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A New Ledin from the Pulp of Punica granatum: Isolation, Charactemation and Antiproliferative Activities

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A New Lectin from the Pulp of *Punica granatum*: Isolation, Characterization and Antiproliferative Activities

M. Phil. Thesis

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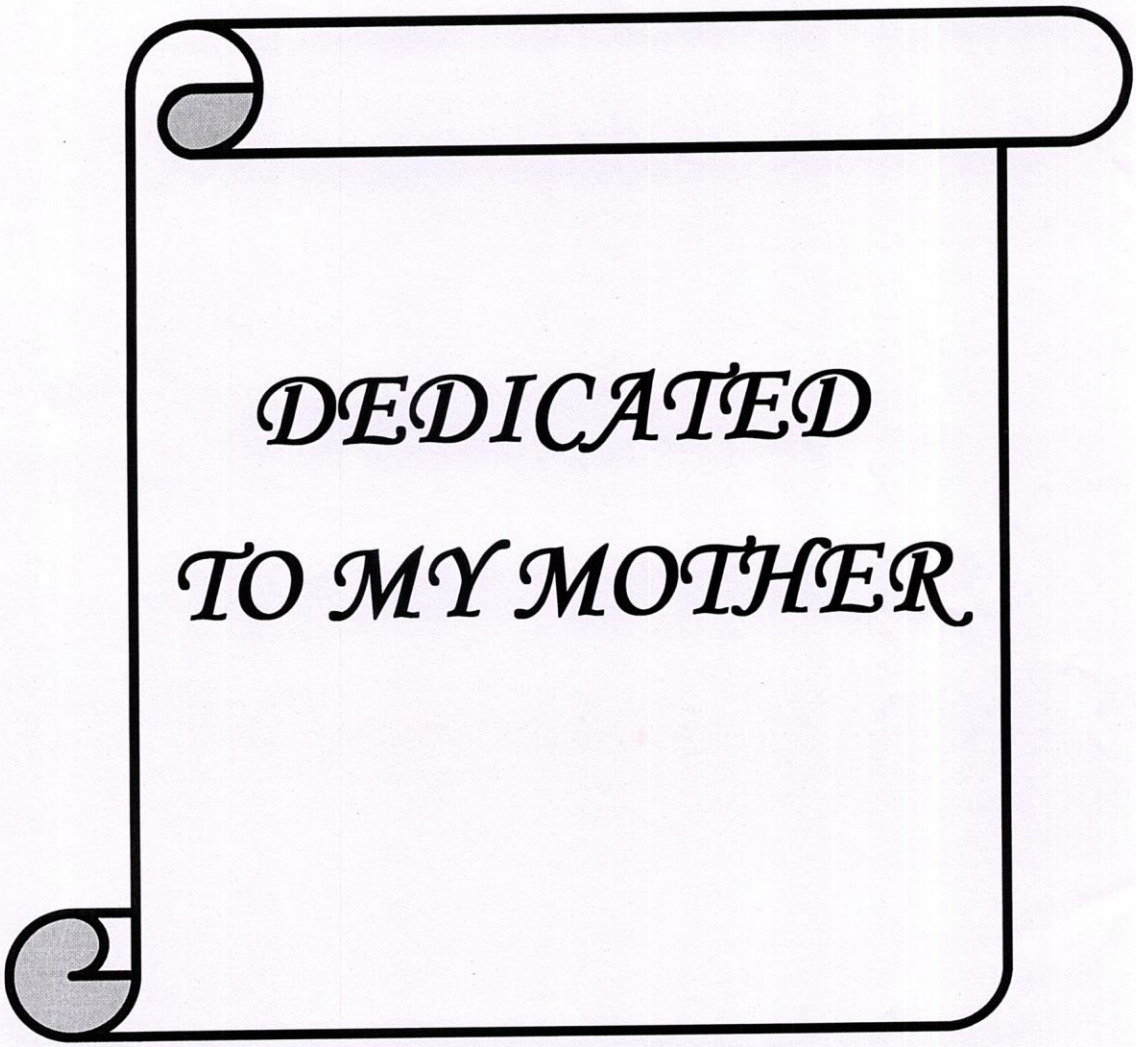
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DECLARATION

I do hereby declare that the materials embodied in this thesis entitled "**A New Lectin from the Pulp of *Punica granatum*: Isolation, Characterization and Antiproliferative Activities**" 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 Bilogy, are the original research works of mine and have not been previously submitted for the award of any degree or diploma anywhere.

(Md. Nurujjman)

Signature of the M.Phil. Fellow

CERTIFICATE

This is to certify that the thesis entitled "**A New Lectin from the Pulp of *Punica granatum*: Isolation, Characterization and Antiproliferative Activities**" has been prepared by Md. Nurujjman 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. A.K.M. Asaduzzman)

Signature of the supervisor

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(Dr. Syed Rashel Kabir)

Signature of the co-supervisor

ABSTRACT

A lectin (designated as PgL) was purified from the pulp of pomegranate (*Punica granatum)* by using Ion-exchange chromatography on DEAE cellulose column with the molecular mass of 28.0 ± 1.0 KDa as estimated by SDS-PAGE both in the presence and absence of β- mercaptoethanol. The lectin agglutinated rat blood cells along with different groups of human blood cells. The hemmaglutination activity was inhibited by 4-nitrophenyl-α-D-manopyranoside and 2-nitrophenyl-β-D-glucopyranoside. The lectin showed the maximum agglutination activity within the pH range of 6.0-8.0 and temperatures range of 30-80°C. PgL was glycoprotein in nature containing 40% neutral sugar. The lectin was a divalent ion-independent glycoprotein that lost 75% of its activity in the presence of 8M of urea. PgL exerted no toxic effect against brine shrimp nauplii and did not show any agglutination activity against five pathogenic bacteria (*Escherichia coli, Listeria monocytogenes, Salmonella enteritidis, Bacillus subtilis* and *Salmonella typhi*). MTT assay showed the growth of Ehrlich ascites carcinoma (EAC) cells to be inhibited upto 6.9-19.8% at a protein concentration of 12.5-100 μg/ml. PgL showed 18.0-33.0% growth inhibition against Ehrlich ascites carcinoma (EAC) cells *in vivo* in mice when administered at 1.5-4.5 mg/kg/day (i.p.), respectively, for five consequent days.

Keywords: lectin; anti tumor; fluorescence spectroscopy; MTT assay; agglutination; sugar inhibition

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CHAPTER ONE

INTRODUCTION

1.1 Introduction

Lectins are a class of ubiquitous proteins or glycoproteins that can agglutinate erythrocytes and interact reversibly with sugar moieties of glycoconjugates (Goldstein et al., 1980). In the past, sugar–protein interactions were ignored in favour of other interactions. It is well-known that despite of their small sizes, sugars play roles in storage and in relaying information within or between cells. Lectin–carbohydrate interactions have gained much attention since they may be employed to improve delivery and in targeting of active compounds to their sites of action (Neumann et al., 2004). They recognize sequences of two or more saccharides with specificity toward both inter residue glycoside linkages and also anomeric configuration, so that they demonstrate antibacterial and antitumor activities by recognizing residues of glycoconjugates on surface of cells. These molecules have been extracted from plants, funji, bacteria and animals. Various lectins have been extracted from plants, such as ASAI and ASAII from *Allium sativum L,* rice, legumes and cucurbitaceae (Van Damme, 1998). Although the amount of lectins in edible plants vary, the daily injestion of lectins by both humans and animals is significant. Since lectins are often resistant to heat and proteolytic enzymes, including those of intestinal microflora, the effects of consumption of these proteins deserves special attention. In an *ad hoc* survey, 53 edible plants were shown to contain lectins and approximately 30% of fresh and processed foods regularly consumed by humans had significant hemagglutination activity.

In the last two decades , there has been increased interest in the potential health benefits of bioactive proteins from plants, including lectins because of the extensive studies which showed that lectin exhibits antiproliferative, antitumor, immunomodulatory, antifungal, antiviral and HIV -1 reverse transcriptase inhibitory activities (Zhang *et al.*, 2009). Lectins are proteins that selectively and reversibly bind carbohydrates. This property gives them a diversity of biological functions, many of which are still unknown. Even for lectins with homologous amino acid sequences, as those from legumes, a common function cannot be ascribed to them because individual parameters such as carbohydrate specificity and other influencing factors differ. In addition to increasing sophisticated descriptions of the occurrence and structural characteristics of

lectins, their potential to enhance health has been a driving force for the expanding interest in lectinology.

Fruits that exhibit lectin activity are apple, banana, plum, cherry, grapefruit, orange, lemon, watermelon, cantaloupe, strawberry, jackfruit, papaya a and pomegranate (Zhang *et al*., 2009).

The pomegranate, locally known as 'Dalim' in Bangladesh *Punica granatum L., an* ancient, mystical, and highly distinctive fruit, is the predominant member of two species comprising the Punicaceae family. Pomegranate fruit is one of the most popular, nutritionally rich fruit with unique flavor, taste, and heath promoting characteristics. Along with sub-arctic pigmented berries and some tropical exotics such as mango, it too has novel qualities of functional foods, often called as "super fruits." This unique fruit borne on a small, long-living tree cultivated throughout the Mediterranean region, as far north as the Himalayas, in Southeast Asia, and in California and Arizona in the United States. In addition to its ancient historical uses, pomegranate is used in several systems of medicine for a variety of ailments. The synergistic action of the pomegranate constituents appears to be superior to that of single constituents. In the past decade, numerous studies on the antioxidant, anticarcinogenic, and anti-inflammatory properties of pomegranate constituents have been published, focusing on treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction, bacterial infections and antibiotic resistance, and ultraviolet radiation-induced skin damage. In addition to its ancient historical uses, pomegranate is used in several systems of medicine for a variety of ailments. In Ayurvedic medicine the pomegranate is considered "a pharmacy unto itself" and is used as an antiparasitic agent a "blood tonic," and to heal aphthae, diarrhea, and ulcers. Pomegranate also serves as a remedy for diabetes in the Unani system of medicine practiced in the Middle East and India. The traditional medicinal value of pomegranate was supported as the fruit extract could inhibit the proliferation of mouse mammary cancer cell line (WA4), derived from mouse MMTV-Wnt-1 mammary tumors in a time and concentration-dependent manner through an arrest of cell cycle progression in the G_0/G_1 phase. Pomegranate extract has also been found to

induce apoptosis by increasing caspase-3 activity in a mouse mammary cancer cell line (WA4).

Though many lectins were purified from various pulps of fruits and other parts of plant, in the present paper, we are reporting the purification and characterization of a 4-nitrophenyl α-D manopyranoside-specific lectin from the pulp of *Punica granatum.*

1.2 Etymology of Pomegranate

The name Punica is the feminized Roman name for Carthage, the ancient city in northern Tunisia from which the best pomegranates came to Italy. It was initially known as *Malum punicum*, the apple of Carthage. But Carl Linneaus a Sweedish botanist called "The Father of Taxonomy" selected the current name, with the specific epithet *granatum,* meaning seedy or grainy. Its common name in the United States, therefore, means "seedy apple".

1.3 Botany of Pomegranate

An attractive shrub or small tree growing 6 to 10 m high, the pomegranate has multiple spiny branches, and is extremely long-lived, with some specimens in France surviving for 200 years. *Punica granatum* leaves are opposite or sub-opposite, glossy, narrow oblong, entire, 3–7 cm long and 2 cm broad. The flowers are bright red, 3 cm in diameter, with three to seven petals. Some fruitless varieties are grown for the flowers alone.

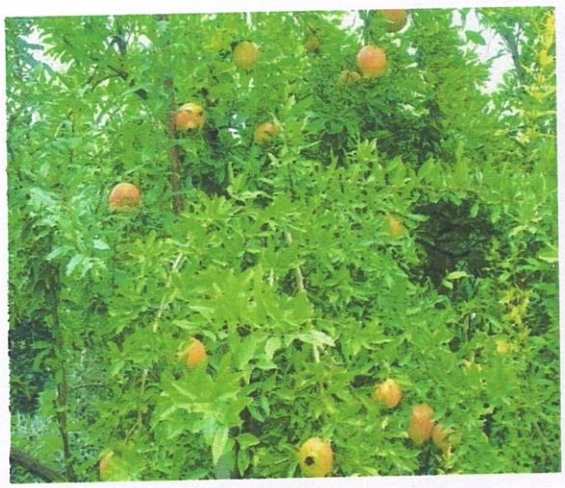


Fig. 1A: Pomegranate tree with fruits

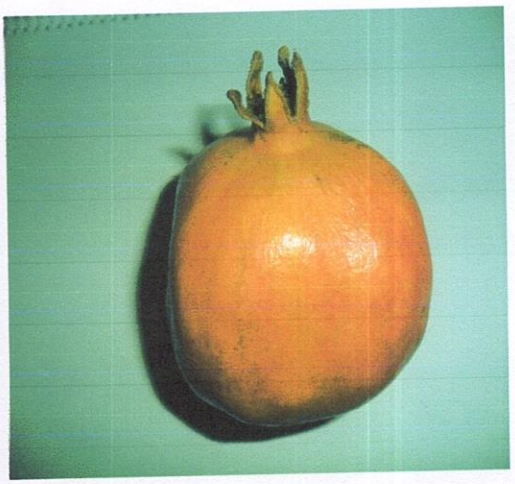


Fig. 1B: A Pomegranate fruit

Fig. 1C: Seeds with pulp of Pomegranate

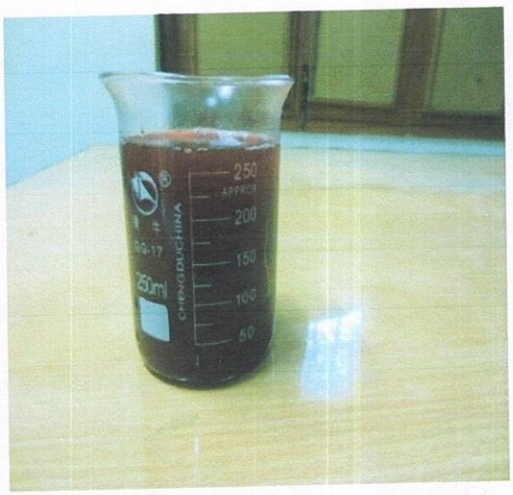


Fig. 1D: Pomegranate Juice extracted from pulp

Source: (From Wikipedia, the free encyclopedia):
Protein and Enzyme Laboratory, Department of Biochemistry and Molecular Biology, University of Rajshahi.

