2 Isolation of Chitin-binding Lectins

2.2.1 Preparation of Crude Protein Extract

In order to isolate the chitin-binding proteins from the potato tubers in biologically active form, all the operations were performed at 4°C. The potatoes were peeled off and sliced. The sliced potatoes were blended to produce the potato juice with the addition of 10 mM Tris-HCl (pH 8.2) buffer containing 1% NaCl and 0.02% $Na_2S_2O_5$ (sodium metabisulfite) as an antioxidant. After a while, the homogenate was filtered through a clean muslin cloth. Finally the homogenate was centrifuged at 17,000 rpm for 15 minutes and the supernatant obtained was collected and dialyzed for about three hours against 10 mM Tris-HCl (pH 8.2) at 4°C. The crude protein extract was stored in a deep freezer and was centrifuged every time before use.

2.2.2 Affinity Column Chromatography

The crude protein samples were subjected to affinity chromatography on a chitin column (2×25 cm) previously equilibrated with 10 mM Tris-HCl

buffer, pH 8.2. The column was first washed by 10-15 volume of 10 mM Tris-HCl buffer, pH 8.2 and then followed by 2 volume of distilled water. Finally the proteins were eluted by 0.5 M acetic acid. The eluted fraction was dialyzed against distilled water for overnight. Fractions (2.5 ml/tube) were collected at a flow rate of 1 ml/min. Absorbance of each fraction was measured at 280 nm and the protein concentration was determined by the Lowry method (Lowry, O. H. *et al.* 1951).

2.3 Test of Purity

2.3.1 SDS-PAGE Method

Principle: Polyacrylamide gel electrophoresis method is commonly used for checking the purity of proteins and their molecular weight determination. Sodium dodecyl sulfate (SDS) is an anionic detergent that binds to most proteins in amounts roughly proportional to molecular weight of the protein, about one molecule of SDS for every two molecules of amino acid residues. The bound SDS contributes large net negative charge, rendering the intrinsic charge of the protein insignificant. In addition, native conformation of the protein is altered when SDS is bound and most protein assumes similar shape and thus similar ratio change to mass. Slab gel electrophoresis in presence of SDS therefore separates proteins almost exclusively on the basis of mass, with smaller polypeptides migrating more rapidly. Protein-SDS complexes will therefore all move towards the anode during electrophoresis and their movements are inversely proportional to their molecular weights. If standard proteins of known molecular weights are also run, the molecular weights of the sample proteins can be determined by comparing them with proteins of known molecular weights. The protein pattern of the selected

fractions was determined by 16% SDS-PAGE according to the method of Laemmli (Laemmli, U. K., 1970).

2.3.2 Reagents and Solutions:

(i) Preparation of 30% acrylamide solution

33.3 g of acrylamide and 0.9 g N, N-methylene-bis-acrylamide were dissolved in 70 ml of distilled water in a 100 ml of volumetric flask and the final volume was made up to the mark by adding distilled water. The solution was filtered by whatman No. 1 filter paper and stored in a refrigerator.

(ii) Preparation of 1.5 M Tris-HCl buffer (pH 8.8)

18.7 g of Tris base was dissolved in 90 ml of distilled water in a conical flask and mixed well. The pH of the solution was adjusted to 8.8 by adding concentrated HCl. The final volume was made up to 100 ml with distilled water.

(iii) Preparation of 0.5 M Tris-HCl buffer (pH 6.8)

6 g of Tris base was dissolved in 90 ml of distilled water in a conical flask and mixed well. The pH of the solution was adjusted to 6.8 by adding concentrated HCl. The final volume was made up to 100 ml with distilled water.

(iv) Preparation of 10 % SDS (Sodium dodecyl sulphate) solution

10 % SDS solution was prepared by dissolving 5 g of SDS in 40 ml of distilled water. After dissolving, the final volume was made up to 50 ml with distilled water.

(v) Preparation of 10 % APS (Ammonium per sulphate) solution

10 % APS solution was prepared by dissolving 0.5 g of APS in 4 ml of distilled water. After dissolving, the final volume was made upto 5 ml with distilled water. The solution was stored in eppendorf tubes (500 µl in each tube) at 20° C.

(vi) TEMED

The commercially available preparation from Sigma Chemicals Co. U.S.A was used without modification.

(vii) Preparation of sample buffer

Components	Amounts
0.5 M Tris-HCl buffer (pH 6.8)	1 ml
10% SDS	2 ml
Glycerol	1 ml
Distilled Water	5.4 ml
Bromophenol Blue	0.5 mg

(viii) Preparation of Coomassie brilliant blue (CBB) staining solution

Components	Amounts
CBB R 250	1 gm
Glacial acetic acid	25 ml
Methanol	225 ml
Distilled Water	250 ml

(ix) Preparation of distaining solution

Components	Amounts
Glacial acetic acid	75 ml
Methanol	50 ml
Distilled Water	875 ml

(x) Preparation of electrophoresis buffer (Chamber buffer)

Components	Amounts
Tris base	6 g
Glycine	28.8 g
SDS	1 g
Distilled water	980 ml

(xi) Preparation of sample

20µl aliquot of the protein sample was mixed with equal amount of sample buffer (1:1, v/v) in an eppendorf tube and heated for 2-3 minutes at 100° C. The sample was then used for SDS-PAGE.

2.3.3 Procedure for SDS-PAGE

Clean and dry plates (7 cm × 10 cm) were assembled with a spacer (1.5 cm thick) and were hold together on a gel-casting stand. The assembly was checked for leakage.

(i) Preparation of separating or running gel

The following solutions were taken in a test tube. Then the tube was swirled gently to mix. To avoid instantaneous polymerization, the tube containing the solution was kept in an ice bath. The solution was used immediately.

Components	Amounts (16%)
30 % acrylamide solution	7 ml
1.5 M Tris-HCl buffer, pH 8.8	3.4 ml
Distilled Water	2.8 ml
10 % SDS solution	120 µl
10 % APS solution	80 µl
TEMED	8 µl

(ii) The separating gel solution was applied to the sandwich

(iii) The top of the gel was covered slowly with a layer of water. It was then allowed to polymerize the gel solution for about one hour at room temperature.

(iv) A layer of water was poured.

(v) Preparation of stacking gel/spacer gel

The following solutions were taken in a conical flask. Then the flask was swirled gently to mix. To avoid immediate polymerization, the flask containing the solution was kept in an ice bath. The solution was used instantly.

Components	Amounts
Acrylamide solution	1 ml
0.5 M Tris-HCl buffer, pH 6.8	1.2 ml
Distilled Water	2.7 ml
10 % SDS solution	50 µl
10 % APS solution	25 µl
TEMED	5 µl

(vi) The stacking gel was poured on the separating gel. Then the Teflon comb was inserted immediately into the layer of the stacking gel solution. Additional stacking gel was added to fill completely the space in the comb. It was taken not to trap air bubbles. The gel solution was allowed to polymerize for about 30 minutes.

(vii) The Teflon comb was carefully removed without tearing the edges of the polyacrylamide wells. After the comb was removed, the wells were rinsed with electrophoresis buffer to remove unpolymerized monomer. The gel wells were filled with electrophoresis buffer.

(viii) The gel sandwich was then attached to upper buffer chamber and the lower buffer chamber was filled with the recommended amount of electrophoresis buffer. The upper buffer chamber was partially filled with the electrophoresis buffer so that the top of the gel sandwich was sunk into the electrophoresis buffer.

Applying electric power supply at a current of 30 mA carried out the electrophoresis. The power supply was disconnected when BPB dye was reached at the mark point of the gel.

(ix) Recovery of the gel

The gel sandwich was removed from the upper buffer chamber, and laid on a sheet of absorbent paper or paper towels. Slide one was removed carefully. Then the gel was removed from the lower plate.

(x) Staining of the gel

After recovery, the gel was stained with staining solution for 12 h at room temperature.

(xi) Distaining of the gel

After 12 h, the gel was removed from the staining solution and the gel was soaked in the distaining solution. When the gel became transparent, it was taken out and rinsed with water.

2.4 Determination of the Protein Concentration by Lowry Method

Reagents

a. Alkaline sodium carbonate solution (20 g/l Na_2CO_3 in 0.1M NaOH solution).

b. Freshly prepared copper sulphate and sodium potassium tartrate solution (5 g/l $CuSO_4.5H_2O$ in 10 g/l Na-K tartrate).

c. Alkaline solution: Mixture of solution a and solution b in the proportion of 50:1 respectively.

d. Folin-Cicolteau's reagent (diluted with equal volume of H_2O, just before use).

e. Standard protein (Bovine serum albumin 10 mg/100 ml in dist. H_2O) solution.

Procedure

For the construction of standard curve 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 ml of the standard protein solution were taken in different test tubes and made up to 1 ml by distilled water.

The protein solution (1 ml) was also taken in duplicate in different test tubes and 5 ml of the alkaline solution (solution-c) was added to the standard protein solution in different test tubes and mixed thoroughly. It was allowed to stand at room temperature for 10 minutes. Then 0.5 ml of diluted Folin-Ciolteau's reagent was added rapidly with immediate mixing and left for 30 minutes. The dark blue color formed was measured at 650 nm against the appropriate blank. By applying the same procedure described above, the absorbance of protein solution was measured and a graph was constructed by plotting the concentrations against their corresponding absorbance (O.D) and from the graph the concentration of protein was determined.

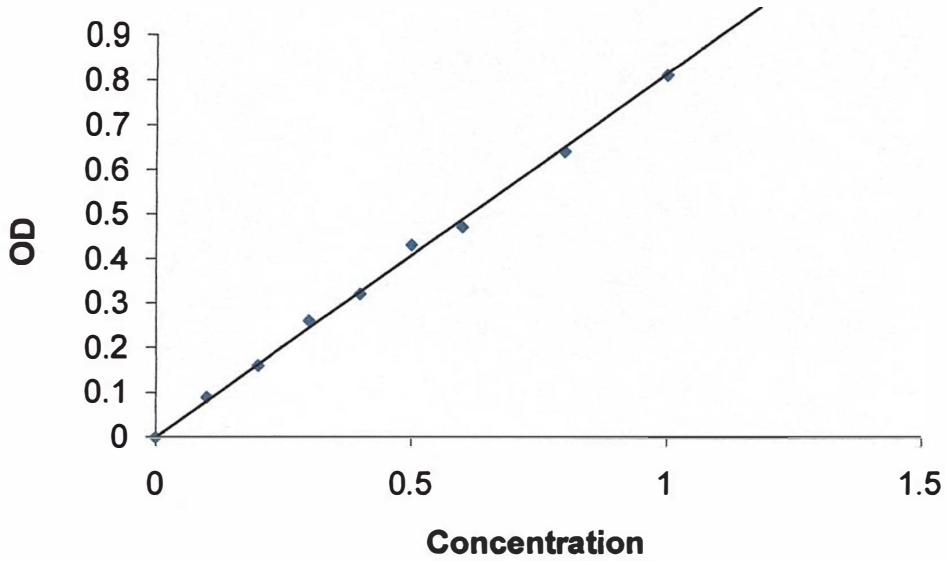


Fig-5: Standard curve of BSA for the determination of protein concentration

2.5 Hemagglutination and Blood Group Specificity Assay

2.5.1 Collection and Preparation of 2% RBCs

Human blood of all groups available (A, B, O and AB) were collected from donors (about 1 ml for every group) in 1% NaCl solution. Blood samples were also collected from mice and hens. All the blood samples were centrifuged at 3, 000 rpm for 4 mins to get 2% RBCs.

2.5.2 Preparation of Hemagglutination Buffer

20 mM Tris-HCl buffer was prepared containing 1% NaCl and 10 mM $CaCl_2$, the pH was maintained to 7.8.

2.5.3 Procedure of Hemagglutination Assay

The hemagglutination assay was performed in 96-well microtiter U-bottomed plates in a final volume of 100 µL containing 50 µL of 2%

suspension of albino rat erythrocytes previously washed with 0.15 M NaCl and 50 µL of two-fold serially diluted lectin solutions. After gentle shaking, the plate was kept at room temperature for 30 minutes; the agglutination titer of the maximum dilution giving positive agglutination was recorded. (Atkinson, H. M, Trust, T. J. *et al.,* 1980).

2.6 Bacterial Agglutination Study

2.6.1 Materials

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

(b) Protein solution

(c) Bacterial cells: *Salmonella enteritidis, Escherichia coli (0157:H18), Listeria monocytogenes* and *Shigella boydii*

2.6.2 Procedure

Bacteria were grown at 37^0C overnight in nutrient broths (liquid nutrient media). After centrifuging at 4000 rpm for 3 mins the precipitate containing the bacterial cells were collected, washed with 20 mM Tris-HCl buffer saline (pH 7.8) and re-suspended in the same buffer. The turbidity of the bacterial cell suspensions must be at least 2.0 at 640 nm.

50 µl of each bacterial suspension was mixed with serial dilution of the partially purified proteins to a final volume of 100 µl in 96 well microtitre plates. The plates were agitated for 2 mins and the mixture was kept at room temperature for 60 mins. Finally bacterial agglutinating activity was monitored by a light microscope.

2.7 Antibacterial Assay

Microorganisms are frequently a cause of prevailing diseases in developing countries. The economic crisis, inefficient public access to medical and pharmaceutical care, emergence of resistant strains, drug toxicity and the side-effects caused by synthetic drugs are some of the main factors influencing the search for the natural products having antimicrobial and antifungal activities. A number of compounds were later synthesized and introduced in clinical practices. Synthesized compounds are necessary to determine their spectrum against various types of gram positive and gram-negative bacteria. The prime objective of performing the antibacterial screening is to determine the susceptibility of the microorganism to any agent and which can be measured *in vitro* by a number of techniques of which antibacterial assay is the most acceptable.

2.7.1 Test Organisms Used for the Study

The pure cultures were collected from the Center for Advanced Research in Sciences (CARS), University of Dhaka, Bangladesh. The following pathogenic bacteria were used for the assay.

Name of test organisms:

Gram positive bacteria: *Listeria monocytogenes*

Gram negative bacteria: *Escherichia coli (0157:H18)*, *Salmonella enteritidis* and *Shigella boydii*

2.7.2 Preparation of Media

The following media were used for the study of antibacterial activity of the above test bacteria.

Ingredient	Amount
Bacto yeast extract	1.0 g
Sodium chloride	0.5 g
Bacto peptone	0.5 g
Distilled water	100 ml
pH	7.2±0.1 at 25 ^{0}C

2.7.3 Procedure

Antibacterial activity of the chitin binding proteins was investigated by the disc diffusion method (Cole, M. D., 1994), performed using sterile petridishes (100 × 15 mm) containing 30 mL nutrient agar. Four species of pathogenic bacteria were seeded separately onto the surface of nutrient agar plates, followed by the placement of a sterile double filter paper disc (5 mm diameter) on the agar surface of each plate. An aliquot (10 µL) of each sample was then added to each disc and the bacterial cells were allowed to grow at 30^{0}C for 12 h. A transparent ring around the paper disc revealed the antibacterial activity.