The edible fruit is a berry and is between a lemon and a grapefruit in size, 5–12 cm in diameter with a rounded shape and thick, reddish skin (Morton *et al.*, 1987). The number of seeds in a pomegranate can vary from 200 to about 1400 seeds. Each seed has a surrounding water-laden pulp the edible sarcotesta that forms from the seed coat ranging in color from white to deep red or purple. The seeds are "exarillate" i.e., unlike some other species in the order, Myrtales there is no aril. The sarcotesta of pomegranate seeds consists of epidermis cells derived from the integument. The seeds are embedded in a white, spongy, astringent membrane.

1.4 Taxonomic hierarchy of Pomegranate

Kingdom	Plantae – plantes, Planta, Vegetal, plants
Subkingdom	Viridaeplantae – green plants
Infrakingdom	Streptophyta – land plants
Division	Tracheophyta – vascular plants, tracheophytes
Subdivision	Spermatophytina – spermatophytes, seed plants, phanérogames
Infradivision	Angiospermae – flowering plants, angiosperms, plantas com flor, angiosperma, plantes à fleurs, angiospermes, plantes à fruits
Class	Magnoliopsida
Superorder	Rosanae
Order	Myrtales
Family	Lythraceae – loosestrife
Genus	Punica L. – pomegranate
Species	Punica granatum L. – pomegranate

Source:
http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=27278

1.5 Nutritional facts of Pomegranate

In general, the pomegranate is a good source of potassium and has modest amount of vitamin C. It is quite low in calories and is very low in sodium. According to US Department of Agriculture Research Service ,100 gm of edible portion of the pomegranate gives about 68 Kcal of energy and contain 0.95 gm protein, 0.6 gm fibre, 6 mg vitamin C, calcium 3 mg, magnesium 3 mg, phosphorus 8 mg, iron 0.3 mg, potassium 259 mg, zinc 0.12 mg and selenium 0.6 mg.

1.6 Chemical Constituents

Pomegranate contains chemical components in its different compartments, which may possess various pharmacological and toxicological activities. Table 1 lists the chemical constituents of pomegranate.

Table-1 A: Chemical Constituents of pomegranate.

Plant Component	Constituent
Pomegranate juice:	anthocyanins (Du *et al.*, 1975), glucose, ascorbic acid, ellagic acid, gallic acid, caffeic acid(Amakura *et al.*, 2000), catechin, EGCG, quercetin, rutin (Artik *et al.*, 1998), numerous minerals, particularly iron amino acids (Lansky *et al.*, 2007).
Pomegranate seed oil:	95-percent punicic acid (Schubert *et al.*, 1999), other constituents, including ellagic acid (Amakura *et al.*, 2000), other fatty acids sterols.
Pomegranate pericarp: (peel, rind)	phenolic punicalagins; gallic acid and other fatty acids (Amakura *et al.*, 2000), catechin, EGCG, quercetin, rutin, and other flavonols (Artik *et al.*, 1998), flavones, flavonones, anthocyanidins.
Pomegranate leaves:	tannins (punicalin and punicafolin); and flavone glycosides, including luteolin and apigenin.
Pomegranate flower:	gallic acid, ursolic acid (Huang *et al.*, 2005), triterpenoids, including maslinic and asiatic acid, other unidentified constituents.
Pomegranate roots and bark:	ellagitannins, including punicalin and punicalagin (Tanaka *et al.*, 1986), numerous piperidine alkaloids.

1.7 Properties of Pomegranate in Toxicological, Pharmacological, Cellular and Molecular Biology researches

1.7.1 Antioxidant properties

The presence of antioxidants has been reported in *Punica granatum* (pg) juice. Pg contains some species of flavonoids and anthocyanidins (delphinidin, cyaniding and pelargonidin) in its seed oil and juice (Seeram *et al.*, 2006) and shows antioxidanst activity three times greater than green tea extract. Pg fruit extracts exhibit scavenging activity against hydroxyl radicals and superoxide anions, which could be related to anthocyanidins (Seeram *et al* 2006). The antioxidant action of Pg is observed, not only through its scavenging reactions, but also by its ability to form metal chelates (Kulkarni *et al.*, 2007). Studies have indicated that methanolic extracts from the peel of Pg has a broad spectrum of antioxidant activities which were evaluated by 1,1-diphenyl 2-picrylhydrazyl (DPPH) free radical scavenging, phosphomolybdenum, Ferric (Fe3+) Reducing Antioxidant Power (FRAP), and Cupric (Cu2+) Reducing Anti oxidanrt Capacity (CUPRAC) assays (Kulkarni *et al.*, 2007 ;Zahin M *et al.*, 2010).

1.7.2 Anti-inflammatory effect

Acute inflammation is a beneficial host response for prevention of tissue injury, but it may also cause immune-associated diseases such as rheumatoid arthritis, inflammatory bowel disease and cancers (Balkwill *et al.,* 2005). Interestingly, *Punica granatum (*Pg) has been shown to inhibit inflammation by different mechanisms.

Cyclooxygenase (COX) and lipooxygenase (LOX), which are key enzymes in the conversion of arachidonic acid to prostaglandins and leukotrienes (important inflammatory mediators), respectively, are inhibited by Pg (Schubert *et al.*, 1999). Ahmed *et al.* have shown that Pg has a significant inhibitory effect on osteoarthritis (OA) by suppressing the expression of matrix metalloproteinases (MMPs) in OA chondrocyte cultures and preventing collagen degradation. It may also inhibit joint destruction in OA patients (Ahmed *et al.*, 2005). Shukla *et al.* showed that pretreatment with 13.6 mg/kg of Pg extract decreased the arthritis incidence and lowered IL-6 and IL-1β levels in animal model of rheumatoid arthritis (Shukla *et al.*, 2008).

1.7.3 Carcinogenesis

Pomegranate possesses inhibitory effects on different type of cancers such as prostate, breast (Sturgeon *et al.*, 2010), colon and lung cancers (Khan *et al.*, 2009). Different mechanisms have been outlined for pomegranates anti-cancer activities in these studies. Pg inhibits NF-kB and cell viability of prostate cancer cell lines in a dose-dependent manner in the LAPC4 xenograft model, in-vitro. Pg polyphenols, ellagitannin-rich extract and whole juice extract inhibited gene expression of HSD3B2 (3beta-hydroxysteroid dehydrogenase type 2), AKR1C3 (aldo-ketoreductase family 1 member C3) and SRD5A1 (steroid 5alpha reductase type 1), which are key androgen-synthesizing enzymes in LNCaP, LNCaP-AR, and DU-145 human prostate cancer cells (Seeram *et al.*, 2006). Because Pg inhibits CYP activity/expression which is necessary for activation of procarcinogens, it may have anti-carcinogenesis effects. Some metabolites of pomegranates chemical components such as 3, 8-dihydroxy-6H-dibenzo [b, d] pyran-6-one (urolithin A, UA) which is produced from Ellagitannins (ETs) may also possess anti-cancer effects (Seeram *et al.*, 2006).

1.7.4 Angiogenesis

Angiogenesis is a possible target for cancer prevention strategy. Interestingly, recent studies have shown the ability of Pg to inhibit angiogenesis (Toi *et al.*, 2003). Toiet *et al.* evaluated the anti-angiogenic potential of Pg by measuring vascular endothelial growth factor (VEGF), IL-4, and migration inhibitory factor (MIF) in the conditioned media of estrogen sensitive (MCF-7) or estrogen resistant (MDA-MB-231) human breast cancer cells, and immortalized normal human breast epithelial cells (MCF-10A). VEGF was strongly decreased in MCF-10A and MCF-7, however, MIF was increased in MDA-MB-231, showing significant potential for inhibitory effects of angiogenesis by Pg fractions on human umbilical vein endothelial cells (HUVEC) (Toi *et al.*, 2003).

1.7.5 Cell cycle arrest

Cell cycle may be altered following exposure to Pg. Previous studies have suggested several mechanisms for these effects, such as modulation of cell signaling molecules in the cell cycle machinery. *Punica granatum* extract (PE) inhibited the proliferation of mouse mammary cancer cell line (WA4), derived from mouse MMTV-Wnt-1

mammary tumors in a time and concentration-dependent manner through an arrest of cell cycle progression in the G0/G1 phase (Dai *et al.*, 2010). Ellagitannins, derived from Pg juice, and their metabolites, urolithins exhibit dose and time-dependent decreases in cell proliferation and clonogenic efficiency of HT-29 cells through cell cycle arrest in the G0/G1 and G2/M stages of the cell cycle followed by induction of apoptosis. Pg pretreatment of normal human epidermal keratinocytes (NHEK) has been found to increase the cell cycle arrest induced by UVA in the G1 phase of the cell cycle.

1.7.6 Apoptosis

Pg causes apoptosis by different mechanisms. Pomegranate extract has been found to induce apoptosis by increasing caspase-3 activity in a mouse mammary cancer cell line (WA4) (Dai *et al.*, 2010). In addition, Pg extracts and punic acid, an omega-5 long chain poly unsaturated fatty acid derived from Pg, have been shown to induce apoptosis in both an estrogen in sensitive breast cancer cell line (MDA-MB-231) and an estrogen sensitive cell line developed from MDA-MB-231 cells (MDA-ERalpha7) through lipid peroxidation and the PKC (Protein kinase C) signaling pathway. They also cause disruption to the cellular mitochondrial membrane. Pg treatment of normal human epidermal keratinocytes (NHEK) inhibited UVB-mediated activation of MAPK and NF-kB pathways, as well as other signal transducers and activators of the apoptosis pathway including transcription 3 (STAT3), PKB/AKT, ERK1/2, mTOR, PI3K, Bcl-X(L) (antiapoptotic protein), Bax and Bad (proapoptotic proteins).

1.7.7 Effects on vital enzymes

Pg has been shown to inhibit different enzymes including phospholipase A2 (PLA2) (that catalytically hydrolyzes the bond releasing arachidonic acid and lysophospholipids) (Lansky *et al.*, 2005), cyclooxygenase (COX), lipooxygenase (LOX) (30), cytochrome P450 (Kimura *et al.*, 2010) and ornithine decarboxylase (ODC) (Bachrach *et al.*, 2004) which plays a role in the urea cycle and catalyzes the decarboxylation of ornithine to polyamines such as putrescine. Aromatase is enzyme responsible for a key step in the biosynthesis of estrogens and catalyzes the formation of estrone and estradiol, which is inhibited by Pg. One of the possible mechanisms in

which Pg can inhibit breast cancer is its inhibitory effect on aromatase and 17 beta hydroxysteroid dehydrogenase enzymes (17β-HSDs), as well as its anti-estrogenic activity (Sturgeon *et al.*, 2010). Furthermore, ellagitannins (ET) and urolithin B (UB), which are found in relatively high quantities in Pg, have been shown to most effectively inhibit aromatase activity in a live cell assay (Adams *et al.*, 2010). Serine protease (SP) is another enzyme which is inhibited by Pg. Ellagic acid and punicalagin, from Pg, have shown lower inhibitory effects on alpha secretase (TACE) and other serine proteases such as chymotrypsin, trypsin, and elastase, thus indicating that they are relatively specific inhibitors of beta-secretase (BACE1) (Kwak *et al.*, 2005).

1.7.8 Cellular differentiation

Study has shown that Pg stimulates the differentiation of osteoblastic MC3T3-E1 cells and affects the function of these cells (Kim *et al.*, 2000). Pg seed oil (but not aqueous extracts of fermented juice, peel or seed cake) has been shown to stimulate keratinocyte proliferation in monolayer culture, without effecting fibroblast function, and as a result facilitates skin repair and promotes regeneration of dermis and epidermis (Aslam *et al.*, 2006). Furthermore, the effect of Pg on differentiation has been observed in breast and prostate cell lines (Kawaii *et al.*, 2004).

1.7.9 Anti-mutagenicity

It has been shown that Pg peel fractions, especially methanol, has anti-mutagenic activities as was detected by the Ames Salmonella/microsome assay against sodium azide (NaN3), methyl methane sulphonate (MMS), 2-aminofluorene (2-AF), and benzo pyrene induced mutagenicity in Salmonella typhimurium (TA97a, TA98, TA100 and TA102) tester strains (Zahin *et al.*, 2010). Methanolic extract of Pg (15 mg/plate) shows the highest anti-mutagenic activity in TA 100 cells (Wongwattanasathein *et al.*, 2010).

1.8 Clinical Application

1.8.1 Prostate Cancer

In vitro studies shows pomegranate fruit extract (PFEs) inhibit prostate cancer cell growth, induce apoptosis of several prostate cancer cell lines (including highly aggressive PC-3 prostate carcinoma cells), suppress invasive potential of PC-3 cells, and decrease proliferation of DU-145 prostate cancer cells (Lansky *et al., 2005* ;Albrecht *et al., 2004* ;Malik *et al.*,2006).

1.8.2 Other Cancer Types

Numerous in vitro studies have investigated the therapeutic effect of pomegranate extracts against several other cancer cell lines. In HT-29 colon cancer cells, cyclooxygenase-2 (COX-2) expression is increased via activation of nuclear factor kappa-B (NFκB) by tumor necrosis factor-alpha (TNF-α), an inflammatory cell signaling process that may be a cause of cancer initiation and progression. Treatment of HT-29 colon cancer cells with PJ, total pomegranate tannins, or concentrated pomegranate punicalagin induced a significant decrease in COX-2 expression. Research utilizing breast cancer cell lines MCF-7 and MB-MDA-231 demonstrates pomegranate constituents effectively inhibit angiogenesis (Toi *et al.*, 2003), tumor growth, proliferation, and invasiveness and induce apoptosis.

Research in mice has shown PFE (pomegranate fruit extract) inhibits tumorigenesis in lung cancer and skin cancer models.

1.8.3 Atherosclerosis

In vitro, animal, and human trials have examined the effects of various pomegranate constituents on prevention and attenuation of atherosclerosis.

Aviram *et al* investigated the antiatherosclerotic effects of a pomegranate by product (PBP) extract after the juice was removed. Four month old E° mice with significant atherosclerosis were given PBP extract (containing 51.5μg gallic acid equiv/kg/day) with an eight-fold higher polyphenol concentration than PJ for three months. This resulted in a significant reduction in MPM oxidative status as evidenced by a 27-percent decrease in total macrophage peroxide levels, a 42-percent decrease in cellular

lipid peroxide levels, and a 19-percent decrease in peritoneal macrophage uptake of oxidized LDL.

1.8.4 Hypertension

A small clinical trial demonstrated PJ inhibits serum ACE and reduces systolic blood pressure in hypertensive patients. Ten hypertensive subjects (ages 62-77; seven men and three women) were given 50 ml/day PJ containing 1.5 mmol total polyphenols for two weeks. Two of seven patients were also diabetic and two were hyperlipidemic. Seven of 10 subjects (70%) experienced a 36-percent average decrease in serum ACE activity and a small, but significant, five-percent decrease in systolic blood pressure (Aviram *et al.*, 2001).

1.8.5 Myocardial Perfusion

In a double-blind, randomized, placebo-controlled trial, 39 patients were given either 240 mL PJ (polyphenol content not specified) (n=23) or a sports beverage of similar color, flavor, and caloric content daily for three months (n=16). Although both control and treatment patients demonstrated similar levels of stress-induced ischemia at baseline, at three months stress-induced ischemia increased in the placebo group (from 5.9±4.3 to 7.1±5.5) but decreased in the treatment group (from 4.5±3.1 to 3.7±3.7). In addition, angina episodes increased 38 percent in the placebo group but decreased 50 percent in the treatment group (a net change of 88 percent). These results demonstrate a reduction in myocardial ischemia and improved myocardial perfusion (as measured by stress-induced ischemia) in patients consuming pomegranate juice (Sumner *et al.*, 2005).

1.8.6 Diabetes

In an animal model of diabetes, Huang *et al* demonstrated the favorable effect of pomegranate flower extract on lipid profiles and cardiac fibrosis of Zucker fatty diabetic rats. Rosenblat *et al* investigated the effect of 50 ml/day Pomegranate juice for three months on oxidative stress, blood sugar, and lipid profiles in 10 type 2 diabetic patients (history of diabetes for 4-10 years) and 10 healthy controls (ages 35-71). In diabetic patients, triglyceride levels were 2.8 times greater, HDL cholesterol was 28-percent lower, and hemoglobin A1C (HbA1C) values were 59-percent higher than in control patients. Insulin was only slightly lower in patients than controls, and C-peptide

(a proinsulin metabolite marker for endogenously secreted insulin) was slightly higher in diabetic patients than in healthy controls at baseline (indicating slight hyperinsulinemia). Consuming PJ for three months did not significantly affect triglyceride, HDL cholesterol, HbA1C, glucose, or insulin values, but did lower serum C-peptide values by 23 percent compared to baseline in diabetic patients a sign of improved insulin sensitivity. PJ consumption also significantly reduced oxidative stress in the diabetic patients. Researchers concluded that despite the sugars naturally present in pomegranate juice, consumption did not adversely affect diabetic parameters but had a significant effect on atherogenesis via reduced oxidative stress.

1.8.7 Dental Conditions

Topical applications of pomegranate preparations have been found to be particularly effective for controlling oral inflammation, as well as bacteria and fungal counts in periodontal disease and Candida associated denture stomatitis (Menezes *et al.*, 2006; Sastravaha *et al.*, 2003).

1.8.8 Bacterial Infections

The only human trials examining the antibacterial properties of pomegranate extracts have focused on oral bacteria (Menezes *et al.*, 2006; Sastravaha *et al.*, 2003). However, several in vitro assays demonstrate its bacteriocidal activity against several highly pathogenic and sometimes antibiotic resistant organisms. Brazilian researchers evaluated the synergistic effect of a P. granatum methanolic extract with five antibiotics on 30 clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA) and methicillin-sensitive S. aureus(Machado *et al.*,2002). Antibiotics tested were chloramphenicol, gen-tamicin, ampicillin, tetracycline, and oxacillin. Although synergistic activity between the pomegranate extract and all five antibiotics was noted in the S. aureus isolates, synergy with ampicillin was the most pronounced. A combination of the two increased the lag time to bacterial growth by three hours (over that of ampicillin alone) and was also bacteriocidal as evidenced by a 72.5-per-cent reduction in methicillin-sensitive organisms and a 99.9-percent reduction in MRSA. Based on earlier research and the results of this study, the ellagitannin, punicalagin, is thought to be the primary constituent responsible for the observed antibacterial effects.

1.8.9 Ultraviolet Radiation

In vitro studies using normal human epidermal keratinocytes and pomegranate fruit extract (PFE) demonstrate, PFE incubation with cell cultures ameliorates UV A anb B radiation induced cell damage in a dose and time dependent manner, providing evidence at a cellular level that PFE may be an effective photo chemopreventive agent (Afaq *et al.*, 2005).

1.8.10 Male Infertility

Research in rats demonstrates pomegranate juice consumption improves epididymal sperm concentration, spermatogenic cell density, diameter of seminiferous tubules, and sperm motility, and decreases the number of abnormal sperm compared to control animals. An improvement in antioxidant enzyme activity in both rat plasma and sperm was also noted (Turk *et al.*, 2008).

1.8.11 Neonatal Hypoxic-Ischemic Brain Injury

Neonatal hypoxic-ischemic (HI) brain injury in severely preterm, very low birth-weight infants is a major cause of infant illness and death and has been associated with an increase in reactive oxygen species. Two studies in which pregnant mice were given pomegranate juice in drinking water revealed the neonatal offspring, when subjected to experimentally-induced HI brain injury, had significantly less brain tissue loss (64% decrease) and significantly decreased hippocampal caspase-3 activity (84% decrease) compared to neonates with experimentally induced HI brain injury from dams who consumed a control beverage (West *et al.*, 2007). These results suggest PJ has an antioxidant-driven neuroprotective effect conferred from mother to neonate.

1.8.12 Alzheimer's Disease

The neuroprotective properties of pomegranate polyphenols were evaluated in an animal model of Alzheimer's disease. Transgenic mice with Alzheimer's-like pathology treated with pomegranate juice (PJ) had 50-percent less accumulation of soluble amyloidbeta and less hippocampal amyloid deposition than mice consuming sugar water, suggesting PJ may be neuroprotective. Animals also exhibited improved learning of water maze tasks and swam faster than control animals (Hartman *et al.*, 2006).

1.8.13 Obesity

Pomegranate flower extract (400 or 800 mg/kg/day) given to obese hyperlipidemic mice for five weeks caused significant decreases in body weight, percentage of adipose pad weights, energy intake, and serum cholesterol, triglyc-eride, glucose, and total cholesterol/HDL ratios. Decreased appetite and intestinal fat absorption were also observed, improvements mediated in part by inhibition of pancreatic lipase activity (Lei *et al*., 2007).

1.9 Plant as a source of protein

No long ago, the word protein would likely have conjured an image of a thick steak. But in these more enlightened times; informed eaters are learning that protein comes in variety forms, many deriving from the plant world. Aromatic grain and nut pilafs, grilled tofu and vegetable kebabs, whole-grain pancakes, and black bean enchiladas are just a few examples. The coming of age of vegetarian cookery, coupled with the ongoing passion for ethnic cuisines, has stretched the definition of protein sources. Although animal kingdom is the main source of protein but plant kingdom also possesses a lot of protein 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 contained about 18-26% and oil seeds contain approximately 30-55% protein. 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 sources of plant protein.

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.

1.10 Proteins and glycoprotein

Proteins are the biomolecules composed of amino acids that are combined with each other by peptide bonds. Proteins are the main constituents of all the living matter. Proteins are found most abundantly in the animal sources and the proteins of these groups are known as first class proteins. Meat, fish, egg, milk, dairy products etc. are animal proteins.

The glycoproteins which contain carbohydrate group attached covalently to the polypeptide chains represent a large group of wide distribution with considerable biological significance. The percent by weight of carbohydrate group in different glycoproteins may vary from less than 1% in ovalbumin to as 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 dozen of monosaccharide residues usually of two or more kinds. Some glycoproteins also contain oligosaccharide units. The sugar residues are generally mannose, galactose, lactose, xylose, glucose, raffinose, arabinose, glucosamine etc (Lis and Sharon, 1981). Among them mannose and glucosamine are predominant.

1.11 Lectins

Lectins were first described in 1888 by Stillmark working with castor bean extracts. W. C. Boyd proposed the term 'Lectin' in 1970 to a group of protein, which possesses the unique ability to agglutinate erythrocyte cells as well as other types of cells. Lectins can be define as group of proteins found in all types of living organisms, either in soluble or in membrane-bound form that recognizes specific carbohydrate structures and thereby agglutinate cells by binding to cell-surface glycoproteins and glycoconjugates (Lis & Sharon, 1998). Another definition is, Lectins are a class of proteins that bind sugar specifically and reversibly and that agglutinate cells, are widely distributed in nature, being found in animals, insects, plants and microorganisms.

For example, some viruses use lectins to attach themselves to the cells of the host organism during infection. Lectins may be disabled by specific mono- and oligosaccharides, which bind to them and prevent their attachment to cell membranes (Lis and Sharon, 1981)

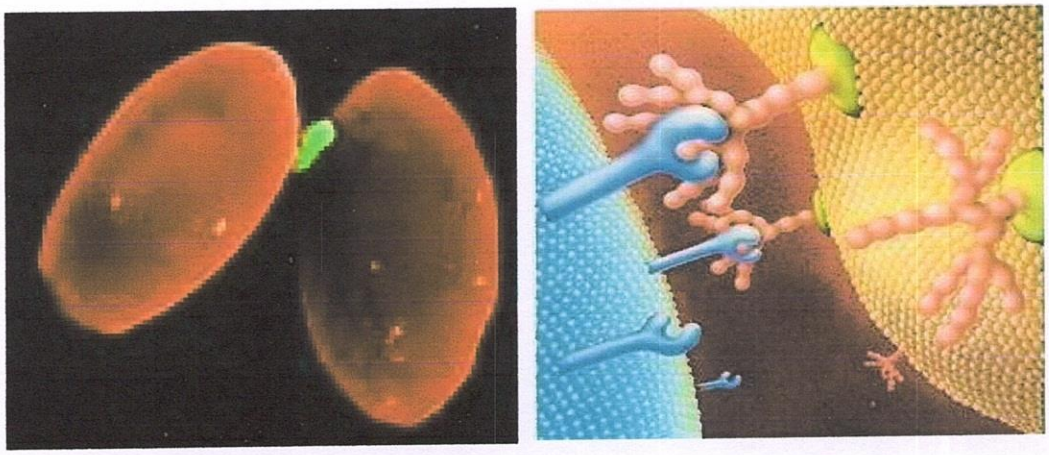


Fig.1E. The mechanism of lectin action

1.12 Etymology

The name "Lectin" is derived from the Latin word legere, meaning, among other things, "to select".

1.13 History

Although they were first discovered more than 120 years ago in plants, they are now known to be present throughout nature. It is generally believed that the earliest description of such a hemagglutinin was by Peter Hermann Stillmark in his doctoral thesis presented in 1888 to the University of Dorpat, (one of the oldest universities in czarist Russia, ested 1632). This hemagglutinin, which was also highly toxic, was isolated by Stillmark from seeds of the castor tree (*Ricinus communis*) and was named ricin. The first Lectin to be purified on a large scale and available on a commercial basis was concanavalin A, which is now the most used Lectin for characterization and purification of sugar-containing molecules and cellular structures.

1.14 Classification and properties of lectins

In the modern classification, there are 3 main types of Lectins: - (1) C-type (2) S-type and (3) legume.

(1) C- type Lectins: - C-type plants Lectins are characterized by a calcium dependent carbohydrate recognition domain. There are two main types of C-type Lectins. (a) Mannose-binding protein and (b) Selectins.

(2) S-type Lectins:- S-type Lectins (S for soluble) are small, soluble proteins with calcium independent affinity for lactosamine and β- galactoside. There are 3 main S-type Lectins: (a) S-Lectins (b) galactose binding and (c) gaLectin.

(3) Legume Lectins:- They have both calcium and manganese binding sites.

On the basis of structural and evolutionary development, most of these plant lectins have been classified as legume lectins, chitin-binding proteins; type 2 ribosome-inactivating proteins, monocot mannose-binding lectins, amaranthins, cucurbitaceae phloem lectins and jacalin-related lectins (Van *et al.,* 1998)

The rich sources of lectins in plants are particularly their organs such as seeds, tubers, bulbs, rhizomes, bark etc. and they have attracted great interest on according of their various biological activities, such as cell agglutination (Medicinal plants), antifungal, antiviral (Tian *et al.,* 2008) and antiproliferative activities (Liu *et al.,* 2010; Kaur *et al.,* 2005). Especially 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 (Wang *et al.,* 2000). But Lectins are usually classified on the basis of source. They are of various types: plant Lectin, vertebrate Lectin, invertebrate Lectin, Lectin of slime mold, Lectin of protozoa, viral Lectin and bacterial Lectin according to their respective sources. Some of the properties of each group of Lectin are given below:

1.14.1 Vertebrate lectins

In the past decade many Lectins have been discovered in vertebrate tissues. This work has been stimulated by an interest in the biological functions of these proteins. These Lectins can be classified into two groups- soluble Lectins that may play a role in the secretion or organisation of extracellular glyconjugates and membrane Lectins that are integrated into membranes and are apparently involved in translocation of glycoconjugates in cells. The soluble Lectins are extracted from tissues with simple buffers, often fortified with complementary sugars that may dissociate them from membrane bound glycoconjugates. The membrane Lectins requires detergents for solubilization, although they may then remain soluble in their absence.