2.8 Bacterial Growth Inhibition Assay

The bacterial growth inhibition was performed by measuring the bacterial nutrient broths (liquid nutrient medium) in the presence and absence of different concentrations of the protein mixtures by using a titer plate reader at A_{630}. Four bacteria - *Salmonella enteritidis, Escherichia coli (0157:H18), Listeria monocytogenes* and *Shigella boydii* were used for this study. Bacteria were grown for 18 hours at 37^0C in the nutrient broths and the absorbance was adjusted to 0.3-0.35 at A_{630} with liquid nutrient medium. 50 µl of each bacterial suspension was mixed with a serial dilution of the protein samples to a final volume of 100 µl in 96-well microtiter plates. A control without the lectins for each bacterium was used. The plate was agitated at 28^0C and the reading was taken after 8 hours at A_{630}. Finally the percent of bacterial growth inhibition in presence of the potato lectin mixtures was determined according to the formula:

% inhibition = Absorbance of (control-test)/Absorbance of control × 100

2.9 Antifungal Assay

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

2.9.1 Experimental Apparatus and Materials

(a) Filter paper discs (b) Petri dish

(c) PDA and Seaboard media (d) Sterile cotton

(e) Sterile forceps (f) Micropipette (10-100µl)

(g) Bunsen burner (h) Autoclave (KT-30L)

(i) pH meter (j) Incubator (9639A, OSK)

2.9.2 Preparation of Culture Media

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

2.9.3 Preparation of Potato Dextrose Agar (PDA) Media

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

2.9.4 Test Organisms

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

(i) Penicillium species

(ii) Rhizopus species

(iii) Aspergillus nigar

2.9.5 Preparation of Discs

(a) Sample discs: Filter paper discs (5mm diameter) were taken in sterile petri dishs. The fungal mycelia were placed over the solid potato dextrose agar. The sterile filter paper discs were soaked with 50 µl of the lectin samples that is at a dose of 10µg/ml and were distributed over the plates.

(b) Control discs: The control disks were soaked with 50 mM Tris-HCl buffer saline, pH 8.0 and applied on the same plates.

2.9.6 Procedure

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

2.10 Cytotoxic Activity Assay

This bioassay indicates toxicity as well as a wide range of pharmacological activities (e.g. anticancer, antiviral, insecticidal, pesticidal, AIDS etc.) of the compounds. Here *in vivo* lethality bioassay is conducted by using the simple

zoologic organism; brine shrimp naupli (*Artemia salina*, Leach). This bioassay can be used as a convenient monitor for screening and fractionation in the discovery and monitoring of bioactive natural products (McLaughlin, J. L. *et al.,* 1991; Perssone, G. *et al.,* 1980; Meyer, B. N. *et al.,* 1982).

2.10.1 Experimental Design

Materials

a) *Artemia salina* leach (brine shrimp eggs)

b) Sea salt (non-ionized NaCl)

c) Small tank with perforated dividing dam to grow shrimp, cover and a lamp to attract the shrimps.

d) Pipettes

e) Micropipette (10µl- 100µl)

f) Vials (4 ml).

g) Magnifying glass (3×)

2.10.2 Procedure

a) Preparation of simulated seawater

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

b) Hatching of brine shrimp

Artemia salina leach (brine shrimp eggs) collected from the pet shop was used as the test organism. Simulated sea water was taken in the small tank and the shrimp eggs (1.5 g/l) were added to one side of the tank and this side was covered. The shrimps were allowed for one days to hatch and immature as nauplii (larvae). Constant oxygen supply was carried out and constant temperature (around 37°C) was maintained during the hatching time. The hatched shrimps were attracted to the lamp on the other side of the divided tank through dam. These nauplii were taken for this bioassay.

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

Twenty one clean vials were taken for the sample in six concentrations (Three vials for each concentration) and another three vials were also taken for control. With the help of a Pasteur pipette 10 living shrimps were taken to each vial and then 1mg/ml lectin mixtures from both the potato species were added to each vial at the final concentration of 1000, 500, 250, 125, 90 and 60 µg/ml. the volume of each vial was adjusted to 4 ml by the addition of artificial sea water (prepared by dissolving 38.0 gm of NaCl in 1 liter DW). All the tests were performed at around 30°C, under the continuous light regime. Three replicate were used for each experiment. The overall procedure carried in presence and absence of 0.4 mm methyl--α-D-mannopyranoside in artificial sea water.

d) Counting of nauplii

After the incubation of 24 hours, the vials were observed using a magnifying glass. The numbers of survivors in each vial were counted and recorded.

From this, the percentage of mortality was calculated for each dilution. The concentration-mortality data were analyzed by using Probit analysis (Finney, D. J. *et al.,* 1971).

2.11 Antineoplastic Activity Assay

2.11.1 Methods and Materials

The tumor cell concentration per ml were determined using the following procedure with the help of Haemocytometer

$$\text{Cells per ml} = \frac{\text{The average count per square} \times \text{Dilution factor}}{\text{Depth of fluid under cover slip} \times \text{Area counted}}$$

$$= \frac{\text{The average count per square} \times \text{Dilution factor}}{(0.1\ \text{mm})^3}$$

$$= \text{The average count per square} \times \text{Dilution factor} \times 10^4$$

2.11.2 Experimental Animals

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

Animal Care

(i) Cage: Mice were kept in iron cages with wood dust bedding which was changed once a week.

(ii) Temperature, light and humidity: A constant room temperature of 28-30^{0}C and a controlled day length, 14 hours light and 10 hours dark were maintained in the laboratory.

(iii) Food: Pellet diet was collected from ICDDR'B, Dhaka. The nutrient composition of the diet (Per hundred grams of diet) is given below:

Nutrient	In grams
Starch	66
Casein	20
Fat	8
Standard vitamins	2
Salt	4
	Total = 100

2.11.3 Experimental Tumor Model

Transplantable tumor (Ehrlich's Ascites Carcinoma) used in this experiment were obtained from Indian Institute of Chemical Biology (IICB), Calcutta - 700032, West Bengal, India and were maintained in our laboratory in Swiss Albino mice by intraperitoneal (i,p) transplantation.

2.11.4 Transfusion of Ascitic Tumors:

Ascitic fluid was drawn out from different tumor bearing Swiss albino mouse at the respective log phases of tumor cells. A 5 ml syringe fitted with 20 gauge needle was used for this tumor cell aspiration. The freshly drawn fluid was diluted with normal saline and the numbers of tumor cells were adjusted to approximately 3 $\times 10^6$ cells/ml with the help of a Haemocytometer. The viability of tumor cells was checked by the Trypan blue dye (0.4%) exclusion assay. Cell samples showing above 90% viability were used for transplantation.

Tumor suspension of 0.1 ml was injected intraperitoneally to each Swiss Albino mouse. Strict aseptic condition was maintained throughout the transplantation process.

2.11.5 *In vivo* Assessment of the Chitin-binding Lectins as an Antineoplastic Agent

In vivo antineoplastic activity of the isolated lectins was determined by measuring the effect of the proteins on tumor cell growth inhibition.

Procedure

The Ehrlich Ascites Carcinoma (EAC) cells are used to be propagated intraperitonealy in our research laboratory once in two weeks from a donor swiss albino mouse bearing 6-7 days old ascites tumors. The cells were diluted with normal saline and then adjusted to a number of 3×10^6 cells/ml by the help of a haemocytometer. The viability of the tumor cells was above 90% and 0.1 ml of those was injected intraperitoneally to each swiss albino

mouse. After 24 hours the mice were randomly distributed into three groups with at least five mice per group. Two groups of the mice were treated for five days with the lectin mixtures at a concentration of 1.38 mg/kg/day for both the protein samples from Deshi and Sheelbilatee potatoes and the remaining group was used as a control. Mice in each group were sacrificed on the sixth day. The haematological parameters viz. WBC, RBC, Hemoglobin (Hb) content, differential counts etc. were determined by the standard methods (Hudson, L. and Hay, F.C., 1989) using cell dilution fluids and haemocytometer. For this purpose, blood was collected from the mouse by tail puncture method. The total number of viable cells in every mouse of the treated groups was compared with those of control (EAC treated only). Treatment started after 24 hours of tumor transplantation and was continued for 10 consecutive days. On day 12, the blood parameters were used to assay the percentage of inhibition that was obtained by the following formula:

% of inhibition = 100 - {(cells from potato lectin - treated mice/cells from control mice) ×100}

2.12 Biofilm Microtitre Assay of the Sheelbilatee and Deshi Potato Lectins

A biofilm is a structured community of bacterial cells (single or mixture of species) enclosed in a self-produced polymeric matrix. Biofilms are highly hydrated structures, consisting predominantly of water and only 10-20 % bacteria. They have a clear structure of polysaccharide penetrated by minute anatomising water channels as a primitive circulation (Costerton *et al.*, 1994) and gradients of oxygen, nutrients, waste and signalling factors exist. The environmental heterogeneity creates a heterogeneous population of bacterial cells (Mah and O'Toole, 2001), although they typically show

reduced growth compared with planktonic bacteria (Brown *et al.*, 1988). In fact the complexity of biofilm structure and metabolism has led to the analogy of biofilms to tissues of higher organisms (Costerton *et al.*, 1995).

Whilst recognized for about 100 years, detailed molecular study of biofilms only began three decades ago and *Pseudomonas aeruginosa* is among the best studied biofilm formers (O'Toole, 2003). *Pseudomonas aeruginosa* is an opportunistic human pathogen causing a variety of infections and is regarded as a primary cause of death in immunocompromised patients and those with cystic fibrosis or cancer. There is an urgent need to develop novel therapeutic agents effective against *Pseudomonas aeruginosa* because this bacterium exhibits multiantibiotic resistance. This resistance is mediated in part by the formation of surface attached biofilms, which form a physical barrier to antibiotic penetration and provide an altered microenvironment (Wagner and Iglewski, 2008).

Beyond cystic fibrosis, *Pseudomonas aeruginosa* is an important respiratory pathogen in pneumonia and chronic obstructive airways disease. It is also a major colonizer of chronic wounds such as burns and diabetic foot ulcers. Beyond the clinical setting, *Pseudomonas aeruginosa* biofilm formation leads to contamination of industrial and commercial waste pipes.

Method

A modified version of the method described by Stepanovic *et al.* (2000) was employed.

Sterilized, clear, ninety-six well, U-bottomed polystyrene microtitre plates were prepared to decrease evaporation from test wells.

Pseudomonas aeruginosa bacteria were grown at 37^0C overnight in nutrient broths (liquid nutrient media). After centrifuging at 4000 rpm for 3 minutes the precipitate containing the bacterial cells were collected, washed with 20 mM Tris-HCl buffer saline (pH 7.8) and re-suspended in the same buffer. The turbidity of the bacterial cell suspensions must be at least 2.0 at 640 nm.

50 µl of the bacterial suspension was mixed with serial dilution of the partially purified proteins to a final volume of 100 µl in 96 well microtitre plates. The plates were then incubated in a humid environment for 48 h at 37°C.

20 µl of 0.1 % (w/v) Crystal violet solution (filtered through 0.45µm filter paper) was added to each well and stained for 10 minutes at room temperature. The plantonic bacteria were pipetted out by touching the top of the tip at the corner of each well and the wells were washed three times successively with PBS buffer and the plate was air dried for 15 minutes. 150 µl of 95% ethanol was added to each stained well. The dye was allowed to solubilize by covering the plate and was incubated for 10 - 15 minutes at room temperature. The contents were transferred to another polystyrene flat bottomed 96 well plate and the optical densities of the microtitre wells were determined using a plate reader at 570 nm.

2.13 Measurement of β-lactamase Efficiency of the Sheelbilatee and Deshi Potato Lectins Against Two Bacterial Species by Fluorescent End Product Emission

Principle

β-lactamase is produced by some bacteria like E. coli, Acinatobacter sp., Klebsiella sp. Shigella sp. etc. This β -lactamase breaks down the β-lactam

ring of the antibiotic that it cannot destroy the bacteria or inhibit their actions. These bacteria become resistant to the β-lactams (Penicillin, Cephalosporin, Carbapenems and Monobactams).

Some bacteria such as staphylococcus species, Haemophilus influenzae, Gonococci, and most gram-negative enteric rods produce β-lactamases (penicillinases). These enzymes can break the β-lactam ring of the antibiotics producing 6-amino Penicillanic acid. It consists of a five-membered thiazolidine (penam) ring, four-membered beta-lactam ring and a side chain. It gives fluroscence when exposed to UV lamp.

If a β-lactamase inhibitor such as Clavulanic acid is combined with the antibiotics, they can prevent the destruction of the β-lactam ring. Lectins are added with the antibiotics instead of Clavulanic acid to check their β-lactamase efficiency and applied it to the bacteria. If the lectin can prevent the activity of the β-lactamase, then the β-lactum ring of the antibiotic will not be degraded and the degradation product will not give fluorescence in UV light.

Method

E. coli and *Pseudomonas pneumoniae* were grown on the agar plates following conventional methods.

50 μg/μl of each β-lactam antibiotic were separately placed in a microcentrifuge tube. Potato lectins were mixed in a 20 μg/ml concentration to the bacterial solution. Approximately half of a loopful (diameter, 2 mm) of growth of each strain was removed from the agar plate, dispensed in each substrate by brief agitation on a vortex apparatus, and incubated for 1 h at

37°C. After incubation, the tubes were centrifuged in a Microfuge for 1 min to remove bacterial cells. Supernatant fluid from each tube, including each uninoculated substrate control tube, was applied separately onto Whatman 3mm paper and heated at 120°C in an oven for 5 min. The fluorescent intensity of each test spot was then compared with its uninoculated substrate control spot under a long-wave UV lamp and classified as negative, weakly positive, or positive.

2.14 Study of Fluorescence Emission Spectra of the Chitin-binding Lectins from Shilbilati and Deshi Potatoes

Fluorescence measurements of Deshi and Sheelbilatee potato lectins were performed at a protein concentration of 64 μg/ml and 52 μg/ml on a Shimadzu Spectrofluorometer RF-5301 PC at room temperature. The native and treated samples (with NaCl, Ca^{2+} and N-acetylglucosamine) were placed in a 1 cm × 1 cm × 4.5 cm quartz cuvette for measurement. Samples were λex = 280 nm and the λem was recorded in the range of 300 – 400 nm and widths for the excitation and emission monochromators were maintained at 5 nm.

The fluorescence intensity of both the lectins was measured when these were in distilled water (native form) and when 75 mM NaCl, 2 mM $CaCl_2$ and 5 mM N-acetylglucosamine were added to those one after the other.

Chapter-Three

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1 Isolation of Chitin-binding Lectins from Deshi and Sheelbilatee Potato Tubers

The mixtures of chitin-binding lectins were isolated from the two varieties of potatoes by the ion-exchange chromatography followed by affinity chromatography.

Crude proteins were applied to the DEAE-cellulose column and eluted with the same buffer with a linear gradient of NaCl (0.0 to 0.4 M). The eluted fractions having the lectin activity were again applied on a chitin column and were eluted from that column by 0.5 M acetic acid. The eluted fraction was dialyzed against distilled water for 12 hours and then against 10 mM Tris-HCl buffer (pH 8.2) for another 12 hour at 4°C.

3.2 Chromatographic profiles of Deshi potato lectins

F1, F2, F3 and F4, four fractions were collected from the DEAE cellulose column. F1 and F2, the fractions containing lectin activity were applied to the chitin column. The bound fraction of proteins with lectin activity was eluted by 0.5M acetic acid.

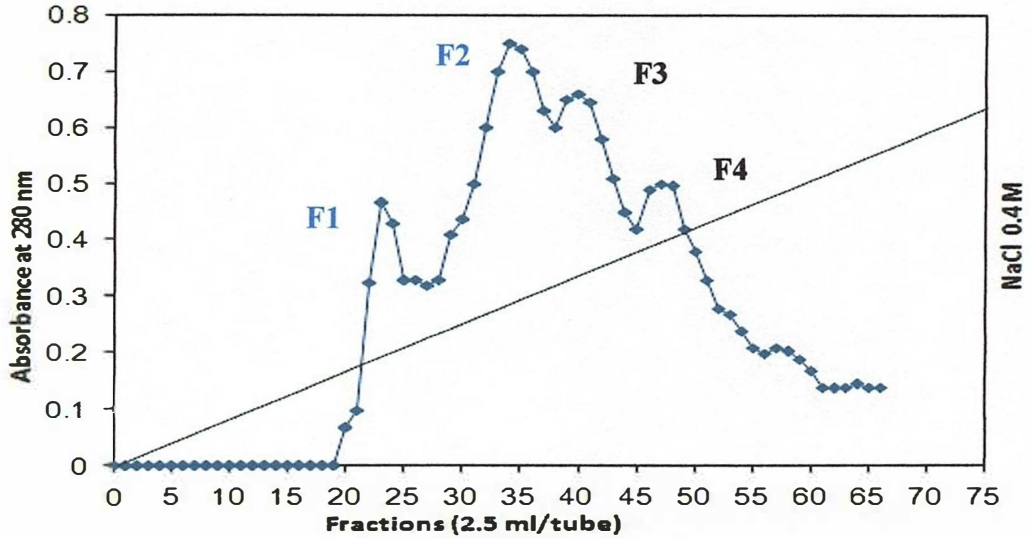


Fig - 6: Crude protein solution from the Deshi potatoes was applied to a DEAE cellulose column (2.5 × 12 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 8.2). Proteins were eluted with the same buffer with a gradually increased NaCl gradient from 0.0 to 0.4 M. The elution profiles were monitored at 280 nm. Fractions (2.5 ml/tube) were collected at a flow rate of 1 ml/min.