Soluble tissue Lectins: Aqueous extracts of many vertebrate tissues contain materials that agglutinate appropriate test erythrocytes. Since this hemagglutination activity can be inhibited by simple sugars or complex glycoconjugates these materials have been classified as Lectins.

1.14.2 Membrane lectins

This group of Lectins is believed to be integrated in membranes, although in most cases this has not been rigorously established. The membrane Lectins are generally assayed by formation of precipitable complexes with soluble glycoconjugates rather than as hemagglutinins. However, the first such protein to be characterized extensively was shown to agglutinate both untreated and sialidase treated erythrocytes and like many classical Lectins. It also induced mitosis in lymphocytes. Most of the known membrane Lectins is Ca^{2+} dependent for their carbohydrate binding activity, where as most of the soluble Lectins do not.

1.14.3 Bacterium lectins

Many intact bacteria possess the ability to bind and agglutinate erythrocytes and other types of cells. While some bacteria, agglutinating activity is exhibited by the cell extracts, where as the intact cells are devoid of such activity. Binding to cells and agglutination are frequently inhibited by simple sugars, indicating that these activities are due to Lectins present on the bacterial surfaces, as first suggested by or due to intracellular Lectins. However, only a small number of bacterial Lectins have been isolated and investigated. From certain bacteria, preparations have been obtained that bind sugars but do not agglutinate cells. Such Lectins like substances are also included here. The best characterized of the bacterial Lectins with response to their molecular properties, carbohydrate specificity to their molecular properties, carbohydrate specificity, and genetics are the mannose–specific surface Lectins of *E. coli*. Other well-studied groups are the surface Lectins specific for Galα4Gal, also produced by many *E. coli* strains. The Lectin like subtances includes those of *Vibrio cholerae* specific for L-fucose, of *Myxococcus xanthus* specific for certain galactose derivatives of oral *actinomycetes* specific for galactose and N - acetyl galactoseamines and of *Mycoplasma sp*. specific for sialic acid. An unusual feature of several of these surfaces Lectin is that they occur in the form of filamentous proteins, known as fimbriae or pili. Of the soluble (intracellular) bacterial Lectins-only those of *Pseudomonus aeruginosa*, which are specific for galactose and L-fucose/mannose, have been investigated in some detail. The bacterial surface Lectins are of special interest for a variety of reason. Those of the *Enterobacteriaceae* mediate the bidding of the bacteria to mucosal surfaces, for example in the gastrointestinal and urinary tracts, and thus appear to play an important

role in the initiation of infection. This seems also to be the case for the sialic acid specific Lectins of *Mycolpasma spp*. The mannose specific surface Lectins may act as determinants of recognition in nonopsonic phagocytosis of the bacteria.

1.14.4 Invertebrate lectins

These Lectins are found impractically all of the approximately 30 phyla and the various classes and sub-classes of invertebrates (Cohen *et al.* 1984) mainly in the hemolymph and sexual organs, e.g. albumin glands and eggs. They are also present in the membranes of hemocytes, cells that function as primitive and rather unspecific immunological protectors (Cheng *et al.,* 1984). Cellular slime molds have favourable properties for biological investigations. *Slime mold* is a broad term describing protists that use spores to reproduce. Slime molds were formerly classified as fungi, but are no longer considered part of this kingdom.

Several species of cellular slime mold (including *D. discoideum* and *P. pallidum*) contain lectin activities that can be extracted and assayed as hemagglutinins. The lectin slime mold activities show significant changes with differentiation. The studies with cellular slime molds are more advanced; and suggest that lectins play a role in developmentally regulated cell cohesion. The possible role of slime mold Lectins in developmentally regulated cell-cell adhesion has been tested with a quantitative in vitro assay. Purification of slime mold Lectins has been facilited by their abundance, which ranges from 1 to 5% of the total soluble proteins of aggregative cells. The Lectins from *Dictyostelium discoideum*, collectively called discoidin, have been studied most extensively. Recent work on the possible function of discoidin-I has been influenced by studies of fibronectin, an extracellular multifunctional protein that like discoidin-I, binds glycocnjugates. Evidence has accumulated over the past few years that fibronectin influences the ordered migration of cells during development. Because of this, it seemed possible that discoidin-I which is synthesized and externalized so prominently as *D. dicoidenum* cells stream into aggregates might also influence ordered cell migration.

1.14.5 Seed lectin

Lectins are particularly abundant in the seeds of many plants, where they have been found to constitute as high as 10% of the total nitrogen of mature seed extracts (Leiner *et al*., 1976). The distribution of Lectin within the seeds differs among representatives of various plant families.

Lectin distribution has been most intensively studied in plants of the *leguminoseae* family. In mature seeds of plants, most of the Lectin is localized in cotyledons which function as storage tissue for food used during germination of the seeds. In addition to the cotyledons, in some cases appreciable amounts of Lectin have been reported in the embryos and small amount have detected in the seed coats. Early investigators noted the absence of Lectin in immature seeds of *Phaseolus multiflorus* and *Vicia cracca* and the correlation of Lectin appearance with the ripening of the seeds. Using a combination of hemagglutination and immunidiffusion against antiserum to the seed Lectin, studied the development of lentil Lectin in the seeds of the plant. No Lectin was detected in immature seeds but was found in ripening mature seeds, thus supporting the earlier studies mentioned above. These studies indicate that the appearance of Lectins in the seeds of legumes occurs during the late stages of maturation of the seeds prior to their dehydration. Several approaches have been used to determine the subcellular localization of Lectin in the cotyledons of mature seeds. Using immunoperoxidase-labeled antibodies to the *Phaseolus vulgaris* Lectin, found that the Lectin was localized in the cytoplasm of cells of the cotyledons. In a cell fractionation study of dry and imbibed *P. vulgaris* seeds using differential sedimentation, found that most of the Lectin was associated with the protein as determined by hemagglutination assays.

Early investigators found no hemagglutinating activity in the vegetative parts of several plants examine found weak hemagglutinating activity in extracts of the stems of *Vicia cracca* detected hemagglutinating activity only at the base of the stem and in the roots of this plants but reported activity in stem extracts of *laburnum alpinum*, *cytisus praecox, cytisus supunus, sophora japonica,* and *Robinica pseudoacacia* and in extracts of leaves and stems of *Evonymus europeae* found weak hemagglutinating activity in 6-

month-old seedlings of *Maclura pomofera* (Osage orange) although in mature tree activity was confined to the developing seeds.

1.15 Proposed roles

1.15.1 Defense mechanisms

An essential feature of any active defense mechanism of a biological system is the ability to specificity recognizes the offensive agent and responds to its presence. The specificities of Lectins and the properties conferred on them by the multivalence 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. Although Lectins may well play a role in the defense system of plants, there is no evidence for the presence in plants of an immune system directly comparable with the animal immune system. Many studies have demonstrated the interaction of plant Lectins with various microorganisms. 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 found that this Lectin, which has a specificity for chitin oligomers (Allen *et al.,* 1970), binds to the hyphal tips and septa of *Trichoderma viridae*, a fungus with chitin-glucan hyphal cell walls; the binding was inhibited by chititriose. The Lectin inhibited growth and spore germination of the fungus and the investigators found evidence that it interfered 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_Golden *et al*., 1978) and has a strong affinity for the zygospores, which are hyphal branches involved in sexual reproduction. 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 *et al.,* 178).

1.15.2 Pathogenesis

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

1.16 Biological properties of lectin

Since virtually all cells come in a sugar coating, it is not surprising that lectins binds readily to cells. Such binding may result in a variety of biological effects.

1.16.1 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 (Goldsteain *et al.,* 1980). For agglutination to occur the bound Lectin must form multiple cross bridges between apposing cells. There is, however, no simple relation between the amount of Lectin bound and agglutination. Where considerable amounts of a Lectin are bound to cells without causing agglutination. This is because agglutination is affected by many factors such as the molecular properties of the Lectin (e.g. number of saccharide binding sites, molecular size), cell-surface properties (for example, number and accessibility of receptor sites, membrane fluidity), and metabolic state of the cells (Nicolson *et al.,* 1976). In addition, agglutination is affected by external conditions of assay such as temperature, cell concentration, mixing and so on. Agglutination is inhibited by appropriate sugar.

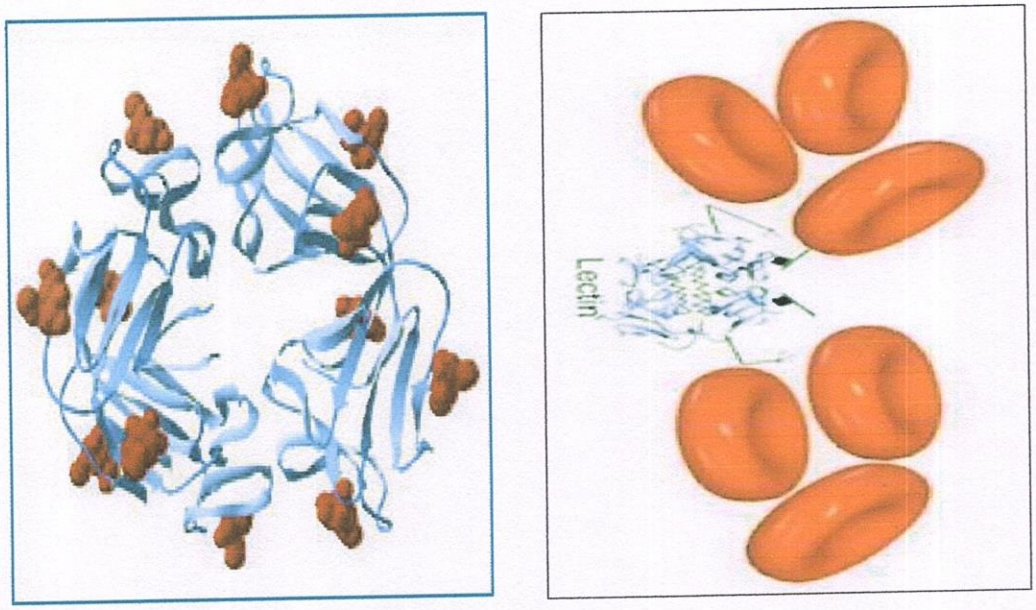


Fig.1F. Agglutination of RBC by lectin

1.16.2 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. The first mitogenic agent to be described was PHA, the Lectin from red kidney bean (*phaseolus vulgaris)*. By 1970 there additional mitogenic Lectins had become known; pokeweed mitogen, a Lectin from *Wistaria floribunda,* and concanavlin A (Lis and Sharon, 1981). Recent examples include the Lectins isolated from *Lathyrus sativus, Ulex europeus* (Yamaguchi *et al.,* 1979) all most all of this mitogen inhibited by simple sugars. Most mitogenic Lectins stimulate only the thymus dependent population of Lymphocytes (T-cells) and are inactive or inhibitory for mitosis of the other class of Lymphocytes, the thymus independent (or B-cell). Exceptions are the Lectins from the crab *Homarus americanus*. From chicken tissue and from the slime mold *Dictyostelium purpureu*, which stimulate mouse B-cell but not T-cell.

1.16.3 Induction of suppressor cells

Treatment of human and murine Lymphocytes with various mitiogens, in particular concanavalin-A, also induces the generation of potent suppressor cells capable of inhibition activities of T and B –cells in vitro. The activities that can be studied include response to mitogens and alloantigens, generation of cytotoxic cells and immunoglobulin production.

In man, the ability to develop suppressor cell activity upon treatment with concanavalin-A seems to be a preorgative of normal Lymphoid cells since in peripheral blood Lymphocytes of patients with immunodeficiency diseases generation of suppressor cells is decreased (Newman *et al.,* 1979).

1.16.4 Lectin dependent cytotoxicity of lymphocytes and macrophages

A) Cytotoxicity of Lymphocytes

The interaction with 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 Clarle & Gold stein. In contrast in the presence of concanavalin–A or other mitogenic Lectins a wide verity of antigenically unrelated target cells are lysed by Cytotoxic T-Lymphocytes, a phenomenon known as Lectin dependent Cytotoxicity.

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, 1983). The lack of immune specificity in the Lectin dependent reaction has been attributed to the ability of the Lectin to bind to both effector and target cells.

B) Cytotoxicity of macorophages

Simillarly to anti-tumor antibodies that can induce macorophage mediated tumor lysis, Lectin 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) possess the ability to mediate carbohydrate specific binding of mouse macrophages and tumor cells and to induce killing of the tumor cells by the macorophages.

Lectin-mediated killing of both syngenic and allogenic tumor cells was observed wheat germ agglutinin, but not soybean agglutinin or concanavalin-A, was shown to initiate lysis of erythrocytes by human blood leukocytes. (Wei and Lindquist, 1981). With guinea Pig macrophages, however, both concanavalin-A and wheat germ agglutinin (but not Soybean agglutinin) induced cytolysis of homologous erythrocytes (Keisari, 1982; Keisari and Pick, 1981).

1.16. 5 Lectin- mediated phagocytosis of target cells

In addition to Lectin dependent killing of tumor cells by macrophages, Lectins mediate binding and occasionally, phagocytosis of other types of cell (Sharon, 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 *et al.*, 1970). Wheat germ agglutinin

markedly enhanced the binding to and phagocytosis of Bacteria such as *Staphylococcus aureus H, Staphylococcus albus* or *Micrococcus luteus* by mous peritoneal macrophages (Gallily *et al.,* 1984).

1.16.6 Insulin immitic activity

Concanavalin-A, wheat germ agglutinin and several other Lectins 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 Lectin to the insulin receptor due to its glycoprotein in nature the competition of binding between the hormone and concanvalin-A or wheat germ agglutitin (Roullier *et al.,* 1986).

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

1.17 Application of lectin

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 glyco-conjugates. The list of application of Lectins is growing rapidly and the potential of these proteins is for from completely explored.

1.17.1 Isolation and structural studies of glycoconjugates

Because of the analogy of Lectin-saccharide interactions with those between antibody and antigen application of Lectins, either in solution or, more commonly, in immobilized form, for the detection and purification of a variety of carbohydrate containing compounds 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.

1.17.2 Detection and identification

The specific interaction of a biopolymer with a Lectin may be taken as evidence that the polymer contains carbohydrate. For example, the first indication that yeast invertase in a glycoprotein was obtained with concanavalin-A (Sumner & Howell, 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 *et al.,* 1982; Hedeo *et al.,* 1981).

Immune electrophoresis has also been adapted for use with Lectins, for example, to study heterogeneity of glycoproteins with respect to carbohydrate content and structure (Bog–Hansen *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. This system (ELLA, Enzyme- linked Lectin assay) is conceptually similar to ELISA.

1.17.3 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 on Lectins of complex biological extracts e.g. from membranes or cell homogenates, usually results in the isolation of mixtures of glycoproteins and polysaccharides. Fraction of glycoproteins and glycopeptides, which differ only slightly in their carbohydrate composition or in the structure of their oligosaccharide units on Lectins. In this way, molecular variants of α-fetoprotein from calf-serum and rat amniotic fluid (Bayerd and kerchaert, 1977), α_1-acid glycoprotein, α-protease inhibitor from human serum and of highly human ceruloplasmin application of Lectins is the isolation of tRNA species containing glycosylated bases tRNA ASP was readily isolated on a column of concanavalin-A Sepharose, and tRNA Try from chromatography on a column of *Ricinus communis* agglutinin –Sepharose.

1.17.4 Structural studies of blood group substances

The contribution of Lectins to our knowledge of the chemical structure of the ABO blood group determinants in humans is well established. Thus, 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 ell (*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 β4GLCNAC for structural elucidation of certain dis-substituted blood group oligosaccharides (Rovis *et al.,* 1973)

1.17.5 Studies of cellular & sub cellular membranes:

The presence of Lectin receptor on cells is readily demonstrated with the aid of suitable Lectin derivative, generally techniques developed by immunologists for the study of cell-surface antigens (Lotan, 1979; Roth, 1980; Schrevel *et al.,* 1981).

Radioactively labeled Lectins may be used to measure the number of Lectin receptor sites of the cell surface and their homogenecity, as well as the affinity of Lectin receptor interactions.

1.17.6 Cell-separation

The wide use of Lectins to separate viable bearing different surface carbohydrates is a recent development. Most of this work has been done with mammalian cells, particularly lymphocytes. In principle any sub-population of cells. Weather 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 damage to 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 separation of leucocytes from erythrocytes in human blood with the aid of PHA. The erythrocytes were selectively agglutinated by the Lectin and were removed from the mixture by centrifugation.

Table 1B. Lectins used for cell separation

Source of Lectin	Source of cell	Examples of cells separation
Peanut	Human	Cortical and Medullary thymocytes, immature and mature cord blood lymphocytes
	Murine	Cortical and Medullary thymocytes, suppressor spleen T cells.
	Chicken	Supressor lymphocytes
Soybean	Human	Helper and suppressor lymphocytes, bone marrow stem cells.
	Murine	B& T splenocytes, stem cells from spleen.
	Hamster	B & T –splenocytes
	Monkey	Bone marrow stem cells
Wheat germ	Murine	B & T-splenocytes
Helix pomatia	Human	Peripheral B & T-lymphocytes
	Murine	B & T –splenocytes
	Rat	B & T – splenocytes
Dolichos biflorus	Human	A_1 O (H) erythrocytes

1.17.7 Identification of microorganisms

Agglutination of a microorganism from a primary isolate with a particular Lectin may constitute a confirmatory identification of the organism making it possible to dispense with subsequent expensive and time consuming culturing or serological testing, e.g. *Neisseria* and related bacteria by its agglutination with wheat germ agglutinin. *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*. These results suggested a rapid and convenient means for identifying *Bacillus arthracis* from primary isolation media. Coagulase positive *Staphylococcus aureus* can be distinguished from coaculase negative *Staphylococci* because only the latter strains are agglutinated by wheat germ agglutinin.

1.17.8 Lectin as drug carriers

The ability of some Lectins to interact preferentially with certain transformed cells has led to attempt 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 *et al.,* 1978) or ricin (Yamaguchi *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 (Kittao and Hattori, 1977), chlorambucil (Lin *et al.,* 2008) and Methotrexate (Lin *et al.,* 2008; Tsuruo *et al.,* 1980), had a higher activity against various cultured tumor cell lines than the equivalent dose of free drug and Lectin.

1.17. 9 Lectin resistant cells

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

1.18 Clinical uses

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

Table 1C. Blood type specific lectins

Specificity	Source of Lectin
Anti-A	*Griffonia simplicifolia I* (A_4) *Helise pomatia* *Phaseotus lunatus* *Viccia cracca*
Anti- A_1	*Dolichos biflorus*
Anti –B	*Griffonia simplicifolia I* (B_4)
Anti –O(H)	*Anguilla anguilla* *Lotus tetragonolobus* *Ulex europeus*
Anti A + N	*Molucell laevis*
Anti- N	*Vicia graminea*
Anti –T	*Arachis hypogaea*
Anti-Tn	*Salvia solaria*

1.18.1 Anti-insect activity of lectins

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

Moreover, at least some transgenic plants can improve the corresponding environments and human health because their production considerably reduces the load of chemical insecticides and herbicides. Lectins demonstrate anti-insect activity. They increase the mortality or delay the development of insect. When incorporated in an artificial diet, *Arisaema jacquemontii* lectin adversely affected the development of *Bactrocera cucurbitae* larvae (Kaur *et al.*, 2006). Arisaema helleborifolium lectin exhibited anti-insect activity towards the second instar larvae of *B. cucurbitae* (Kaur *et al.*, 2006). The insecticidal property of lectins may be due to orchestration of enzymatic activity of larvae. After treatment with different lectins, the activity of esterases in larvae was increased whereas the activity of acid phosphatase and alkaline phosphatase decreased. Galectin-1 treatment of Plutella xylostella larvae brought about disruption of the microvilli and induced abnormalities in these epithelial cells (Chen *et al.*, 2009). *Dioscorea batatas* lectin inhibited the emergence of *Helicoverpa armigera* larvae into adults by avidly binding to larval brush border and peritrophic membrane.

1.18.2 Antifungal activity of lectins

The expression of Gastrodia elata lectins in the vascular cells of roots and stems was strongly induced by the fungus Trichoderma viride, indicating that lectin is an important defense protein in plants. Following insertion of the precursor gene of stinging nettle isolectin I into tobacco, the germination of spores of Botrytis cinerea, Colletotrichum lindemuthianum, and T. viride was significantly reduced. Thus, lectins may be introduced into plants to protect them from fungal attack. Plant lectins can neither bind to glycoconjugates on the fungal membranes nor penetrate the cytoplasm owing to the cell wall barrier. It is not likely lectins directly inhibit fungal growth by modifying fungal membrane structure and/or permeability. However, there may be

indirect effects produced by the binding of lectins to carbohydrates on the fungal cell wall surface. Chitinase-free chitin-binding stinging nettle (Urtica dioica lectin) impeded fungal growth. Cell wall synthesis was interrupted because of attenuated chitin synthesis and/or deposition (Parijs *et al.*, 1991). The effects of nettle lectin on fungal cell wall and hyphal morphology suggest that the nettle lectin regulates endomycorrhizal colonization of the rhizomes. Several other plant lectins inhibit fungal growth. The first group includes small chitin-binding merolectins with one chitin-binding domain, e.g., hevein from rubber tree latex (Parijs *et al.*, 1991) and chitin-binding polypeptide from Amaranthus caudatus seeds. The only plant lectins that can be considered as fungicidal proteins are the chimerolectins belonging to the class I chitinases. However, the antifungal activity of these proteins is ascribed to their catalytic domain.

1.18.3 Lectins–antitumor drugs

It is well documented that lectins have an antitumor effect. Flammulina velutipes hemagglutinin-inhibited proliferation of leukemia L1210 cells. *Haliclona cratera* lectin displayed a cytotoxic effect on HeLa and FemX cells. Dark red kidney been hemagglutinin exerted an antiprolifarative activity toward leukemia L1210 cells. Glycine max lectin impeded proliferation of brest cancer MCF7 cells and hepatoma (HepG2) cells (Lin *et al.*, 2008). Mistletoe lectin used JHeLa cells (Liu *et al.*, 2008), Polygonatum odoratum lectintreated murine fibrosarcoma L929 cells (Liu *et al.*, 2009), Polygonatum cyrtonema lectin-treated human melanoma A375 cells (Liu *et al.*, 2009), Pseudomonas aeruginosa hemagglutinin-treated breast cancer cells (MDA-MB-468, and MDA-MB-231HM cells; *Liu et al.*, 2009), French bean hemagglutinin-treated breast cancer MCF-7 cells (Lam and Ng 2010), and recombinant protease-resistant galectin-9- treated myeloma cells. Although the apoptotic pathways look different, activation of different caspases is usually involved. Caspase-3 plays a central role in apoptosis. It interacts with caspase-8 and caspase-9. Therefore, caspase-3 is usually investigated in apoptotic pathways, except in the case of a caspase-3- deficient cell line (e.g., MCF-7 cells) which was used in the study of French bean hemagglutinin (Lam and Ng 2010). Caspase-8 and -9 are also activated (Liu *et al.*, 2009; Liu *et al.*, 2009; Lam and Ng 2010). Apoptosis can be mediated by death receptors initated by lectins. FAS receptor is the receptor with which lectins often interact (Liu *et al.*, 2009; Lam and

Ng 2010). The interaction is probably by protein–protein interaction. The Bcl family members (anti-apoptotic factors) were down-regulated (Bhutia *et al.*, 2008; Liu *et al.* 2009; Lam and Ng 2010). The sequestration of cytochrome C in mtochondria was interrupted. Cytochrome c release wasobserved (Bhutia *et al.*, 2008, b; Liu *et al.*, 2009; Lam and Ng, 2010). Finally, mitochondrial membrane depolarization was detected (Liu *et al.*, 2009; Lam and Ng 2010). G_0/G1 arrest was frequently observed (Bhutia *et al.*, 2008; Liu *et al.*, 2009; Lam and Ng 2010). It seems that it is the characteristic of lectin-induced apoptosis.

1.18.4 Lectins interaction with intestinal mucosa

A number of years ago, Jaffe and his co-workers had proposed that the toxicity of bean lectins could be attributed to their ability to bind to specific receptor sites on the surface of the intestinal cells lining the intestine. Numerous investigators have since served to establish the validity of this concept by demonstrating that lectins of diverse sugar specificity bind to various regions and to different types of cells the intestinal mucosa (Etzler and Branustra *et al.*, 1986). Binding of the kidney bean Lectin to the intestinal is the appearance of lesions and severe disruption and abnormal development of the microvilli.

1.18.5 Lectins-antiviral drug

The D-mannose-specific lectin from Gerardia savaglia was firstly reported to prevent infection of H9 cells with human immunodeficiency virus (HIV)-1. Further more, the lectin inhibited syncytium formation in the HTLV-IIIB/H9-Jurkat cell system and HIV-1/human lymphocyte system by reacting with the oligosaccharide side chains of the HIV-1 gp120 envelop molecule (high-mannose oligosaccharides). A year later, the lectins concanavalin A, wheat germ agglutinin, *Lens culinaris* agglutinin, *Vicia fabagglutinin, T. dioica* (potol) agglutinin and phytohaem (erythro) agglutinin were found to bind to gp120. They were able to inhibit fusion of HIV-infected cells with CD4 cells by a carbohydrate-specific interaction with the HIV-infected cells. Plant lectins displayed anti-coronaviral activity, especially mannose-binding lectins, in severe acute respiratory syndrome corona virus. They interfered viral attachment in early stage of replication cycle and suppressed the growth by interacting at the end of the infectious virus cycle.

1.18.6 Clinical states in which Lectins have been used to Assess:

1. Immune dysfunction.
2. Acute myeloid leukemia
3. Chronic lymphatic leukemia
4. Lymphoproliferative diseases
5. Hodgkin's disease
6. Malignnant lymphoma
7. Nonlyphoid malignancies
8. AIDS
9. Immune disorders
10. Sezary's syndrome
11. Lupus erythematosus
12. Rheumatoid arthritis
13. Stress
14. Leprosy
15. Hepatitis
16. Diabetes mellitus

1.19 Aim of my present study

The lectin have attracted great interest on according to their various biological activities, such as cell agglutination (Konkumnerd *et al.*, 2010), antifungal (Sitohy *et al.*, 2007), antiviral (Tian *et al.*, 2008) and antiproliferative activities (Liu *et al.*, 2010). They are used as a tool both for analytical and preparative purposes in Biochemistry, Cellular Biology, Immunology and related areas. In agriculture and medicine the use of lectins greatly improved in the last few years. Specially mannose and N-acetyl-D-glucosamine-binding lectins are considered biologically important defense protein, because these sugars are widely distributed in microorganisms, insects and animals (Kaur *et al.*, 2005). Although different sugar specific lectins were purified from

different plant sources e.g., mannose specific lectins from *Ophiopogon japonicas* and *Smilax glabra* Roxb (Wong *et al.*, 2008, Tian *et al.*, 2008), mannose/maltose specific lectin from *Calystegia sepium* (Leiner 1976), N-acetyl-D-glucosamine specific lectin from *Arundo donex* (Kaur *et al.*, 2005), *Curcuma amarissima* (Kheere *et al.*, 2010), chitin binding lectin from *Setcreasea purpurea*. Lectin purification from *Punica granatum* may have significant analytical and clinical applications. First time *Punica granatum* cultivated in Bangladesh is subjected for the source of fruit lectin in this research work. The present thesis comprises the following works:

- Purification and characterization of a lectin from *Punica granatum*.
- Investigation of the physicochemical properties of the purified lectin.
- Cytotoxicity and antimicrobial study of the purified lectin.
- Antitumour study of the purified lectin (*in vitro* and *in vivo*).