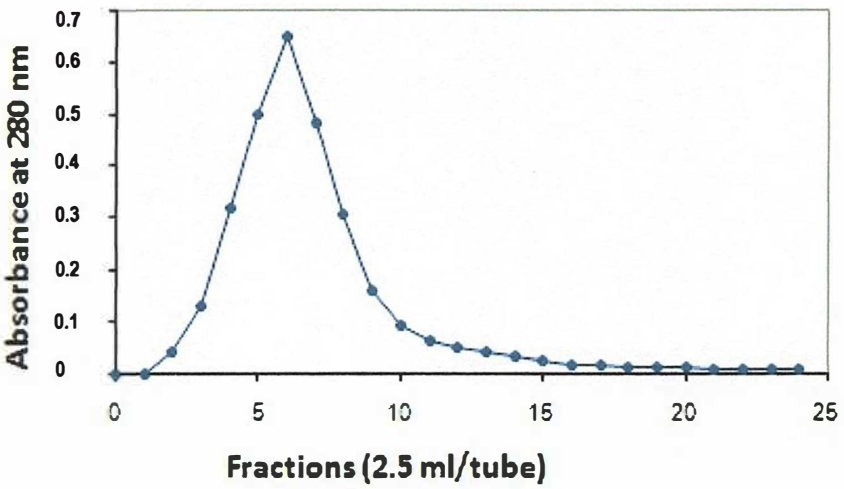


Fig - 7: The eluted proteins were applied to a chitin column (2×25 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 8.2). The proteins were eluted by 0.5 M Acetic acid at a flow rate of 1ml/min (2.5 ml/tube). The elution profiles were monitored at 280 nm.

3.3 Chromatographic profiles of Sheelbilatee potato lectins

F1, F2, F3 and F4, four fractions were collected from the DEAE cellulose column. F1, the fraction containing lectin activity was applied to the chitin column. The bound fraction of proteins with lectin activity was eluted by 0.5M acetic acid.

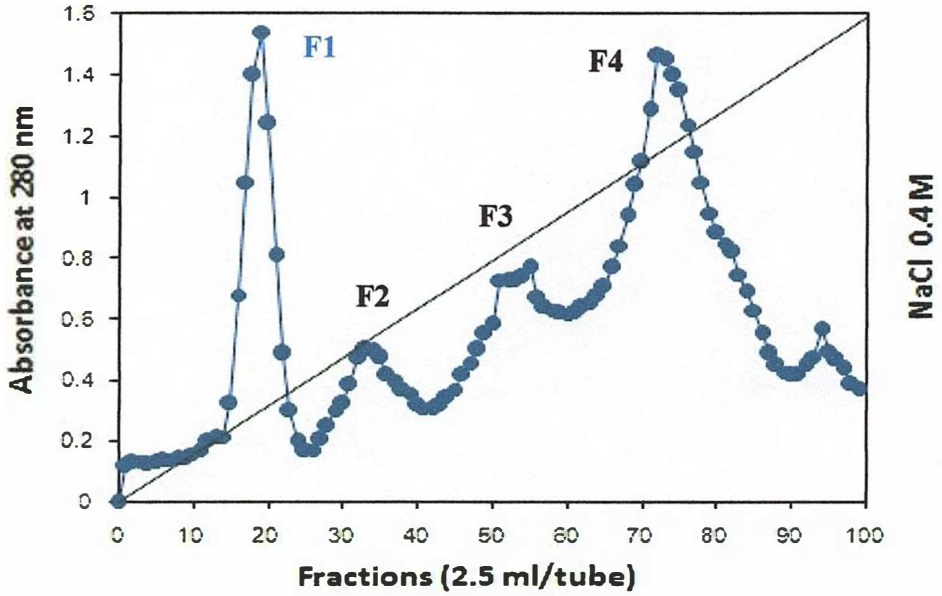


Fig - 8: Crude protein solution from the Sheelbilatee potatoes was applied to a DEAE cellulose column (2.5 × 12 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 8.2). Proteins were eluted with the same buffer with a gradually increased NaCl gradient from 0.0 to 0.4 M. The elution profiles were monitored at 280 nm. Fractions (2.5 ml/tube) were collected at a flow rate of 1 ml/min.

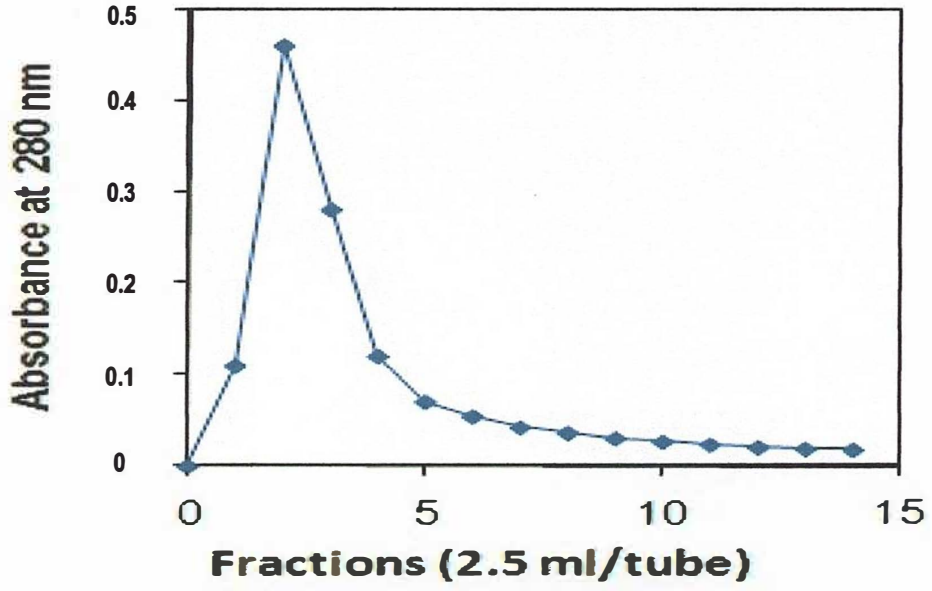


Fig - 9: The eluted proteins were applied to a chitin column (2×25 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 8.2). The proteins were eluted by 0.5 M Acetic acid at a flow rate of 1ml/min (2.5 ml/tube). The elution profiles were monitored at 280 nm.

The isolation of Sheelbilatee and Deshi potato tuber lectins by different chromatographic procedures are shown in a flow chart below (Figure - 10).

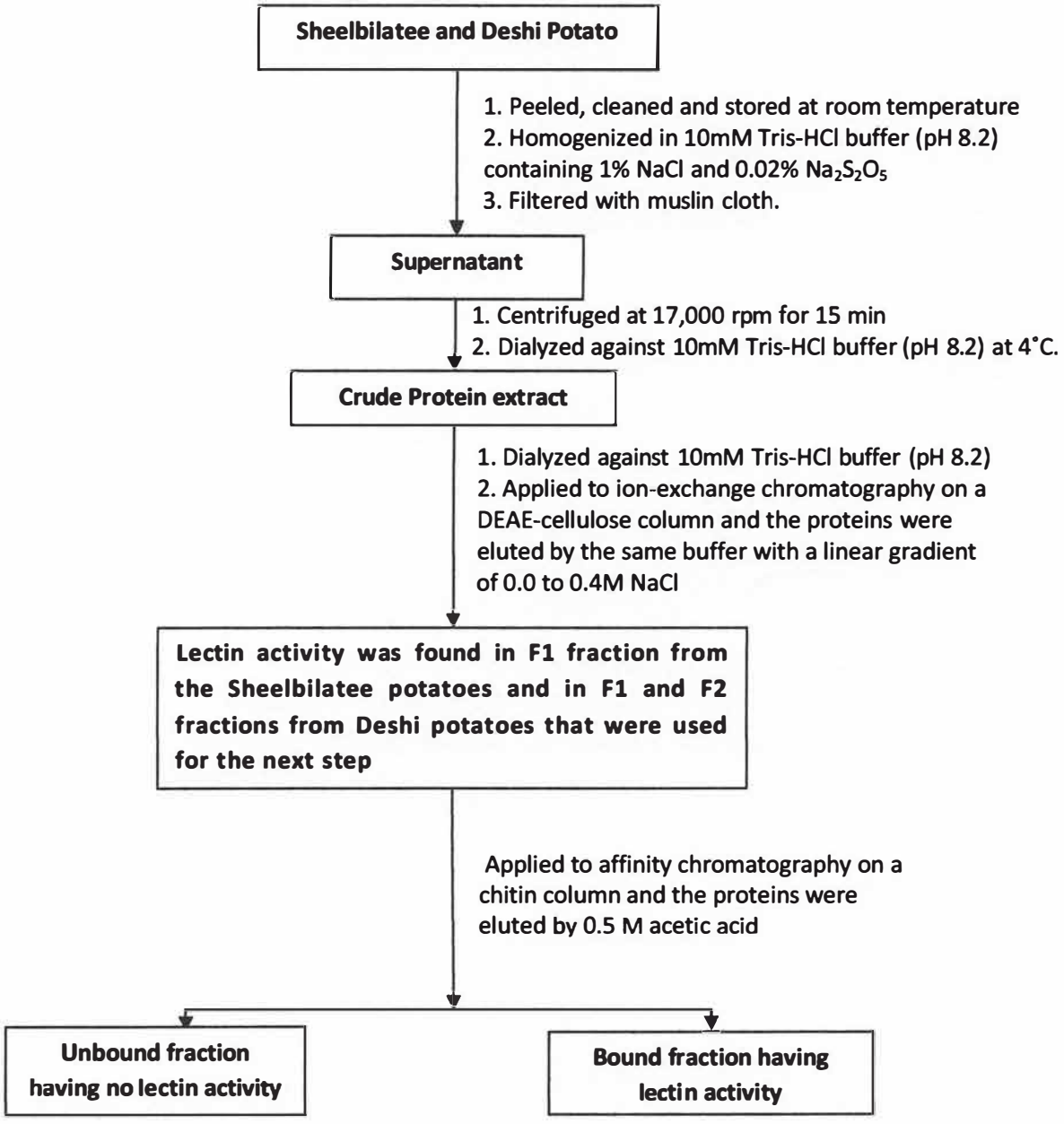


Fig - 10: Flow chart for the isolation of Sheelbilatee and Deshi potato tuber lectins by chromatographic procedures

3.4 Determination of the Molecular Mass by SDS-PAGE

The molecular weight of the isolated protein mixtures were determined by SDS- polyacrylamide gel electrophoresis using Bovine serum albumin (BSA) (MW. 67 kDa), Ovalbumin (MW. 45 kDa), Carbonic anhydrase (MW. 29 kDa), Trypsin inhibitor (MW. 20 kDa) and Lysozyme (MW. 14.6 kDa) as reference proteins. The molecular weight was calculated from the standard curve of reference proteins which was constructed by plotting the molecular weights against relative mobility of the reference proteins. As determined by SDS-PAGE, the molecular weights of the isolated chitin-binding lectins are 22±1 kDa and 24±1 kDa in case of Deshi potato lectins and 21±1 kDa and 23±1 kDa in case of Sheelbilatee potato lectins (Figure - 11).

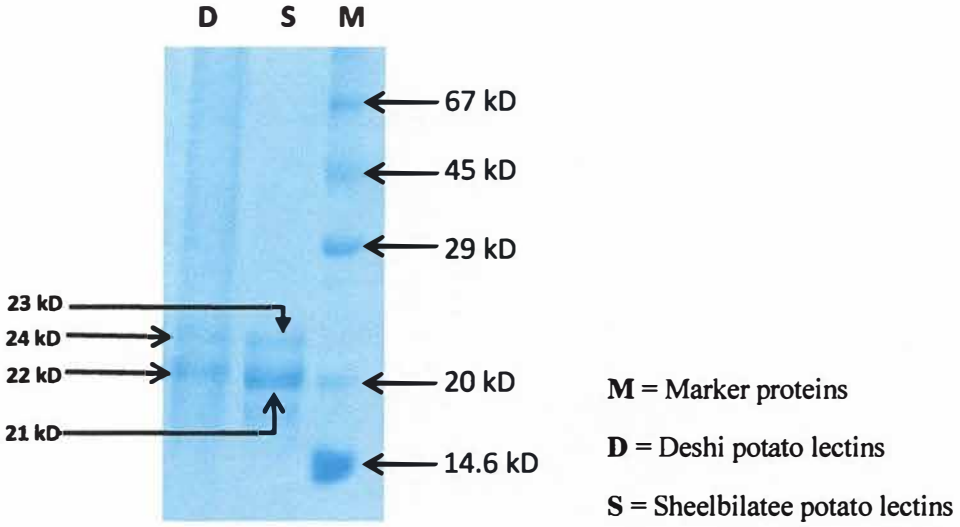


Fig - 11: Molecular weights of the chitin-binding lectins from potatoes shown by SDS-PAGE

Till now, the molecular weight range of all the purified potato lectins was reported to be between 40.0-60.0 kDa (Allen, A. K, Neuberger, A., 1973; Allen, A. K. *et al*, 1978; Park, W. D. *et al*, 1983; Millar, D. J. *et al*, 1992; Kilpatrick, D. C., 1980; Matsumoto, I. *et al*, 1983; Racusen, D. and Foote, M., 1980). Chitinases devoid of lectin activity was also purified from potato and the molecular weight was 16 kDa (Gozia, O. *et al*, 1993). The molecular weights of the isolated chitin-binding lectins are partially different with those studied previously. This result showed that the molecular mass of *Solanum tuberosum* tuber lectin cultivated in Bangladesh does not match with the other potato lectins reported earlier. This difference may be due to the different varieties of potatoes. It was evident that chitinases purified from a variety of potato species showed different molecular weights (Shewry, P. R., 2003) with the diversity of varieties cultivated in different locations.

3.5 Determination of the Protein Concentration by Lowry Method

The absorbance of 1.0 at 280 nm for the chitin-binding lectin mixtures from Deshi potatoes and Sheelbilatee potatoes corresponded to 0.64 mg and 0.62 mg of protein respectively as determined by the Lowry method (Lowry, O. H., 1951) using BSA as standard.

3.6 Hemagglutination and Blood Group Specificity Assay

The chitin-binding lectins showed no specificity to any type of blood. Those proteins agglutinated the RBC cells of every blood type significantly that is are highly active. This result supports the data from the Owen Foundation that the potato lectin agglutinates all human and animal erythrocytes, including bovine, sheep, goat, horse, pig, cat guinea pig, rat, mouse and rabbit. It is not blood group specific(http://www.owenfoundation.com/Health_Science/Lectins_in_Foods.html).