CHAPTER TWO

PURIFICATION AND CHARACTERIZATION

METHODS AND MATERIALS

2.1 Chemicals

The important chemicals used in this study Isolation, Characterization of Lectin from *Punica granatum* juice are mentioned below with their manufacturers:

2.1.1 Chemicals for sample preparation and activity test

Tris (Hydroxymethyl) aminomethene: Fluka Sweden

Sodium Chloride: Mearck, Germany

Hydrochloric Acid: BDH Chemicals Ltd., Poole England

Copper sulfate: BDH Chemicals Ltd., Poole England

Calcium chloride: Mearck, Germany

2.1.2 Chemicals for sugar inhibition test

Tris (Hydroxymethyl) aminomethene: Fluka, Sweden

Hydrochloric Acid: BDH Chemicals Ltd., Poole England

Calcium chloride: Mearck, Germany

Sodium Chloride : Mearck, Germany

L-Arabinose: Siga Chemicals Co., U.S.A

D-Galactose: Siga Chemicals Co., U.S.A

D-Mannose: Siga Chemicals Co., U.S.A

N, N-methylene –bis –Acrylamide: Siga Chemicals Co., U.S.A

D-Rafffinose: Siga Chemicals Co., U.S.A

D- Glucose: Siga Chemicals Co., U.S.A

D-Rhamnose: Siga Chemicals Co., U.S.A

D-Fucose: Siga Chemicals Co., U.S.A

D-Maltose: Siga Chemicals Co., U.S.A

D-Xylose: Siga Chemicals Co., U.S.A

D-Lactose: Siga Chemicals Co., U.S.A

D-Melibiose: Siga Chemicals Co., U.S.A

Methyl- α -D-glactopyranoside: Siga Chemicals Co., U.S.A

Methyl- α -D-glucopyranoside: Siga Chemicals Co., U.S.A

N-Acetyl-D-glucoseamine: Siga Chemicals Co., U.S.A

2.1.3 Chemicals for ion exchange chromatography and purity test

DEAE Cellulose gel

Ethylenediaminetetraacetic acid, EDTA: Fluka, Sweden

Acrylamide: Bio Rad Laboratories, Richmond, U.S.A

N,N-methylene-bis-acrylamide: Bio Rad Laboratories, Richmond, U.S.A

Glycerol: Bio Rad Laboratories, Richmond, U.S.A

Glycine: Bio Rad Laboratories, Richmond, U.S.A

TEMED (N,N,N,N-tetramethylene diamine): Siga Chemicals Co., U.S.A

Lauryl Sulfate (SDS): Siga Chemicals Co., U.S.A

Butanol: BDH Chemicals Ltd., Poole England

Ammonium persulfate(APS) : Bio Rad Laboratories, Richmond, U.S.A

Bromophenol blue: Bio Rad Laboratories, Richmond, U.S.A

Coomassie Brilliant blue-250: Bio Rad Laboratories, Richmond, U.S.A

Glacial Acetic Acid: Mearck, Germany

CBB R- 250: Mearck, Germany

Methanol: Mearck, Germany

Tris (Hydroxymethyl) aminomethene: Fluka, Sweden

Bromophenol blue: BDH Chemicals Ltd., Poole England

Acetic Acid: BDH Chemicals Ltd., Poole England

β-Marcaptoethanol : Bio Rad Laboratories, Richmond, U.S.A

Bovine serum albumin (67 kDa),: Fluka, Sweden

Ovalbumin (45 kDa): Fluka, Sweden

Carbonic anhydrase (29 kDa): Fluka, Sweden

Trypsin inhibitor (20 kDa) : Fluka, Sweden

Lysozyme (14.6 kDa): Fluka, Sweden

2.1.4 Chemicals for physicochemical property test

Ethylenediaminetetraacetic acid (EDTA) : Fluka, Sweden

Urea: BDH Chemicals Ltd., Poole England

Guanidin-HCl: Mearck, Germany

Sodium Acetate: Mearck, Germany

Glacial acetic acid: BDH Chemicals Ltd., Poole England

Sodium biphosphate: Fluka, Sweden

Tris (Hydroxymethyl) aminomethene: Fluka, Sweden

Glycine: Fluka, Sweden

Sodium hydroxide: BDH Chemicals Ltd., Poole England

Magnecium Chloride

Manganese Chloride

2.1.5 Chemicals for other purposes

Sodium azide: Siga Chemicals Co., U.S.A

Sodium bicarbonate: OSK., Japan

Phenol: OSK., Japan

Sea salt (non-iodized): OSK., Japan

Sulphuric acid: OSK., Japan

Trypan Blue: OSK., Japan

2.2 Equipments

The important equipments used throughout this study are listed below:

Equipments for sample preparation and purification

1) Fraction collector-SF-160 (Advantec, Japan)
2) Homogenizer- Model AM-5
3) Cool chamber
4) Micro centrifuge
5) Frize dryer
6) Ion exchange column
7) pH-meter
8) Incubator
9) Micropipette
10) Shimadzu Spectrophotometer
11) Slub gel electrophoresis
12) Magnetic Stirrer
13) Micro Shaker
14) Clean bench
15) Autoclave
16) Fluorescence spectrometer
17) High-speed centrifuge
18) Dialysis tube

2.3. Collection of sample

P. granatum (pomegranate) was collected from local market of Rajshahi, Bangladesh; all over the year.

2.4 Methods of Purification

2.4.1 Preparation of crude protein extract

In order to purify protein from *P. granatum* (pomegranate) fruits in biologically active form, all the operations were performed at 4°C. After peeling, the juice was produced from the pulp with the help of a clean muslin cloth. After production of the juice, 10mM Tris-HCl (pH 8.2) buffer was added to it. The juice was clarified further by centrifugation at 10,000 rpm, at 4°C for 10 minutes. The supernatant was stored at 0°C.

2.4.2 Ion exchange column chromatography

A. Packing of the column: Dried powder was dissolved in distilled water for 1 hour. A column of desired length was packed with the activated column material. Precaution was taken to avoid trapping of air bubbles during packing.

B. Equilibration of column: After packing, the column was equilibrated with 10 mM Tris-HCl buffer pH 8.2.

C. Sample application and wash: Sample was centrifuged at 10,000 rpm, at 4°C for 10 minutes. The centrifuged sample was loaded on ion exchange column at room temperature which was previously equilibrated by the same buffer. After sample application the ion exchange column was washed with 10 Mm Tris-HCl buffer (pH 8.2). Proteins were eluted with the same buffer with linear gradient of NaCl (0.0- 0.5) M.

An automatic fraction collector collected the eluant. Fractions (3ml/tube) were collected at a 1 min flow rate. Absorbance of each fraction was measured at 280nm.

2.5. Test of purity

2.5.1. 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 15% SDS-PAGE according to the method of Laemmli (1970).

2.5.2 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 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 upto 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

It was prepared by mixing the following components:

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

(ix) Preparation of distaining solution

The distaining solution was prepared by mixing the components as given below.

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

(x) Preparation of electrophoresis buffer (Chamber buffer)

Electrophoresis buffer was prepared by the following components.

Components	Amounts
Tris base	3 g
Glycine	14.4 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.

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 (15%)
30 % acrylamide solution	6.6ml
1.5 M Tris-HCl buffer, pH 8.8	3.4 ml
Distilled water	3.2ml
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) The layer of water was poured.

(v) Preparation of stacking gel/Spacers gel

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

Components	Amounts (15%)
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 µ

(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 filled lower buffer chamber 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 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 the sandwich 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 soaking the gel in distaining solution did distaining. When the gel became transparent, it was taken out and rinsed with water.

2.6 Blood type specificity test

2.6.1. Collection and preparation of 2% blood

All types of human blood(A, B, AB, O), hen red blood cells, rat red blood cells were collected, (about 1 ml of every group) in 1% NaCl solution. The rat blood and the hen blood were also taken for the test. All the blood samples were centrifuged at 1,027 g for 10 min to get 2% blood cell.

2.6.2 Preparation of Hemagglutination buffer

20 mM Tris-HCl buffer, containing 1% NaCl and 10 mM $CaCl_2$ (Hemagglutination buffer), pH 7.8.

2.6.3 Procedure of hemagglutination assay

The hemagglutination test was performed in the presence of 50 µl of hemagglutination buffer in every well of the titre plate. Then 50 µl of protein (PgL) solutions were added to the first well of the titre plate and serially diluted upto 8 well . Finally, 50 µl of 2% blood cells (RBC) in saline were added to the wells of titre plate. Then, this plate were shacked by microshaker and incubated at 34^0C for 60 minutes (Atkinson *et al.*, 1980).

2.7. Determination of Optical Density (O.D. at 280 nm) vs. Protein Concentration by Folin-Lowary Method (Lowry *et al.*, 1951).

2.7.1 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 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.

2.7.2 Method

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 upto the volume 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 concentration against absorbance (O.D) and from the graph the concentration of protein was determined.

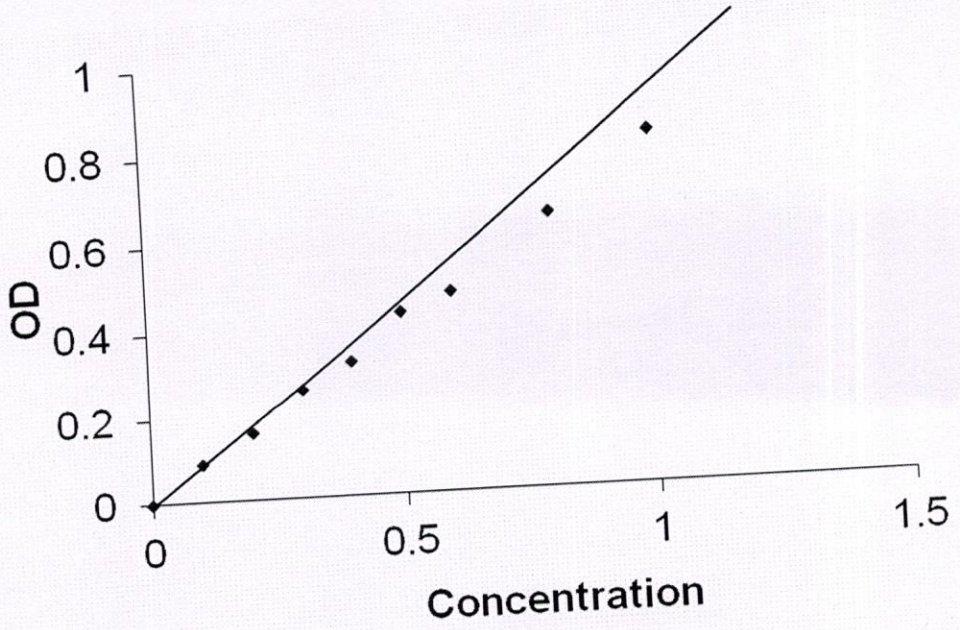


Fig. 2A. Standard curve of BSA for the determination of protein concentration

2.8 Test for glycoprotein and estimation of sugar

Phenol in the presence of sulfuric acid can be used for quantitative colorimetric micro determination of sugars and their methyl derivatives, oligosaccharide and polysaccharides as described by Dubois *et al.,* (1956).

This method was also employed for detecting the presence of sugar in proteins and enzymes.

A. Materials

(i) 5% Phenol (in water)

(ii) Concentrated sulfuric acid

(iii) Protein solution

B. Procedure

The protein solution (0.5 ml from protein solution of 0.40 mg / ml) was taken in a test tube and made upto 2 ml by distilled water. Then 1 ml of 5% phenol was added to it and finally 5 ml of conc. H_2SO_4 was added rapidly. To obtain good mixing, the stream of acid being directed against the liquid surface rather than against the side of the test tube. The tube was allowed to stand for 10 minutes. Then it was shaken and kept in the dark at 25 to 30° C for 20 minutes. It was taken out and the absorbance of the solution was measured at 490 nm.

C. Preparation of standard curve

A standard glucose solution (0.1 mg/ml) was prepared. Then 0.0, 0.1, 0.2, 0.3, 0.4, 0.6, & 0.8 ml of this solution containing 0.0, 0.01, 0.02, 0.03, 0.04, 0.06, & 0.08 mg of glucose, respectively, were taken in different test tubes and made upto 2 ml with distilled water. The solution was treated similarly as described above. A standard graph of glucose was constructed by plotting the concentration of glucose against their absorbance. From the graph, the concentration of sugar in protein was calculated.