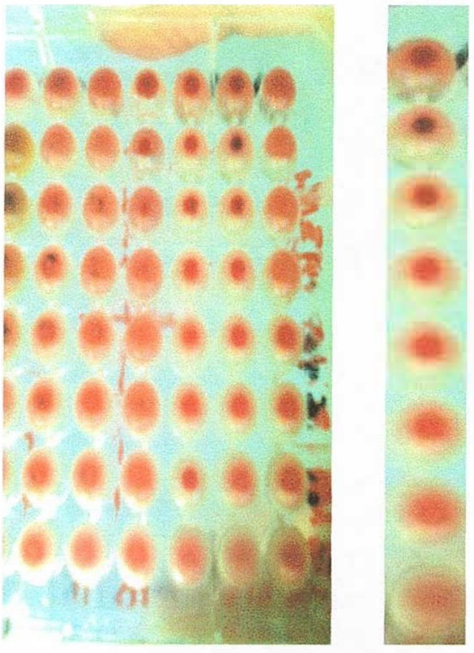


Fig - 12: Hemagglutination activity given by the Deshi and Sheelbilatee potato lectins (serially diluted)

The minimum agglutination concentration was 4 µg/ml for agglutinating the blood of Swiss albino mice. DB1 (*Dioscorea batatas* protein 1) and DB2 (*Dioscorea batatas* protein 2) from *D. batatas* tuber (Gaidamashvili, M. *et al*, 2004) agglutinate rabbit erythrocytes at 2.7 and 3.9 µg/ml respectively; whereas AJL (*Arisaema jacquemontii* lectin) from *A. jacquemontii* tuber (Kaur, M. *et al*, 2006) required 11.5 µg/ml. NNTL showed no blood group specificity as it agglutinated all the blood groups tested. This behaviour was observed in other lectins also, for example, EspecL (*Erythrina speciosa* lectin) and BBL (*Belamyia bengalensis* lectin) and a lectin from *Phaseolus coccineus* seeds (Chen J. *et al* 2009) can agglutinate all human blood groups (Konozy, E. H. E. *et al*, 2003 and Banerjee, S. *et al*, 2004). A different result was also observed for HTTL (*Helianthus tuberosus* lectin), which could not agglutinate any human blood group (Suseelan, K. N. *et al*, 2002) and the

lectin of *Macrotyloma axillare* seeds was observed to agglutinate only human blood group A (Santana, M. A., *et al*, 2008).

3.7 Bacterial Agglutination Study

Agglutination of several bacterial strains confirms the interaction between the lectins and the strains. From the result it became evident that only *Salmonella enteritidis* was sensitive to the presence of Sheelbilatee lectins whereas lectins from the Deshi potatoes could not agglutinate any of bacterial species studied (Figure - 14). The bacteria were strongly agglutinated and the minimum agglutination concentration was 2.0 µg/ml.

Strong agglutination of *Helicobacter pylori* coccids was observed with mannose-specific Concanavalin A (Con - A), fucose-specific *Tetragonolobus purpureas* (Lotus A) and N-acetyl glucosamine-specific *Triticum vulgaris* (WGA) lectins (Khin, M. M. *et al.*, 2000). Concanavalin A also aggregates a variety of Gram negative bacteria specifically *Salmonella typhimurium* (Naughton, P. J. *et al.*, 2000 and Minor, L. L. *et al.*, 1973). It was also reported that EuniS lectin agglutinate *Staphylococcus aureus*, *Streptococcus sp.*, *Klebsiella sp.* and *Pseudomonas aeruginosa* (Oliveira, M. D. L. *et al.*, 2008).

Two distinct levels of antibacterial activity were demonstrated: a low percentage of inhibition, possibly suggesting a bacteriostatic activity and a higher activity that may be bactericidal (Hubert, F. *et al.*, 1996). In fact, observations under the microscope revealed that some bacteria were deformed or showed decreased mobility during the antibacterial assay when compared to control (Tunkijjanukij, S., Olafsen, J. A. 1998). Deshi and

Sheelbilatee lectins did not show this type of low percentage inhibition towards the bacteria. Either they completely agglutinated the bacteria or did not agglutinate at all. It can be compared with the high antibacterial activity exhibited by HSL (Gowda, N. M. *et al*., 2008) which inhibited the complete growth of both gram-positive bacteria (*Staphylococcus sp*. and group D *Streptococci*) and gram-negative bacteria (*Shigella sp*, *Escherichia coli*, *Proteus sp*. and *serratia sp*.).

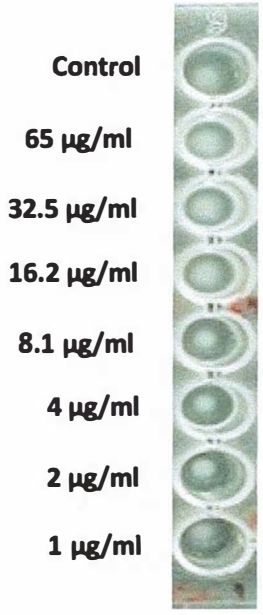
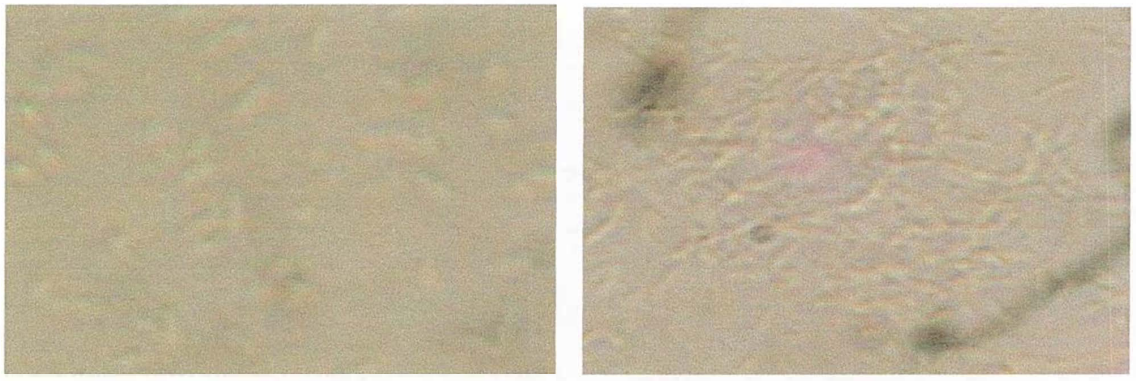


Fig -13: Bacterial agglutination activity given by the serially diluted mixture of Sheelbilatee potato lectins

Concanavalin A aggregates a variety of Gram negative bacteria specifically *Salmonella typhimurium* (Le Minor, L. *et al*, 1973; Naughton, P. J. *et al*, 2000) and the sites of Con A-binding are thought to be exposed to the sugars of the bacterial lipopolysaccharide and the O-antigen factor 1 (Leiner, I. E., 1976). This data is comparable with the results obtained here.

These results indicated that the potato lectins recognized the surface molecules on the gram-negative bacteria. The bacterial agglutination might be due to the presence of cognate glycan antigen on the cell surface of the induced bacteria. It was reported that potato lectin interact with some phytobacteria (Ghanekar, A., Pérombelon, M. C. M., 1980). From these findings we can suggest that Sheelbilatee lectins have lectin properties that are responsible for the agglutination of bacteria.



***Salmonella enteritidis* (control)**

***Salmonella enteritidis* (agglutinated by the Sheelbilatee lectins at the concentration of 65 µg/ml**

Fig - 14: Bacterial agglutination activity of Sheelbilatee potato lectins against *Salmonella enteritidis*

3.8 Antibacterial Assay

Both Deshi and Sheelbilatee potato lectins exhibited notable antibacterial activity against the tested pathogenic bacteria *Escherichia coli (0157:H18)*, *Listeria monocytogenes*, *Salmonella enteritidis* and *Shigella boydii.* From Figure - 15, Figure - 16, Figure - 17 and Figure - 18, it becomes quite evident that the lectins were most active against *Listeria monocytogenes* among the four bacterial species. Each disk did contain 25 µg of the lectin mixtures.

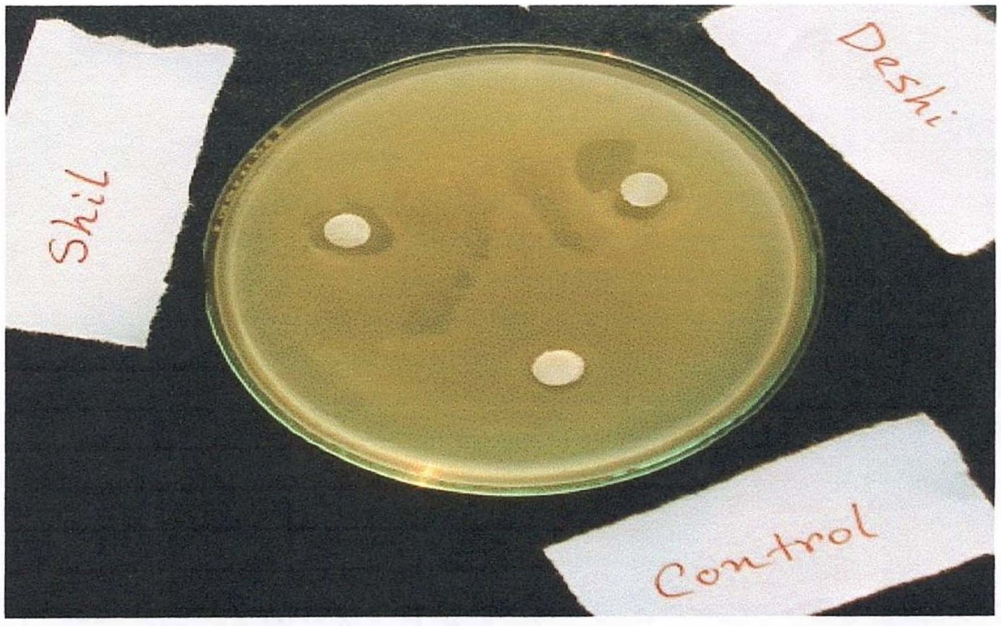


Fig- 15: Antibacterial activities of Deshi and Sheelbilatee potato chitin-binding lectins against *E. coli* (0157:H18)

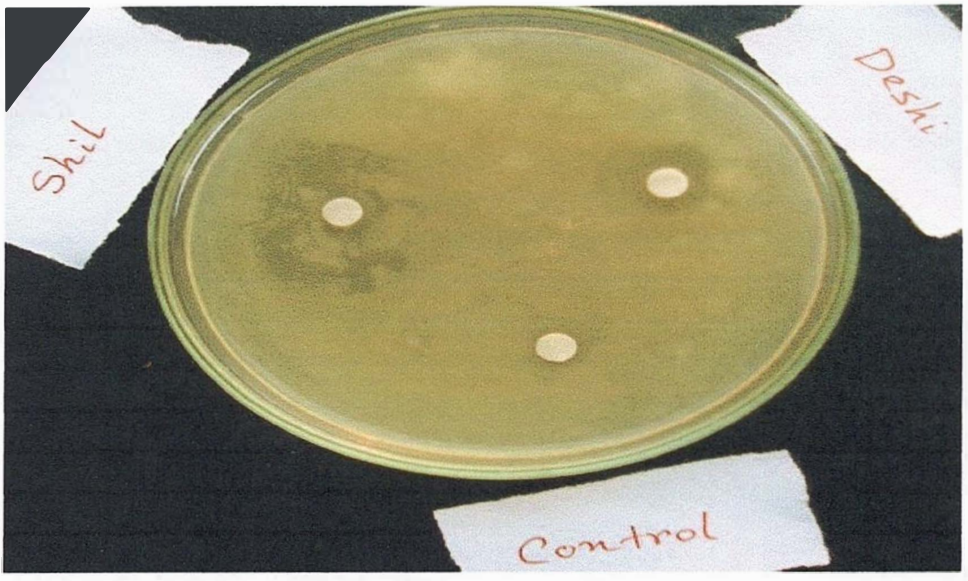


Fig- 16: Antibacterial activities of Deshi and Sheelbilatee potato chitin-binding lectins against *Listeria monocytogenes*

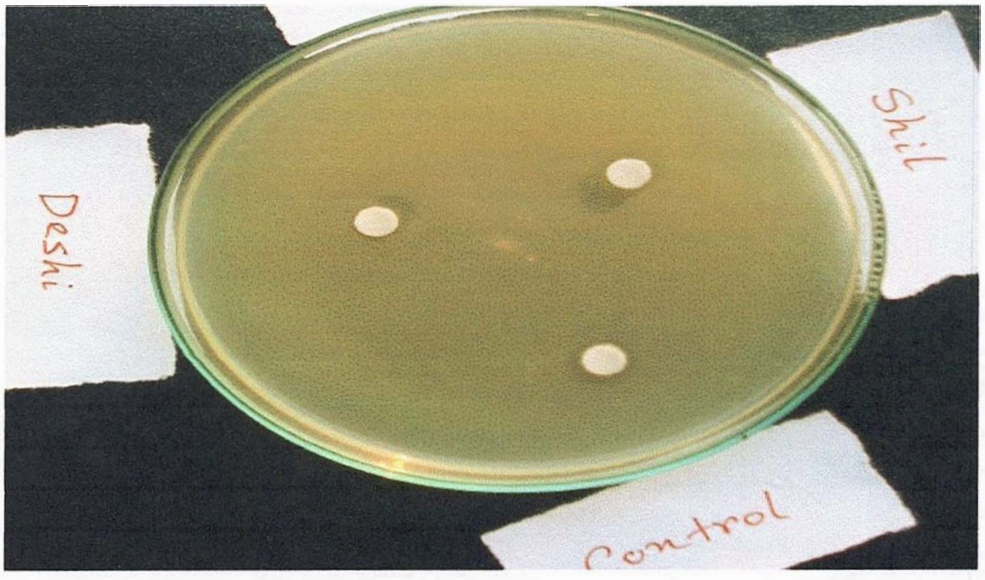


Fig- 17: Antibacterial activities of Deshi and Sheelbilatee potato chitin-binding lectins against *Salmonella enteritidis*

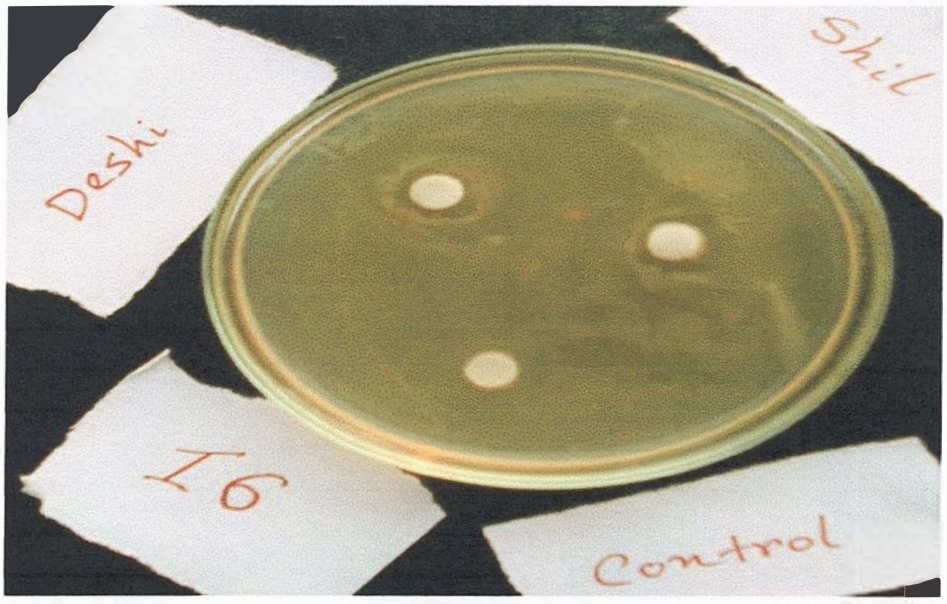


Fig- 18: Antibacterial activities of Deshi and Sheelbilatee potato chitin-binding lectins against *Shigella boydii*

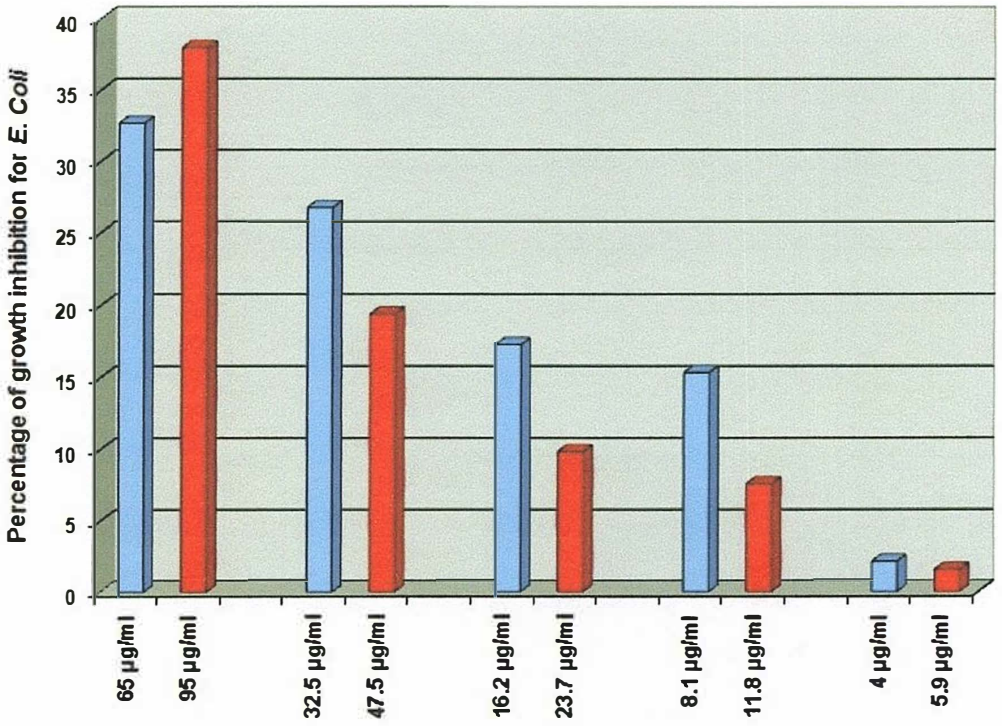
The studied lectin showed a remarkable antibacterial activity against all the tested bacteria. Proteins from seed extracts of some plant species showed higher antibacterial activity than the other parts of the plant (Basile, A. *et al.*, 1997) and have an important role in the protection of seeds against microbial invaders. In recent years, several reports have appeared worldwide concerning reduced activity of ciprofloxacin against *Salmonellae typoid* and *Shigella dysenteriae* (Rahman, M. M. *et al*, 2005; Renuka, K. *et al*, 2005; Taneja, N. *et al*, 2005). Plant extract and other naturally occurring compounds are found to be as an alternative of various drugs. It was reported that various plant extracts showed higher antimicrobial activity (Kim, Y. S. and Shin, D. H., 2005; Saĕdıç *et al*, 2002). Lectins in higher plants defend against pathogenic bacteria and fungi by recognizing the infecting microorganisms via binding, thereby preventing their subsequent growth and multiplication (Etzler, M. E., 1986).

An outstanding feature of the antibacterial activity of the isolated lectins is their nonselective activity against several species of human pathogenic bacteria. By following the disk diffusion method, a lectin from Eugenia uniflora L. seeds showed such strong antimicrobial activities against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella sp.* with a minimum inhibitory concentration (MIC) of 1.5 µg/ ml (Oliveira, M. D. L., 2008).

3.9 Bacterial Growth Inhibition Assay

The tuber lectins from Deshi and Sheelbilatee potato varieties inhibited the growth of a number of pathogenic bacterial strains: *Escherichia coli (0157:H18)*, *Listeria monocytogenes*, *Salmonella enteritidis* and *Shigella boydii* at different concentrations (Figure - 19, Figure - 20, Figure - 21 and Figure - 22).

In Figure - 19, the highest cell growth inhibition for *E. coli* was 32.5% in the presence of 65µg/ml of Sheelbilatee lectins whereas 95µg/ml of Deshi lectins inhibited those bacteria by 37%. So Sheelbilatee lectins were found to be more active in comparison to Deshi lectins.



Blue Colour indicates Sheelbilatee lectins whereas red colour indicates

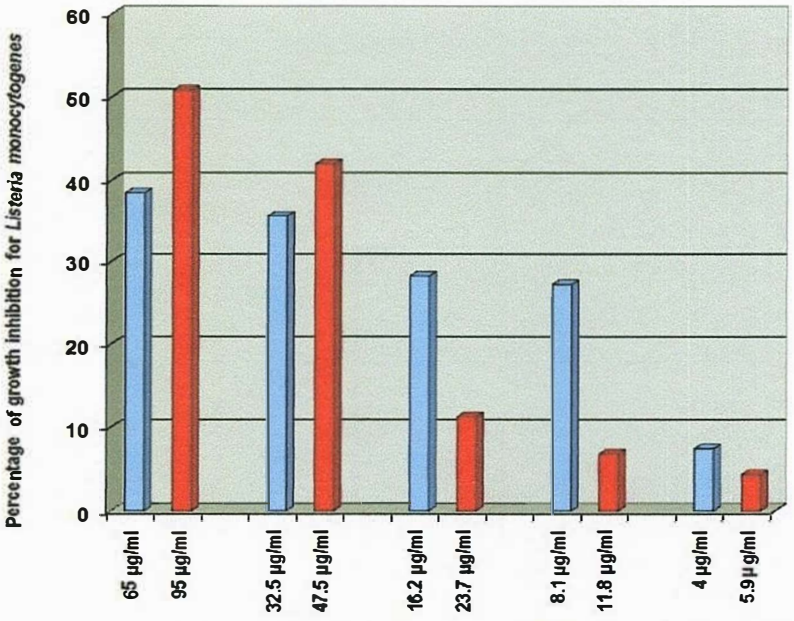
Fig - 19: *E. coli* growth inhibition by Deshi and Sheelbilatee potato lectins

In Figure - 20, the highest cell growth inhibition for *Listeria monocytogenes* was 38% in the presence of 65µg/ml of Sheelbilatee lectins whereas the bacteria were inhibited by 50% in the presence of 95µg/ml of Deshi lectins. In Figure - 21, the highest cell growth inhibition for *Salmonella enteritidis* was 37% in the presence of 65µg/ml of Sheelbilatee lectins whereas 95µg/ml of Deshi lectins inhibited those bacteria by 36%.

In Figure - 22, the highest cell growth inhibition for *Shigella boydii* was 8% in the presence of 65µg/ml of Sheelbilatee lectins whereas the bacteria were inhibited by 27% in the presence of 95µg/ml of Deshi lectins. In this case, Deshi lectins showed more activity than the Sheelbilatee lectins against these bacteria.

It was reported that heat-stable serine protease inhibitor from the potato tuber inhibited growth of a variety of bacterial strains, *Straphylococcus aureus* and *E. coli* (Kim, M. H., 2006); and EuniS lectin showed inhibitory activity against *Staphylococcus sp, Bacillus subtilis, E. coli* and *Pseudomonas aeruginosa* (Oliveira *et al*, 2008).

A novel antimicrobial protein (AP1) was purified from leaves of the potato (*Solanum tuberosum*, variety MS - 42.3) with a procedure involving ammonium sulphate fractionation, molecular sieve chromatography with Sephacryl S-200 and hydrophobic chromatography with Butyl-Sepharose using a FPLC system. The inhibition spectrum investigation showed that AP1 had good inhibition activity against five different strains of *Ralstonia solanacearum* from potato or other crops, and two fungal pathogens, *Rhizoctonia solani* and *Alternaria solani* from potato (Feng, J., *et al,* 2003).

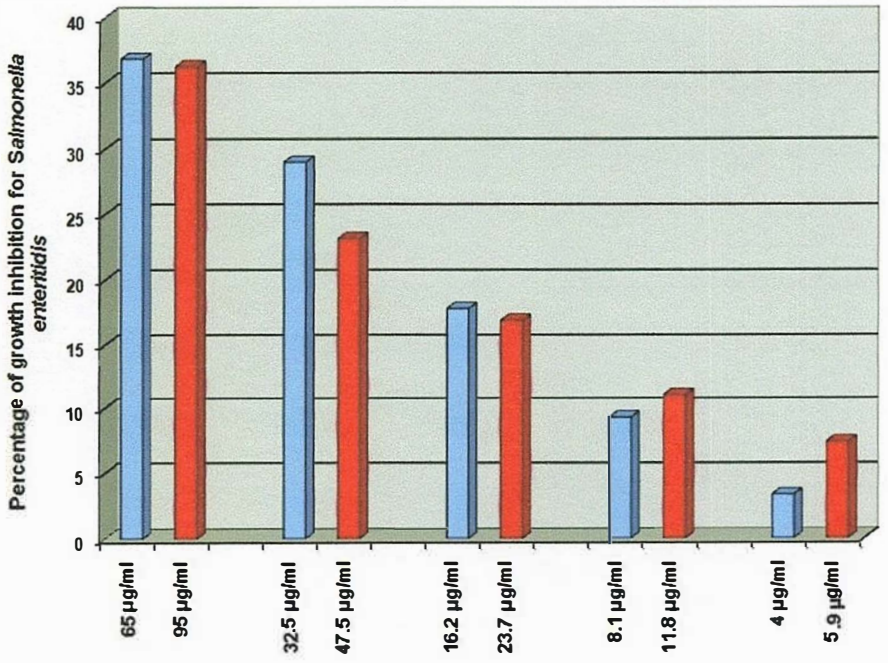


Blue colour indicates Sheelbilatee lectins whereas red colour indicates Deshi lectins

Fig - 20: *Listeria monocytogenes* growth inhibition by Deshi and Sheelbilatee potato lectins

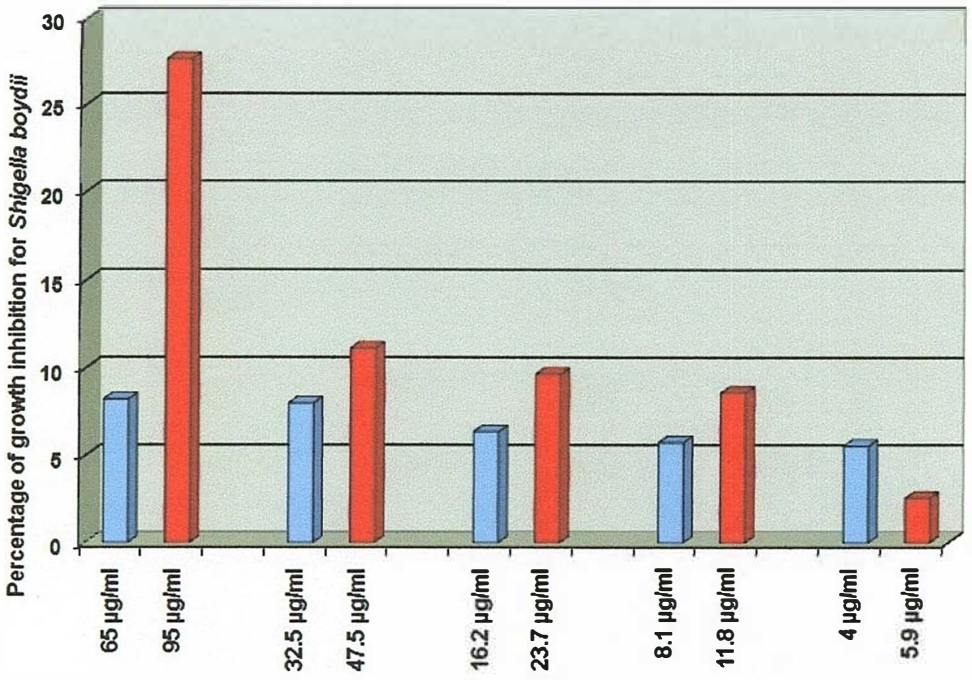
A T-antigen binding lectin from marine invertebrate, sea cucumber (*Holothuria scabra)* showed marked bacterial growth inhibition. About 5 µg of HSL was able to inhibit efficiently the growth of *Staphylococcus sp.* as indicated by decrease in 50% of turbidity, whereas 15 µg inhibited complete growth. HSL also inhibited effectively the growth of other gram-negative (*Serratia sp.*, *Proteus sp.*, *Shigella sp.* and *E. coli*) and gram-positive (Group D Streptococci) bacteria, indicating its broad spectrum antibacterial effect.

The result obtained here supports the above findings and at the same time are in accordance with the bacterial agglutination and *in vitro* antibacterial activity performed earlier.



Blue Colour indicates Sheelbilatee lectins whereas red colour indicates Deshi lectins

Fig - 21: *Salmonella enteritidis* growth inhibition by Deshi and Sheelbilatee potato lectins



Blue Colour indicates Sheelbilatee lectins whereas red colour indicates Deshi lectins

Fig - 22: *Shigella boydii* growth inhibition by Deshi and Sheelbilatee potato lectins

The highest percentage of growth inhibition (50%) was found in case of *Listeria monocytogenes* when a concentration of 95µg/ml of Deshi potato lectins was applied. *Shigella boydii* was the least susceptible against both the lectins whereas the maximum growth inhibition was 27% obtained against Deshi lectins. Based on concentration of the proteins, Sheelbilatee potato lectins were found to be more active than the Deshi potato lectins against most of the bacteria in almost every concentration. A comparative analysis of the growth inhibition percentage of different bacterial species against Deshi and Sheelbilatee lectins are shown in Figure - 23.

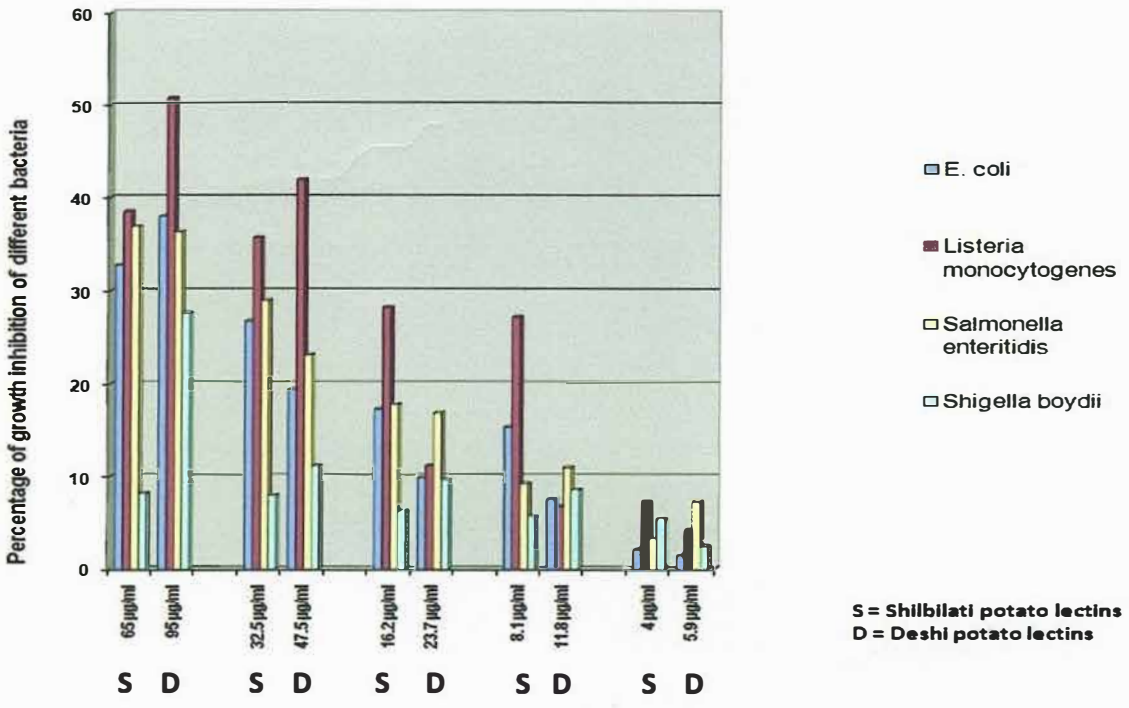


Fig - 23: A comparative analysis of the growth inhibition percentage of different bacterial species against Deshi and Sheelbilatee lectins

3.10 Antifungal Assay

Both Deshi and Sheelbilatee potato lectins exhibited notable antifungal activity against the tested fungal species *Rhizopus species*, *Penicillium species* and *Aspergillus niger*. From Figure - 24, Figure - 25 and Figure - 26, it becomes quite evident that the lectins were most active against *Aspergillus niger* among the four fungal species. Throughout this experiment each disk did contain 50 µg of lectin mixtures.