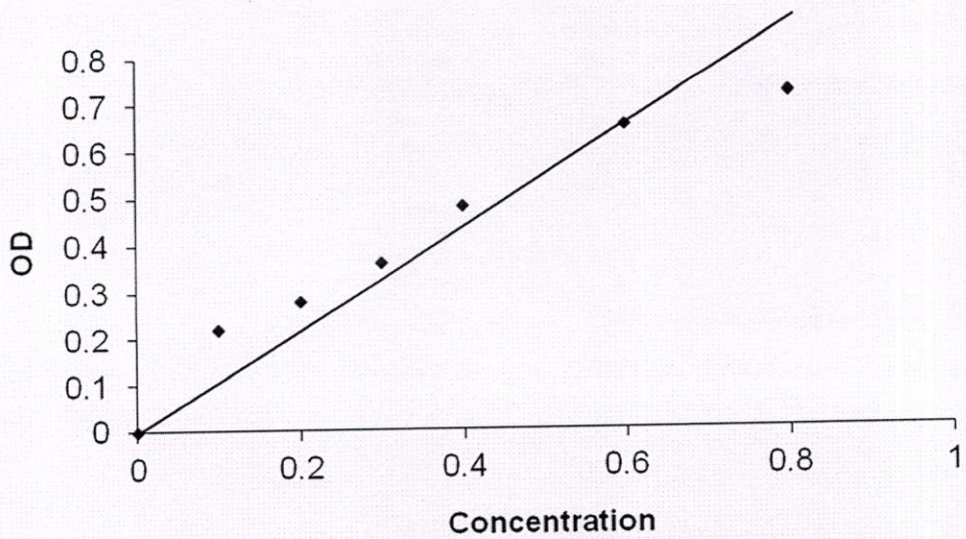


Fig. 2B. Standard curve for estimation of sugar present in glycoprotein

2.9. Hemagglutination inhibition study

2.9.1 Materials

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

(b) 2% RBC in 1% NaCl.

(c) D- mannose, D-maltose, D-raffinose, D-glucose, D-galactose, D-lectose, D-fucose, D-fructose, D-rhamnose, D-melibiose, D-arabinose, methyl-α-D-glucopyranoside, N-acetyl-D-glucosamine, methyl-α-D-galactopyranoside, methyl-β-D-glucopyranoside, 4- Nitrophenyl-β-D-galactopyranoside, 2-Nitrophenyl-β-D-galactopyranoside, 4-Nitrophenyl - β- D- glucopyranoside, 2-Nitrophenyl-β-D-glucopyranoside, 4-Nitrophenyl-α-D-glucopyranoside, 4-Nitrophenyl-β-D-glucopyranoside, 2-Nitrophenyl-β-D-glucopyranoside, 4-Nitrophenyl-α-D mannopyranoside, Inositol, cellulose, at the final concentration of 50 mM.

2.9.2 Procedure

The hemagglutination-inhibition test was performed in the presence of different sugars as described above. 25µl of hemagglutination buffer were added to the selected every well of the 96 well titre plate. Then 25 µl of sugar solutions were added to the

first well of the titre plate and serially diluted upto 8 well and then 25 µl of protein solutions (0.40 mg/ml of PgL) were added to the wells of the titre plate. Finally, 50 µl of 2% blood cells (RBC) in saline were added to the wells of titre plate. Then, this plate were shacked by microshaker and incubated at 34^0C for 60 minutes (Atkinson *et al.,* 1980).

2.10. Determination of pH stability

The pH stability of pomegranate lectin (PgL) was examined by incubating the lectin solutions (1 mg/ml) against different buffer (pH ranges from 4.0-12.0) for 8 hours at room temperature (30^0C). After 8 hours the lectin solutions were dialyzed against 10 mM tris–HCl buffer, pH 7.8 containing 0.15 mM NaCl for 12 hours. Then the hemagglutination activity was checked. The following buffers were used for pH stability assay: 0.1 M sodium acetate (pH 4.0 and 6.0), 0.1 M Tris–HCl (pH 8.2) and 0.1 M glycine-NaOH (pH 9.0 and 12.0).

2.11. Determination of heat stability

To examine the thermostability, PgL (0.40 mg/ml in Tris-HCl buffer saline, pH 7.8) was heated in a water bath for 30 min at different temperatures from 30-80°C and cooled at room temperature. Then 50 µl of PgL was serially diluted with an equal amount of hemagglutination buffer, pH 7.8 and the hemagglutination titer was performed. The non-heated lectin sample was used as a control, which denoted 100% activity.

2.12. Treatment with Urea and divalent cation

For detecting the effect of urea, purified lectin solution(0.40 mg/ml) was incubated at room temperature with 8 M urea for 30 min.PgL in the same buffer without urea was used as control and its activity was considered as 100%.To determine the hemagglutination activity on divalent cataions, PgL was incubated with 0.2 M EDTA for 1 hour at room temperature . After incubation the sample was dialyzed exhaustively against 0.5 M NaCl and 10 mM tris buffer (pH 8.2) for 12 hours at 4 °C and then subjected to hemagglutination assay in the presence and absene of each Ca^{2+}, Mg^{2+} and Mn^{2+} in the hemagglutination buffer.

2.13 Method for structural stability study

Fluorescence measurements of PgL were performed at 25 μg/ml protein concentration and with 1 & 2 mM of 4-Nitrophenyl-α-D-mannopyranoside on a Shimadzu spectrofluorometer RF-5301 at room temperature. PgL was incubated with 5.0 mM of EDTA for 1 hour and 8M of urea for 1 hour and 2 hour and the native and treated samples were placed in a 1 cm × 1 cm × 4.5 cm quartz cuvette. Samples were measured and recorded at the λex of 280 nm and the λem in the range of 300-400 nm and widths for the excitation and emission monochromators were maintained at 5 nm.

2.14 Results and Discussion

2.14.1 Purification

The lectin was purified from *Punica granatum* (Pomegranate) fruit juice by using ion exchange chromatography on DEAE Cellulose column. At first crude sample was centrifuged at 10,000 rpm, 4°C for 10 minutes. The clear supernatant was applied on DEAE Cellulose column previously equilibrated with 10 mM Tris-HCl buffer, pH 8.2. The bound fraction was eluted by linear gradient of NaCl (0-0.5M) in 10 mM tris-HCl, pH 8.2 buffer. The unbound fraction did not show any hemagglutination activity. The eluted fraction showed hemagglutination activity. From 250 ml of pomegranate juice, around 5.0 mg of lectin was purified each time.

2.14.2 Determination of the molecular mass

The molecular weight of the purified protein was determined by SDS- polyacrylamide gel electrophoresis using Aprotinin (MW 6.5), Lysozyme (MW 14.4), Carbonic anhydrase (MW 31.0) and Serum albumin (MW 66.2 kDa) as reference proteins. The molecular weight was calculated from the standard curve of reference proteins which was constructed by plotting molecular weight against relative mobility of the reference proteins on gel after electrophoresis and the molecular weight of purified lectin as determined by SDS-PAGE was estimated to be 28±1 kDa.

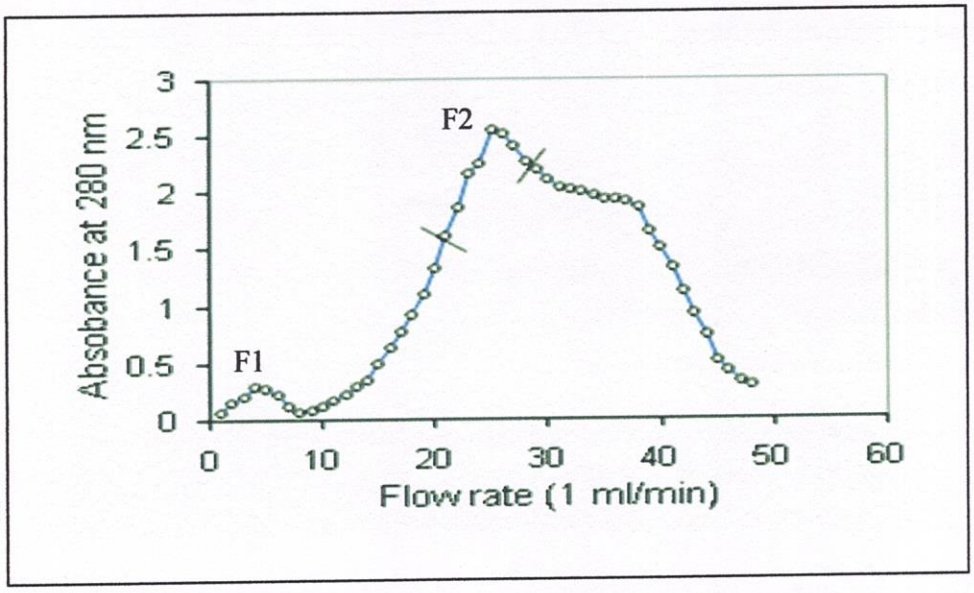
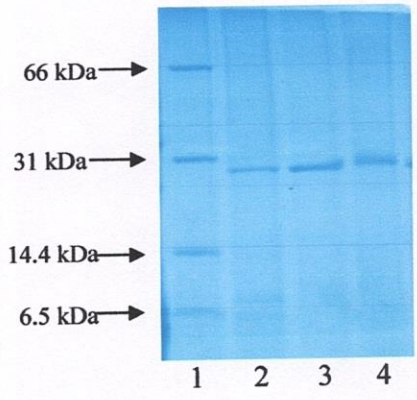


Fig. 2C. Ion exchange chromatography of PgL on DEAE-Cellulose column.

Fraction F2 agglutinated red blood cells whereas fraction-1 did not agglutinated red blood cells. The elution profiles were monitored at 280 nm. Fractions (3 ml / tube) were collected at 1 ml / min flow rate. The sample *P. granatum* possesses a lot of pigment substances; hence lots of pigment compounds were bound with the column. Therefore, we got the graph like above.



Lane 1: Marker proteins
Lane 2: Crude Pg
Lane 3: Pure Lectin
Lane 4: Lectin with β-marcaptoethanol

Fig. 2D. SDS-PAGE of PgL on 15% polyacrylamide gel. Lane 1: Marker proteins Lane; 2: Crude Pgl. Lane 3: Pure Lectin, Lane 4: lactin with β-marcaptoethanol

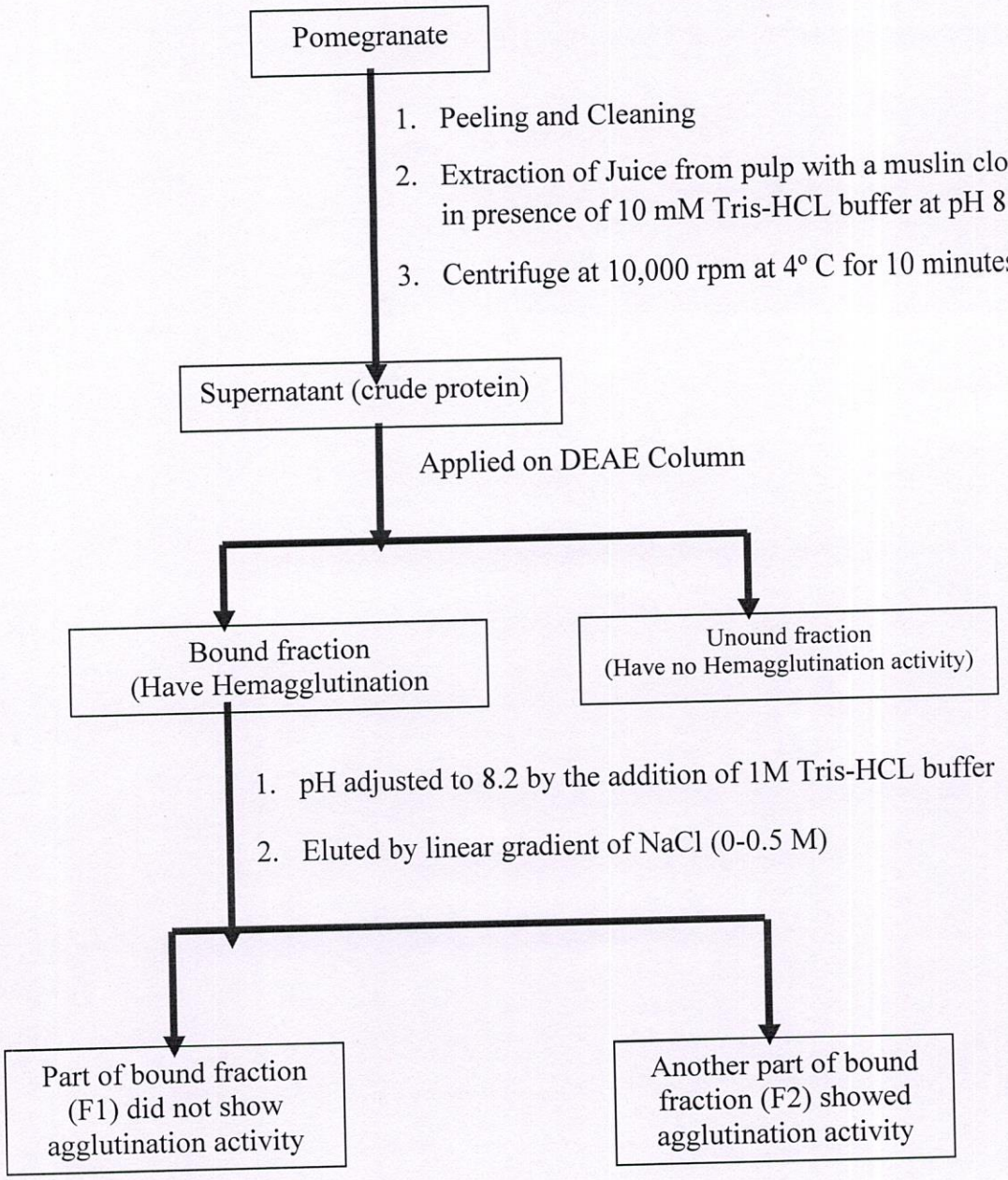


Fig. 2E. Flow chart of Lectin purification from *Punica granatum*

Table 2A: Summary of the purification of Lectin from Pomegranate

Fraction	Total Protein (mg)	Total Hemagglutination activity (titre)	Specific activity	Yield (%)
Crude	9750	1600000.00	164	100
Ion-Exchange Chromatography	58.5	48000.00	820.5	3

Hemagglutination Activity (titre) = Reciprocal of highest dilution showing visible hemagglutination

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

$$\text{Specific activity} = \frac{\text{Observed Specific activity}}{\text{Total protein}} \times 100$$

$$\text{Fold} = \frac{\text{Observed Specific activity}}{\text{Initial Specific activity}} \times 100$$

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

2.14.3 Optical density vs concentration estimation

The absorbance of 0.25 for *Punica granatum* lectin (PgL) at 280 nm was found to be equal to 0.40 mg/ml of protein as determined by Lowry method (1951) using BSA as standard.