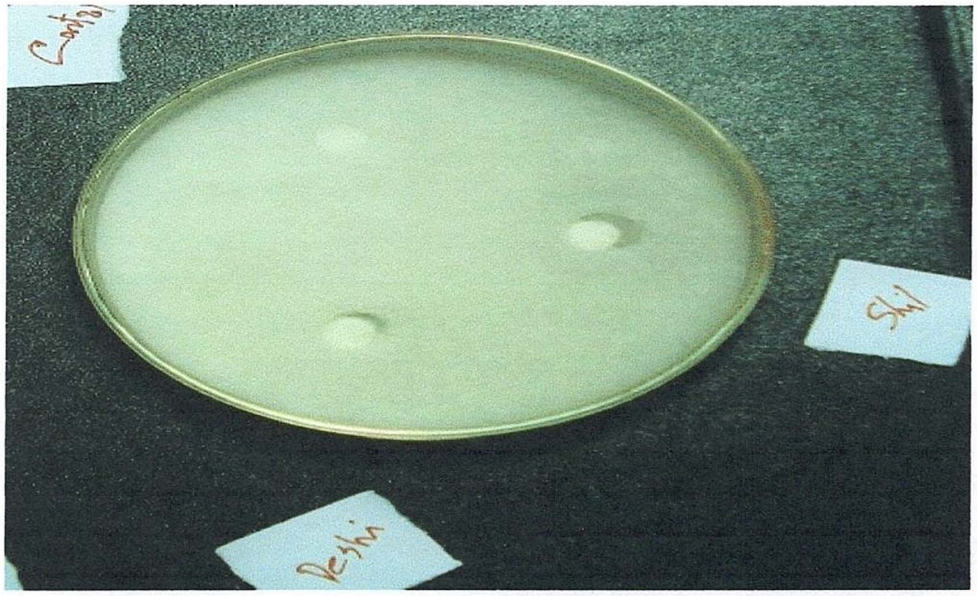


Fig- 24: Antifungal activities of Deshi and Sheelbilatee potato chitin-binding lectins against *Rhizopus species*

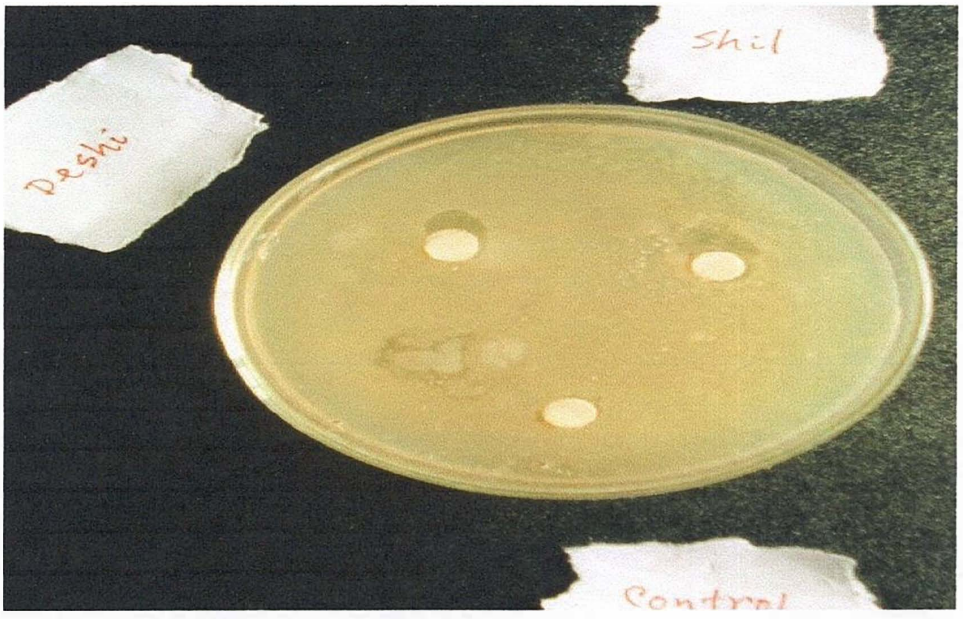


Fig- 25: Antifungal activities of Deshi and Sheelbilatee potato chitin-binding lectins against *Penicillium species*

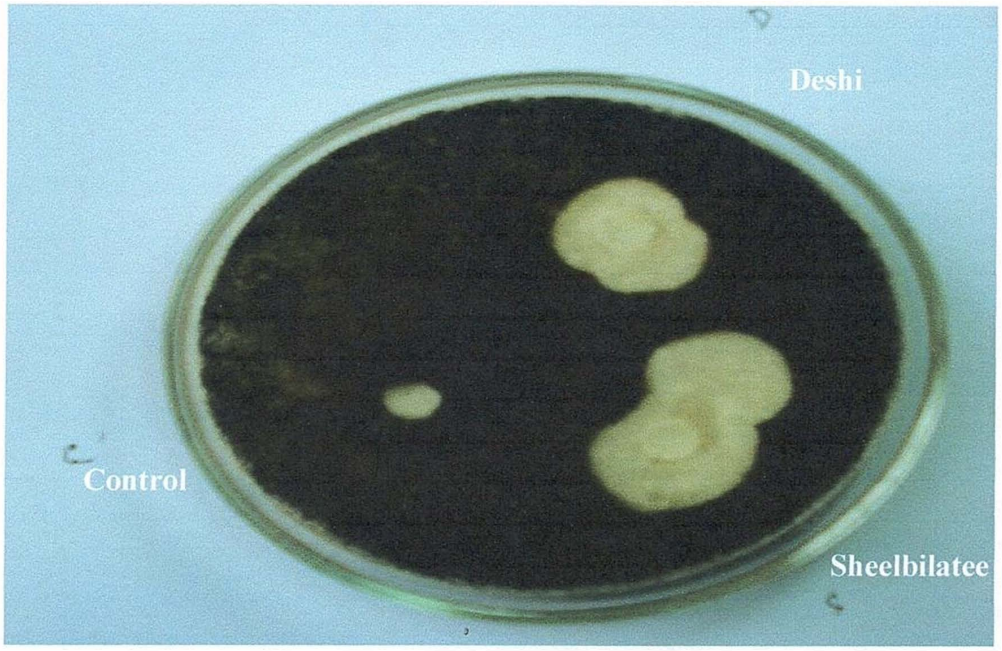


Fig- 26: Antifungal activities of Deshi and Sheelbilatee potato chitin-binding lectins against *Aspergillus niger*

Chitin-binding lectins have been isolated from different sources; including bacteria, insects, plants, and mammals (Campos-Olivas, R. *et al*, 2001; Rebers, J. E. and Willis, J. H., 2001; Suzuki, M. *et al*, 2002; Van Dellen, K. *et al*, 2002) and most of them have shown antifungal activity. It was reported that *Stinging nettle* rhizomes lectin, *Klaxveromyces bulgaricus* lectin, a lectin-like protein from *Amaranthus caudatus* and a chitinase-like lectin from *Urtica diocia* possessed the anti-fungal activities (Broekaert, W. F. *et al*, 1989; Verheyden, P. *et al*, 1995). Several plant proteins capable of inhibiting fungal growth *in vitro* have been isolated and characterized. Among these proteins, called antifungal proteins (AFPs), glucanases and chitinases (Mauch, F. *et al.*, 1988), thaumatin-like proteins (Hejgaard, J. *et al.*, 1992; Vigers, A. J. *et al.*, 1991; Woloshuk, C. P. *et al.*, 1991), several

families of basic-cysteine-rich peptides (Broekaert, W. F. *et al.*, 1997), chitin-binding proteins (Raikhel, N. V. *et al.*, 1993), ribosome-inactivating proteins (Leah, R. *et al.*, 1991), aspartic proteinases (Guevara, M. G. *et al.*, 2002, 2004) and proteinase inhibitors (Terras, F. *et al.*, 1993) have been found. AFPs may be part of the preformed defense barriers or may be induced upon perception of a micro-organism. There is evidence that these proteins may have a direct antimicrobial activity *in vivo*. In particular, previous studies have shown enhanced resistance to microbial pathogens by transgenic plants overexpressing some of these antimicrobial proteins (Dixon, R. A. *et al.*, 1996).

Previously antifungal properties of potato lectin and chitinases were studied by following their effects against early developmental stages of *Fusarium oxysporum* (Gozia, O. *et al*, 1993).

So, the antifungal activities of Deshi and Sheelbilatee potato lectins showed good correlation with the activities of chitinases studied previously.

3.11 Cytotoxic Activity Assay

Till now a lot of chitinases with and without the lectin activity was reported to be purified. Some lectins are toxic and showed cytotoxicity against brine shrimp nauplii (Allen, A. K. *et al*, 1978).

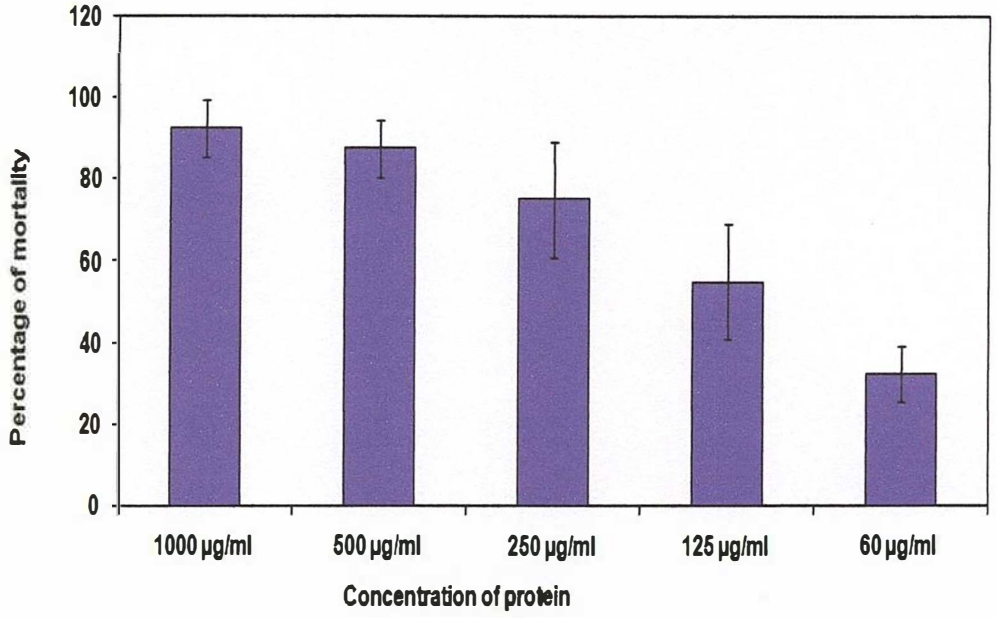


Fig- 27: Percentage of mortality of brine shrimp nauplii treated with Sheelbilatee potato lectins after exposure for 24 hours

Our present data showed the mortality rate of brine shrimp nauplii was increased with the increase of concentration of the lectin. The graph shows the percentage of mortality of the brine shrimp nauplii against different concentrations of Sheelbilatee lectins (Figure - 27). The LC_{50} value is 75 µg/ml (determined by using Probit analysis) indicating the cytotoxicity is quite high. In comparison, the LC_{50} value for Deshi potato lectin is 90 µg/ml (Figure - 28).

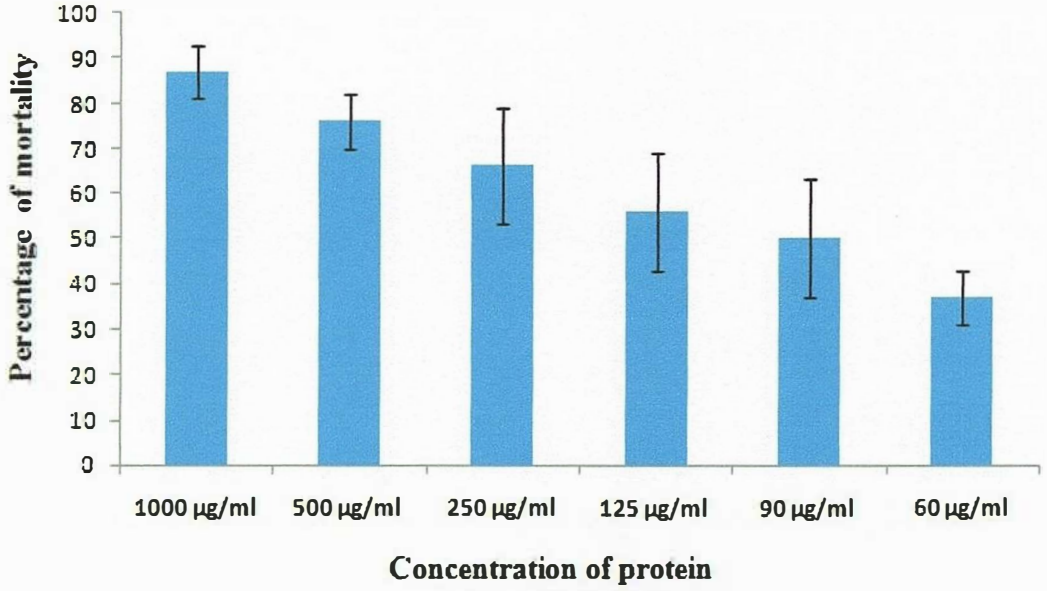


Fig- 28: Percentage of mortality of brine shrimp nauplii treated with Deshi potato lectins after exposure for 24 hours

Plant lectins can induce human peripheral blood mononuclear cells to kill red blood cells (RBC) from different species selectively. Cytotoxicity was induced by both mitogenic components of phytohemagglutinin-P (PHA), erythroagglutinating (E-PHA) and leukoagglutinating (L-PHA), and nonmitogenic lectins wheat germ agglutinin (WGA) (MacDermott R. P. *et al*, 1976). Areas of epithelial cell necrosis and even zones of complete epithelial cell degradation are seen in biopsies of the stomach and intestine of mammals (Lorenzsonn V., Olsen W. A., 1982). In general, lectins alter host resistance to infection, cause failure to thrive and can even lead to death in experimental animals (Nachbar, M. S. *et al*, 1980).

Our present data showed the mortality rate of brine shrimp nauplii was increased with the increase of concentration of the lectin. The graph shows the percentage of mortality of the brine shrimp nauplii against different

concentrations of Sheelbilatee lectins. The LC_{50} value, 75 µg/ml indicates the cytotoxicity quite high. In comparison, the LC_{50} value for Deshi potato lectin is 90 µg/ml (determined by using Probit analysis).

In the combined cytotoxicity study of Sheelbilatee and Deshi potato lectins using the of brine shrimp nauplii bioassay, it was found that the Sheelbilatee potato lectins are more cytotoxic in comparison to Deshi potato lectins (Figure - 29).

The LC_{50} values of *Trichosanthes cucumerina* Seed lectin, *Nymphaea nouchali* tuber lectin and Diamond potato lectin are 261 µg/ml, 120 µg/ml and 65 µg/ml respectively (Kabir, S. R. *et al*, 2012; 2011; 2010).

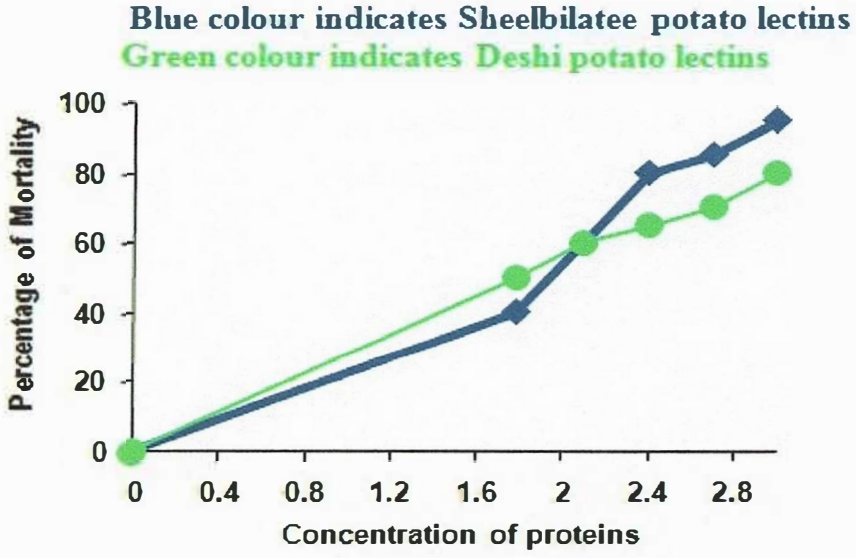


Fig- 29: Combined cytotoxicity study of Sheelbilatee and Deshi potato lectins using the of brine shrimp nauplii bioassay.

3.12 Antineoplastic Activity Assay

Deshi potato lectins and Sheelbilatee potato lectins showed 83.04% and 79.84% activity *in vivo* while inhibiting the Ehrlich's Ascites Carcinoma

(EAC) cell growth at the dose of 1.38 mg/Kg/day. Antiproliferative activity was also found in some other seed lectins, such a lectin from *Phaseolus coccineus* seeds showed *in vitro* antineoplastic activity against L929 cells (Chen, J. *et al.*, 2009). Other galactose binding lectin as for example lectin from the roots of a Chinese herb *Astragalus membranaceus* was found to inhibit the proliferation of HeLa and K562 cell lines (Yan, Q. *et al.*, 2010). Figure - 30 shows the reduction in number of viable EAC cells in Swiss Albino mice treated with the potato lectins.

Several kinds of plant lectins have been reported to prevent the multiplication of cancer cells (Liu, B. *et al.*, 2010) and due to the differences in their sugar specificity each lectin exhibits variation in their antiproliferative effect against tumor cell lines (Yan, Q. *et al.,* 2010).

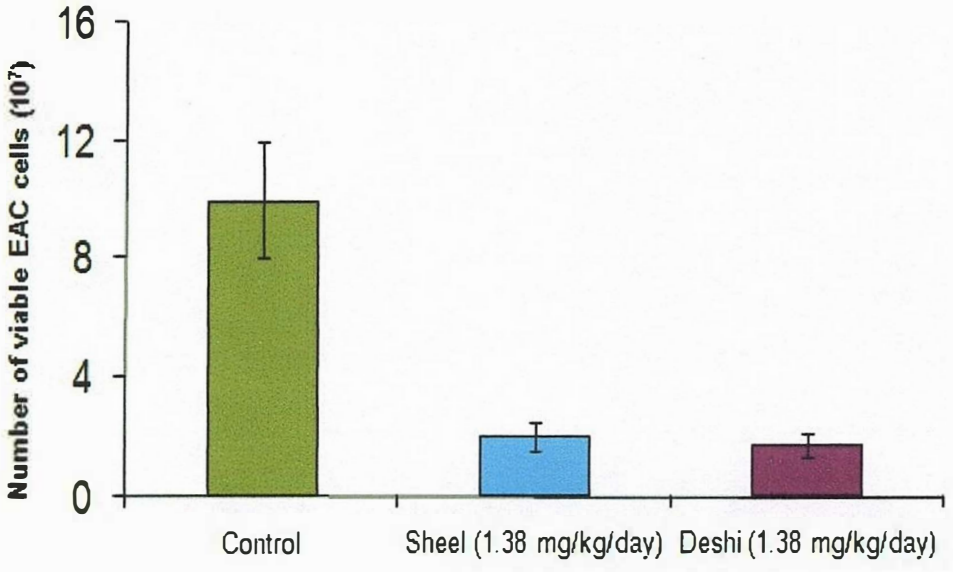


Fig- 30: Numbers of EAC cells counted by a light microscope in the Swiss Albino mice on the 6th day after treating 5 days continuously with the lectins isolated from Shilbilatee and Deshi Potatoes

EAC cell growth was also studied *in vivo* in mice by using jackfruit lectin that inhibited 21.8, 40.2, 57.5 and 83% of growth when administrated as 50, 100, 150 and 200 μg/day respectively (Ahmed, H. *et al.*, 1988). A lectin from *Curcuma amarissima* rhizomes also showed *in vitro* antiproliferative activity against a breast cancer cell line (BT 474) and showed the high antiproliferative activity with an IC50 of approx. 21.1 μg (Kheeree, N. *et al.*, 2010).

Figure - 31 shows the reduction of WBC cells in the control mice in comparison to the lectin-treated mice that supports the event take place in this disease. In Figure - 32, it can be seen that the mice that were treated with Deshi potato lectins have higher number of RBC cells in contrast to the control mice.

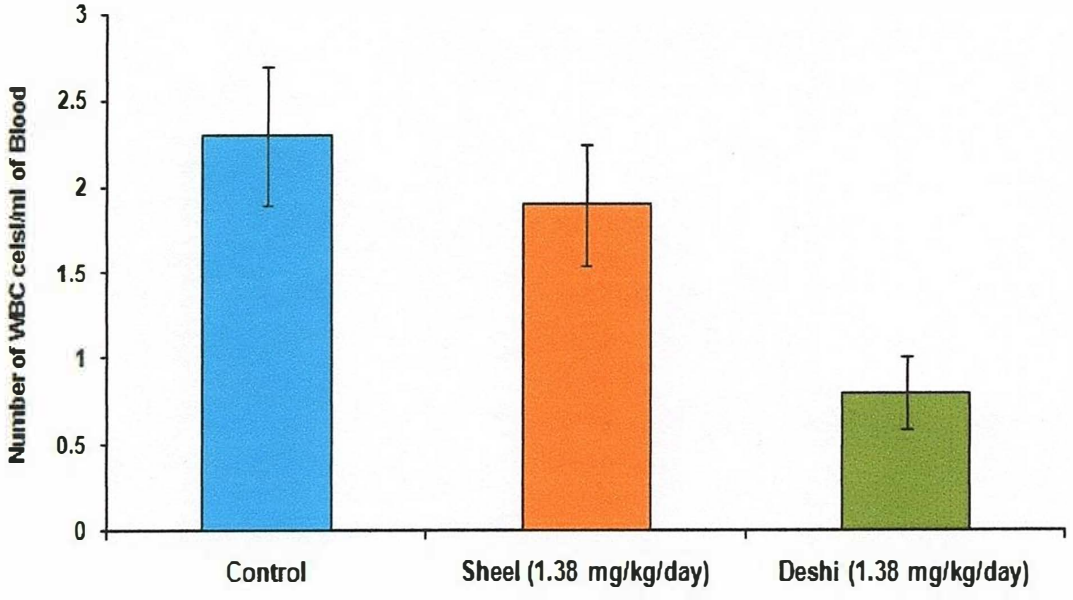


Fig- 31: Numbers of WBC cells counted by a light microscope in the Swiss Albino mice treated with the Sheelbilatee and Deshi Potato lectins lectins on the 12th day after tumor inoculation

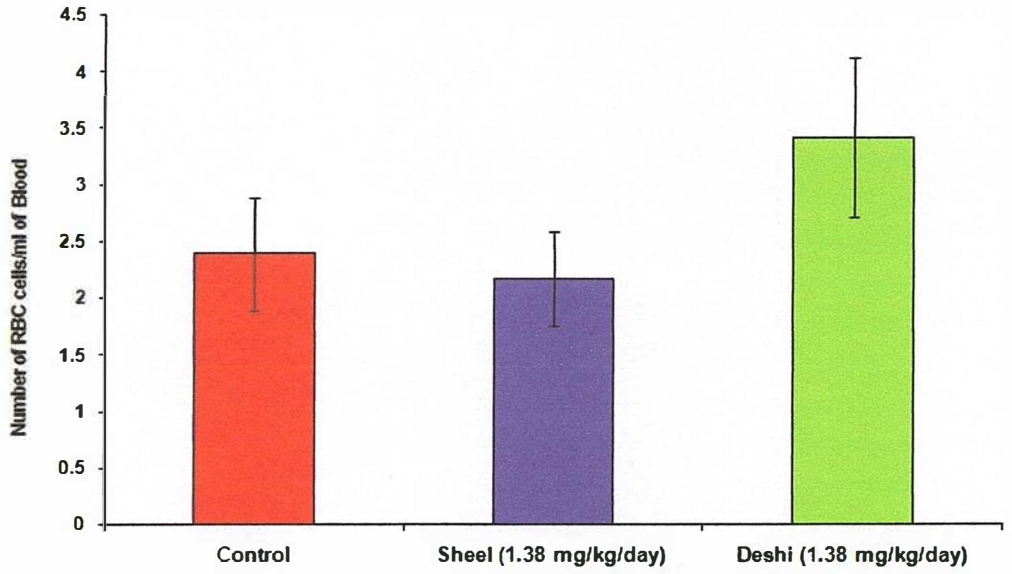


Fig- 32: Numbers of RBC cells counted by a light microscope in the Swiss Albino mice treated with the Sheelbilatee and Deshi Potato lectins lectins on the 12th day after tumor inoculation

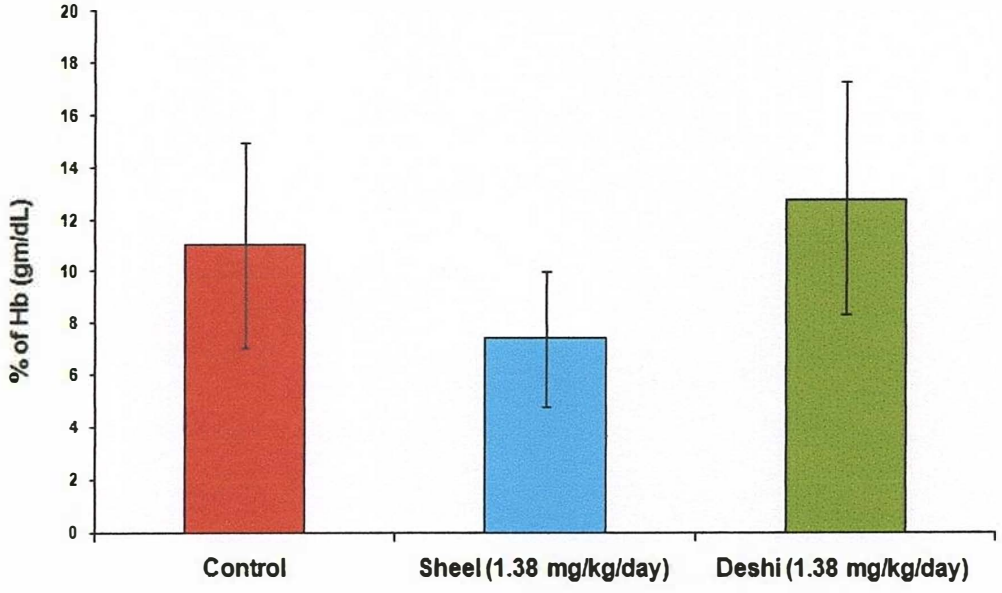


Fig- 33: Percentage of Hemoglobin of the Swiss Albino mice treated with the lectins isolated from Sheelbilatee and Deshi Potato lectins on the 12th day after tumor inoculation

But unexpectedly the mice treated with Sheelbilatee potato lectins were found to have even lesser number of RBC cells than the control mice. This may have happened due to the cytotoxicity of Sheelbilatee lectins compared to Deshi potato lectins. This finding is also supported by the percentages of hemoglobin in the control and treated mice (Figure - 33).

Recent studies on laboratory mice have shown that plant lectins might be employed in the formulation of novel cancer diagnostic and therapeutic approaches in pre-clinical stages (Chang, C. P. and Lei, H. Y., 2008). Several typical lectins such as mistletoe lectin, ricin and Wheat germ agglutinin have been reported to possess remarkable antitumour activities by inducing apoptosis in cancer cells (Liu, B. *et al.*, 2010). Animals with cancer showed an almost complete inhibition of tumour growth when mistletoe lectins were applied to the right side (Zarcovic, N. *et al.*, 2001).

To inhibit the most notorious diseases cancer, a few studies were reported against EAC cells (Ahmed, H. *et al.,* 1988, Akev, N. *et al.*, 1993). The present study was carried out to evaluate the antitumor effects of Potato tuber lectins on EAC bearing mice. The result showed that these two lectin mixtures decreased the EAC cell growth 83.04% and 79.84%. This result was very significant when compared with Jackfruit lectin that inhibited only 21.8, 40.2 and 57.5% of EAC cell growth at 50, 100 and 150 µg/day respectively (Ahmed, H. *et al.,* 1988). The information obtained from the present investigation is insufficient to use the Deshi and Sheelbilatee lectins as an anticancer agent, but the lectins have the possibility to be developed as a powerful anticancer agent due to their potent antiproliferative activity against EAC cells.

3.13 Biofilm Microtitre Assay of the Sheelbilatee and Deshi Potato Lectins

It became quite evident that the lectin mixtures from Deshi potatoes as well as Sheelbilatee potatoes inhibited the formation of biofilm by *Pseudomonas aeruginosa* as compared to the control (Figure - 35 and Figure - 36). The inhibitory effect decreased with the dilution of the protein sample indicating its concentration-dependent nature. The Deshi potato lectin mixture was found to be more active as 65µg/ml of Deshi potato lectins gave the same value for 80µg/ml of Sheelbilatee potato lectins.

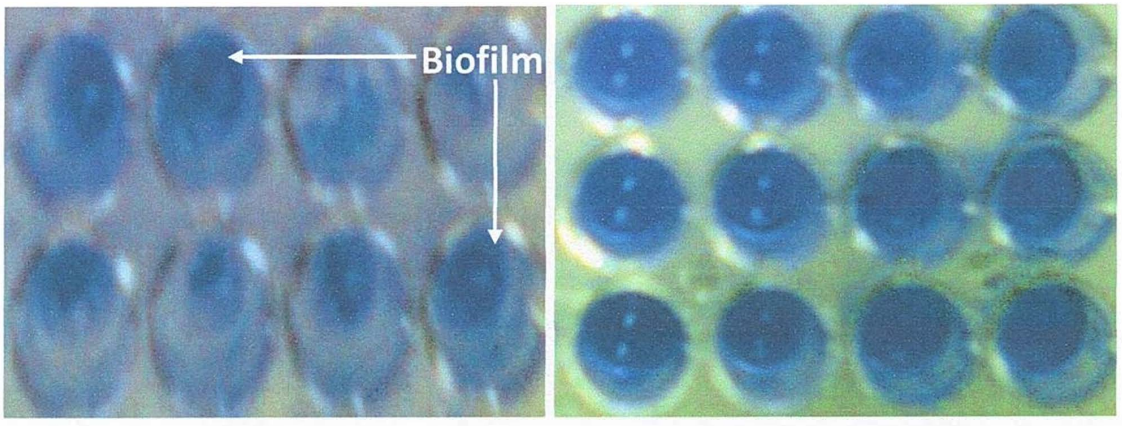


Fig- 34: Formation of biofilm by *Pseudomonas aeruginosa* (before and after the solubilization with the dye).

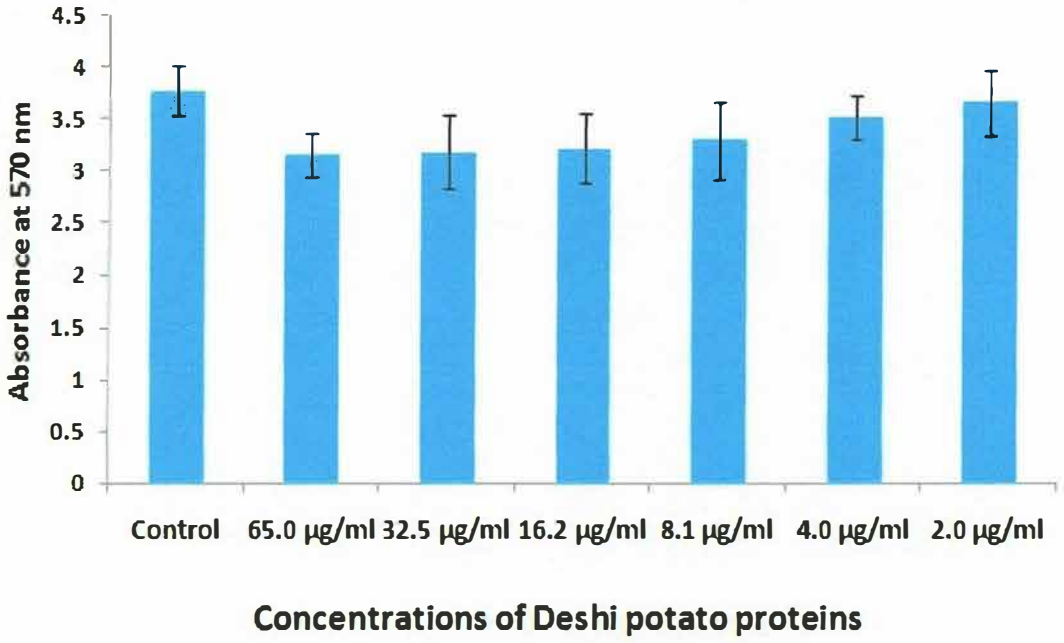


Fig- 35: Formation of biofilm by *Pseudomonas aeruginosa* in the presence of Deshi potato lectins in various concentrations.

A protein from *Labramia bojeri* seeds (Labramin) inhibited the biofilm formation by *Streptococcus mutans* and *Streptococcus sobrinus* (Oliveira, M. R. T. R. *et al.*, 2007). Recently a glycopeptide dendrimer GalAG2 inhibited the biofilm production by *Pseudomonas aeruginosa* as evidenced by using the steel coupon assay (Kadam, R. U. *et al.*, 2011).

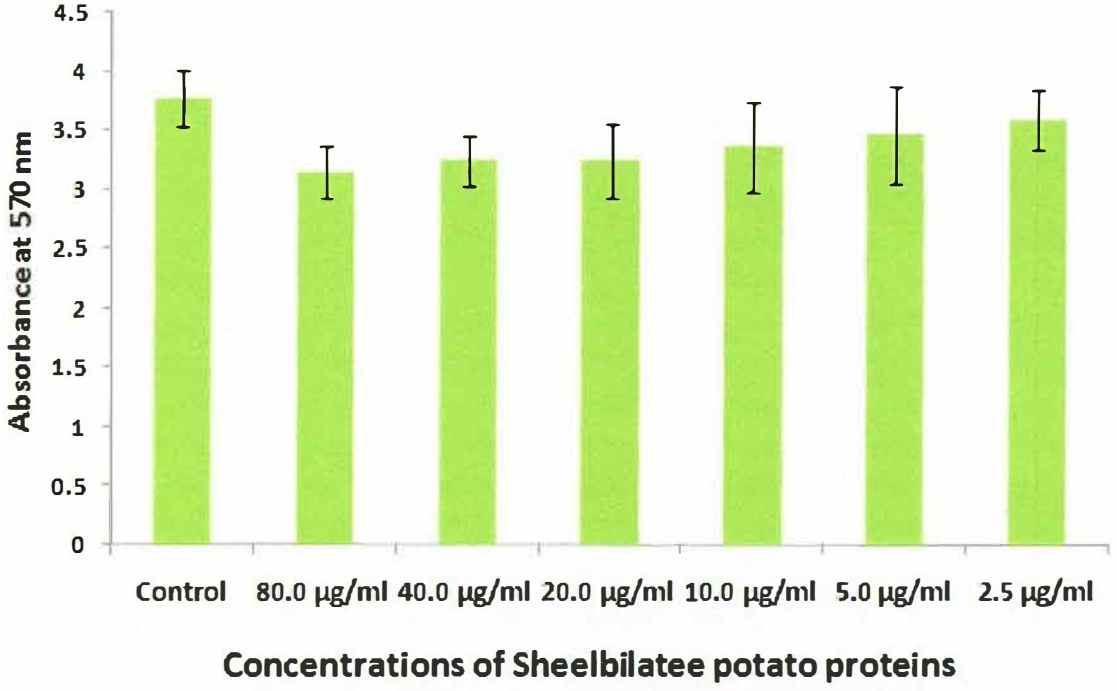


Fig- 36: Formation of biofilm by *Pseudomonas aeruginosa* in the presence of Sheelbilatee potato lectins in various concentrations

Another study showed that six lectins extracted from the seeds of Leguminosae family (ConA, ConBr, DVL, DGL, CFL and VML) by affinity chromatography (Sumner and Howell, 1936; Moreira *et al.*, 1983; Oliveira *et al.*, 1991; Moreira and Cavada, 1984; Moreira *et al.,* 1996; Cavada *et al.*, 1998) inhibited the adherence of five Streptococci species (Teixeira, E. H. *et al*, 2006).

Seven plant lectins were extracted from their source by ammonium sulfate precipitation and were purified by sugar-affinity chromatography on glucose or mannose-derivatized column (Naeem *et al.*, 2007). CCL, Con A and PHA lectins were purified using metal charged EGTA-sepharose (Naeem *et al.*, 2006). All the plant lectins tested, inhibited both the adherence and biofilm

production by *Streptococcus mutans* in a concentration dependent manner (Islam, B. *et al.*, 2009).

In the present experiment, it was shown that interference with Sheelbilatee and Deshi potato lectins can lead to significant inhibition and dispersion of biofilms, which clearly marks these protein mixtures as a valuable target for developing biofilm inhibitors.

3.14 Measurement of β-lactamase Efficiency of the Sheelbilatee and Deshi Potato Lectins Against Two Bacterial Species by Fluorescent End Product Emission

It was found that the end product of each β-lactam substrate could be detected on the filter paper without subsequent electrophoretic separation after brief heating at 120°C for 5 min (Chen J. *et al*, 2009).

Both Sheelbilatee and Deshi potato lectins were weakly positive against *E. coli* but failed to show any positivity against *Klebsiella pneumoniae* (Figure - 37).

For the potato tuber lectins, the β-lactamase activity of the lectin against *E. coli* was classified as weakly positive when the fluorescent intensity of the spot was faint but discernibly greater than the fluorescent intensity of the spot obtained for *Klebsiella pneumoniae* and for the uninoculated substrate control.

Though it is a very preliminary result, it can be assumed that the potato tuber lectins might have acted as weak β-lactamase inhibitors to prevent the destruction of the β-lactam ring.

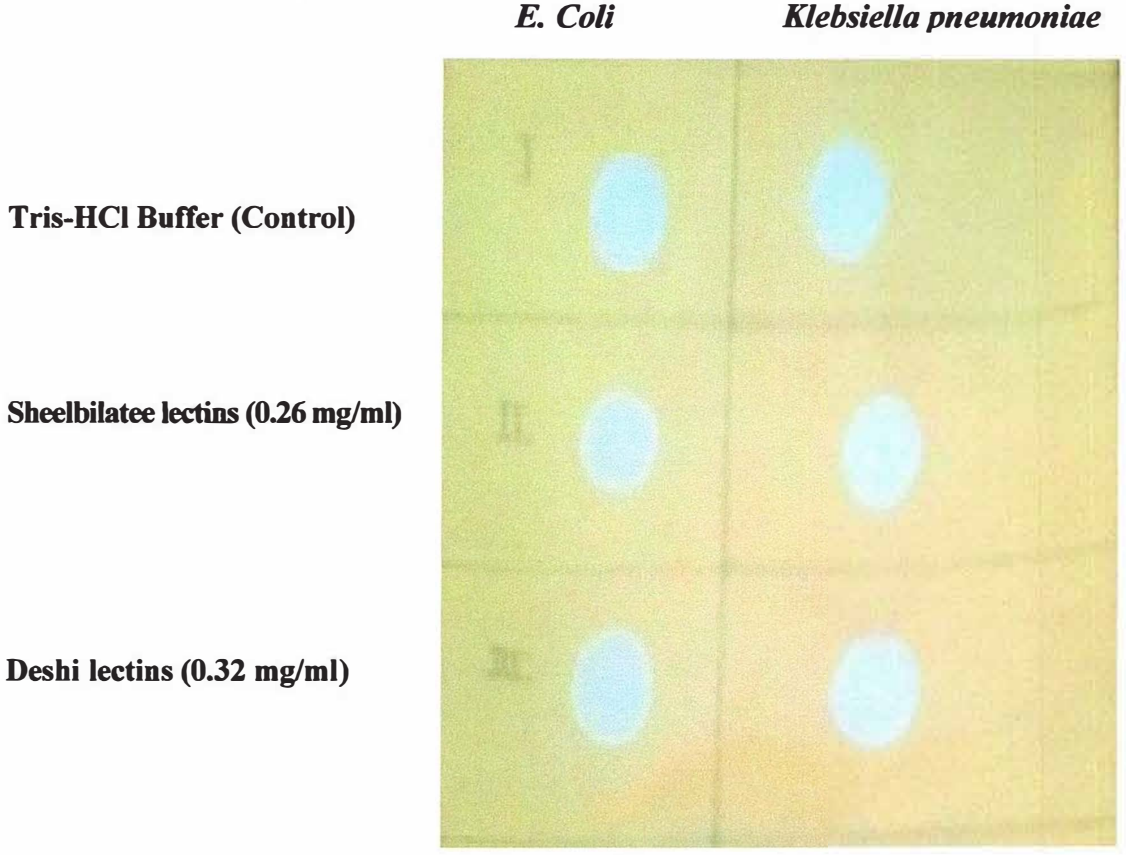
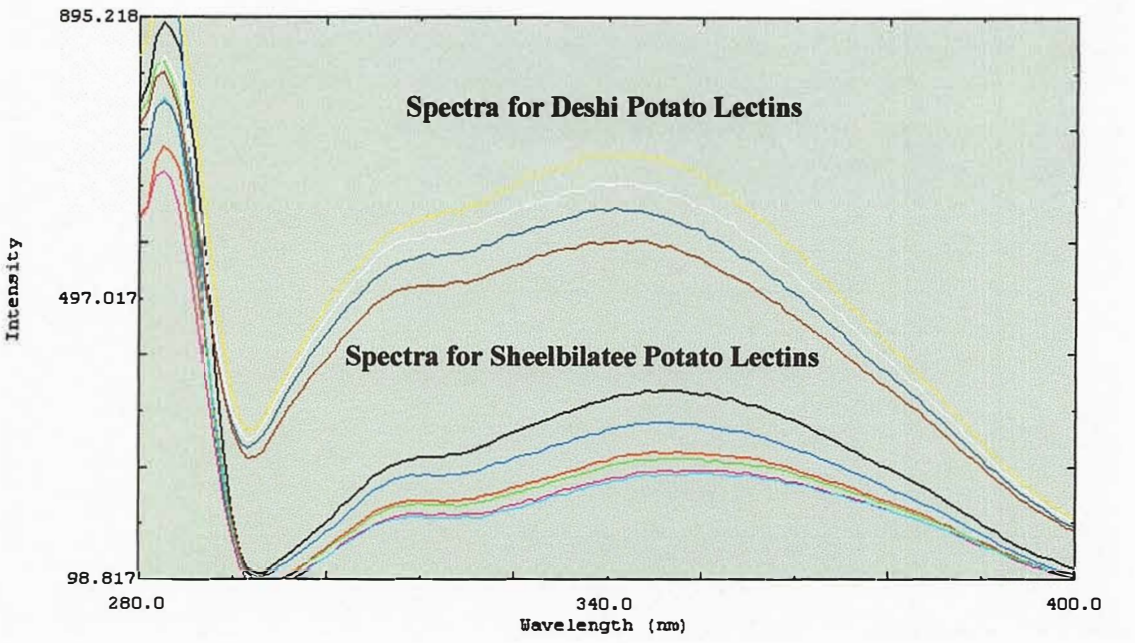


Fig - 37: Measurement of β-lactamase efficiency of the Sheelbilatee and Deshi potato lectins against two bacterial species by fluorescent end product emission

3.15 Study of Fluorescence Emission Spectra of the Chitin-binding Lectins from Sheelbilatee and Deshi Potato



Legends for the Spectra for Deshi Potato Lectins

Deshi potato lectin in DW
Lectin + 75 mM NaCl
Lectin + 75 mM NaCl + 2 mM $CaCl_2$
Lectin + 75 mM NaCl + 2 mM $CaCl_2$ + 5 mM N-acetyl glucosamine

Legends for the Spectra for Sheelbilatee Potato Lectins

Sheelbilatee potato lectin in DW
Lectin + 75 mM NaCl
Lectin + 75 mM NaCl + 1 mM $CaCl_2$
Lectin + 75 mM NaCl + 2 mM $CaCl_2$
Lectin + 3M NaCl + 2 mM $CaCl_2$ + 2.5 mM N-acetyl glucosamine
Lectin + 3M NaCl + 2mM $CaCl_2$ + 5 mM N-acetyl glucosamine

Fig - 38: Fluorescence emission spectra of 13 µg/ml of Sheelbilatee and Deshi potato lectins in the presence of different concentrations of NaCl, $CaCl_2$ and a specific sugar, N-Acetyl glucosamine

To check the structural stability of the chitin-binding lectins from Deshi and Sheelbilatee potato tubers, the measurement of fluorescence spectra were done at different states as shown in Figure - 38. The fluorescence intensity was found higher in case of Deshi potato lectins as compared to Sheelbilatee potato lectins. The fluorescence intensity of both Deshi and Sheelbilatee potato lectins did not increase in the presence of NaCl and Ca^{2+} salts rather decreased a bit. Furthermore, on addition of the N-acetylglucosamine sugar to the lectin solution, the fluorescence intensity became more decreased as compared to that in the presence of the salts.

The study of the conformational changes of those lectins upon binding to different concentrations of ions, metal ions and a sugar by fluorescence spectroscopy indicates the possible structural similarities in these lectins and the number of Tryptophan residues may be higher in the Deshi potato lectins than the Sheelbilatee lectins. These potato lectins may be calcium-independent in nature but further investigation is required to reach in any conclusion.

CONCLUSION

Though it was not possible to purify the lectins from the two potato species studied, the chitin-binding lectin mixtures as expected have marked antibacterial and antifungal activities. These lectins can be developed as antimicrobial agents that inhibit bacterial growth by agglutinating to them. Potato chitin-binding lectins also responded well to the antineoplastic activity study though their cytotoxic effects may become an obstacle to use those as any kind of anticancer agents. The information and knowledge of the antimicrobial properties of these lectins may be implemented in the sector of medical, pharmaceutical science and agriculture in our country that those can be used for the treatment of various infectious diseases as well as cancer.

The biofilm microtitre assay and the β-lactamase efficiency assay by fluorescent end product emission were performed to get an idea about the antimicrobial and antiproliferative role that the lectin mixtures can play. In addition, the study of the conformational changes of those lectins upon binding to different concentrations of ions, metal ions and a sugar by fluorescence spectroscopy gives an indication about the possible structural similarities in these proteins and their possible independence to calcium ions.

It may help to understand the mechanism of action as well as to perform a more detailed structural study of these types of lectins in future.

Chapter-Four

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REFERENCES

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