2.14.4 A test for glycoprotein and estimation of sugar

The purified PgL was glycoprotein in nature as it gave yellow-orange color in the presence of phenol-sulfuric acid (Crueger and Crueger, 1990). The percentage of sugar present in the glycoprotein was calculated from the standard curve of glucose and it was found that the protein contained of 40% neutral sugar.

2.14.5 Hemagglutination activity:

The minimum hemagglutinating activity of PgL was found to be 12.5µg/ml for O type human erythrocytes and albino rat erythrocytes (Fig.2F). The lectin did not show any agglutination activity against chicken erythrocytes.

Table 2B. Agglutination of different Blood types by PgL

Blood	Group	PgL (µg/ml)
Human blood	O^+	12.5
Albino rate blood	-	12.5

* Minimum PgL concentration required for a visible agglutination.

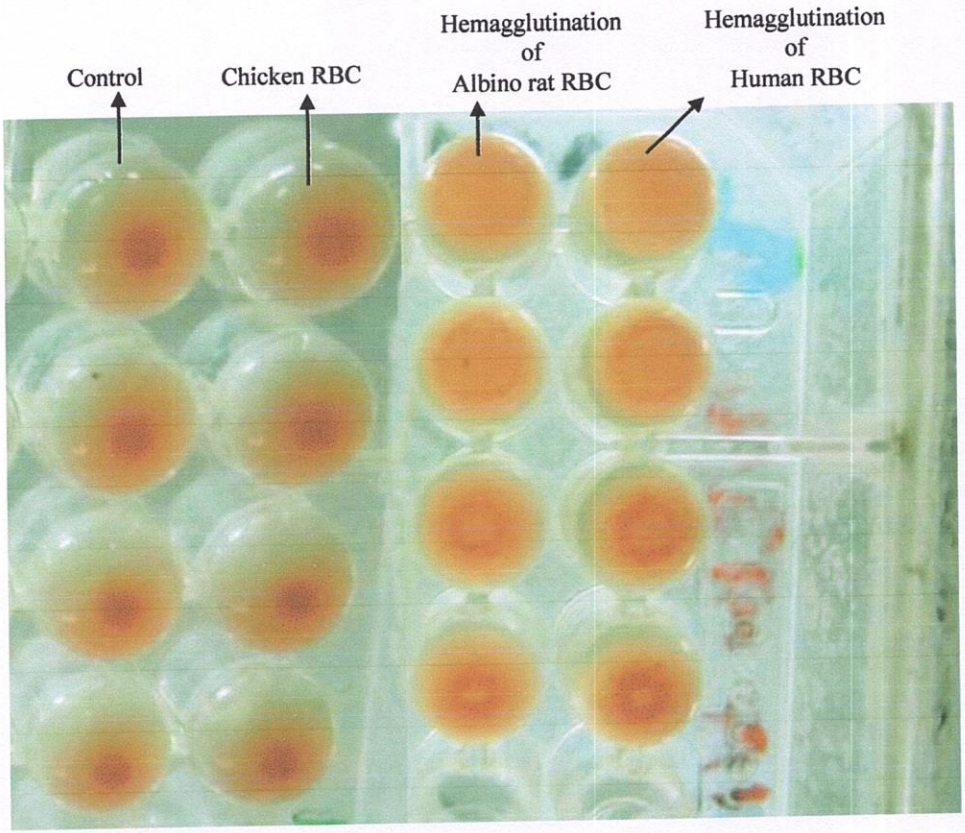


Fig. 2F. Hemagglutination of different blood types by PgL

2.14.6 Effect of pH

PgL was observed to be stable between the pH 6.0 to 12.0 and showed the maximum hemagglutination activity at the pH range from 6.0 to 8.2. The lectin lost its activity 50% at pH 9.0 and 10.0, whereas 87.5% activity lost at pH 11.0 and 12.0 and 100% activity lost at pH 5.0 and bellow 5.0. Percentage activity was calculated assuming the maximum agglutination activity to be 100%. The Fig. 2G demonstrates the overall result.

2.14.7 Effect of temperature

The hemagglutination activity of PgL was not changed with the change of temperature. The lectin was incubated at different temperatures for 30 min and the hemagglutination activity was assessed. Lectin activity (hemagglutinating activity) was not changed between the temperatures 30-80°C as represented in Fig. 2H. The highest agglutination activity observed at room temperature was taken as 100% activity.

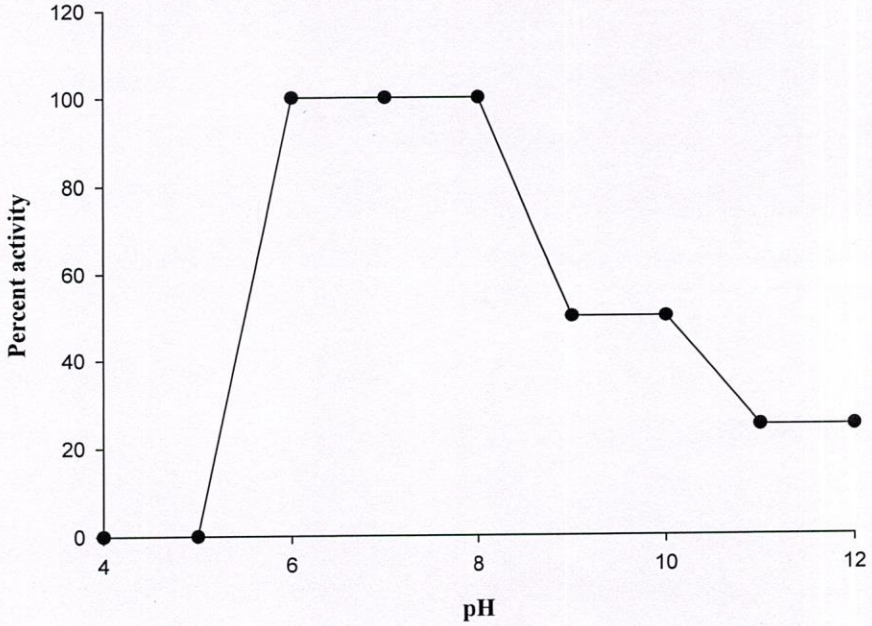


Fig. 2G. Hemagglutination activities of PgL at different pH values

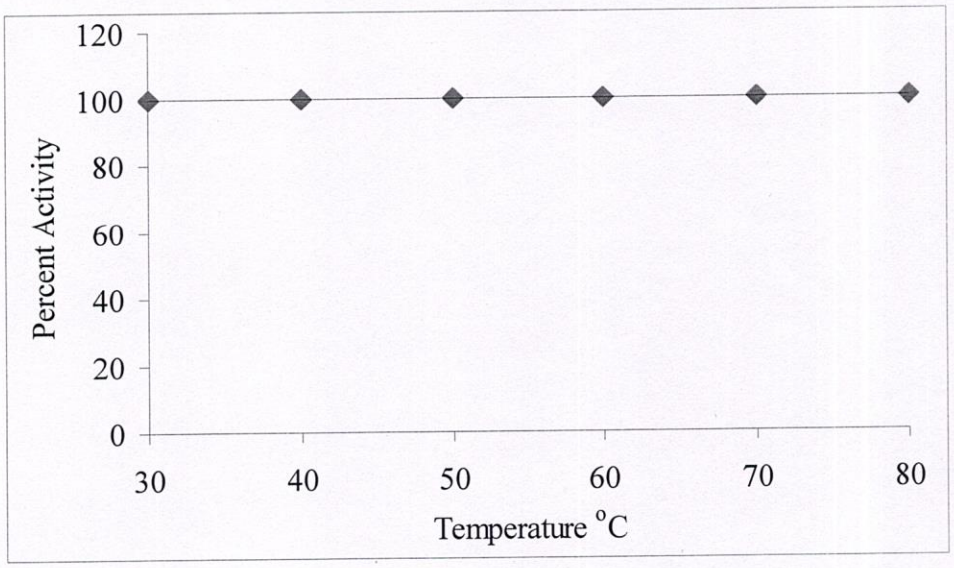


Fig. 2H. Hemagglutination activities of PgL at different Temperature

2.14.8 Effect of denaturant and divalent cations on PgL

The hemagglutination activity of PgL was affected by urea. The lectin lost its activity 75% in the presence of 8 M urea as compared with control. Furthermore, EDTA treated PgL did not show any agglutination activity when divalent ions were absent in the hemagglutination buffer. But after addition of 10 mM of Ca^{2+} or Mn^{2+} to the hemagglutination buffer, PgL showed agglutination activity.

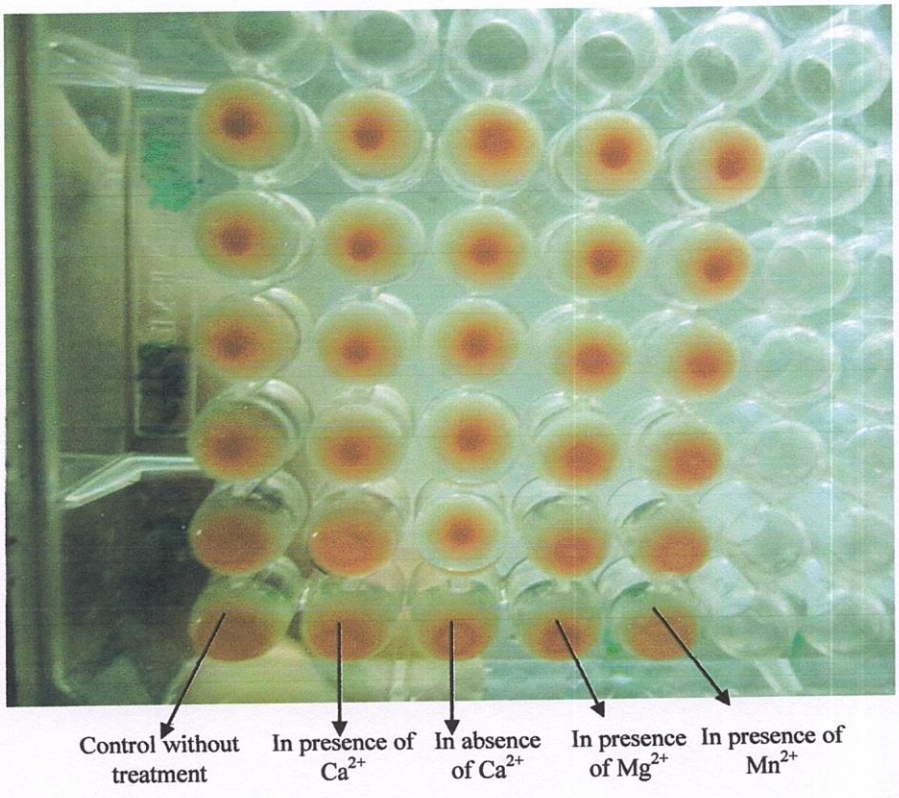


Fig. 2I. hemagglutination of PgL in presence and absence of divalent ions.

2.14.9 Hemagglutination inhibition study

The hemagglutination inhibition of PgL was performed using rat red blood cells in the presence and absence of different sugars. The result of hemagglutination inhibition was presented in Table 2C. It was evident that 4-nitrophenyl-α-D mannopyranoside is the best inhibitor for PgL.

Table 2C: Inhibition of hemagglutination activity of PgL by mono and oligo saccharides: "+" Hemagglutination activity; "-" No hemagglutination activity.

Sl No	Sugar Name	Hemagglutination at different Concentrations(mM)			
		1.48	0.74	0.37	0.185
1	D-mannose	+	+	+	+
2	D-maltose	+	+	+	+
3	D-raffinose	+	+	+	+
4	D-glucose	+	+	+	+
5	D-galactose	+	+	+	+
6	D-lectose	+	+	+	+
7	D-fucose	+	+	+	+
8	D-fructose	+	+	+	+
9	D-rhamnose	+	+	+	+
10	D-melibiose	+	+	+	+
11	D-arabinose	+	+	+	+
12	methyl-α-D-glucopyranoside	+	+	+	+
13	N-acetyl-D-glucosamine	+	+	+	+
14	methyl-α-D-galactopyranoside	+	+	+	+

15	methyl-β-D-glucopyranoside	+	+	+	+
16	4-Nitrophenyl-β-D-galactopyranoside	+	+	+	+
17	2-Nitrophenyl-β-D-galactopyranoside	+	+	+	+
18	4-Nitrophenyl-β-D-glucopyranoside	+	+	+	+
19	2-Nitrophenyl-β-D-glucopyranoside	-	-	+	+
20	4-Nitrophenyl-α-D-glucopyranoside	+	+	+	+
21	4-Nitrophenyl-β-D-glucopyranoside	+	+	+	+
22	2-Nitrophenyl-β-D-glucopyranoside	+	+	+	+
23	4-Nitrophenyl-α-D mannopyranoside	-	-	-	+
24	Inositol	+	+	+	+
25	cellulose	+	+	+	+

2.14.10 Structural stability studied by Fluorescence spectroscopy

Structural changes of PgL were determined based on its binding to inhibitory sugar, chelating agent EDTA and urea. PgL showed standard emission spectra at 346 nm. When 1 mM sugar was added to the lectin, the fluorescence intensity decreased and after increasing the concentration to 2 mM the intensity decreased even more. While 8 M urea treated PgL for 1 hour and 2 hour and also EDTA treated PgL for 1 hour did not show any significance difference against the standard PgL intensity.

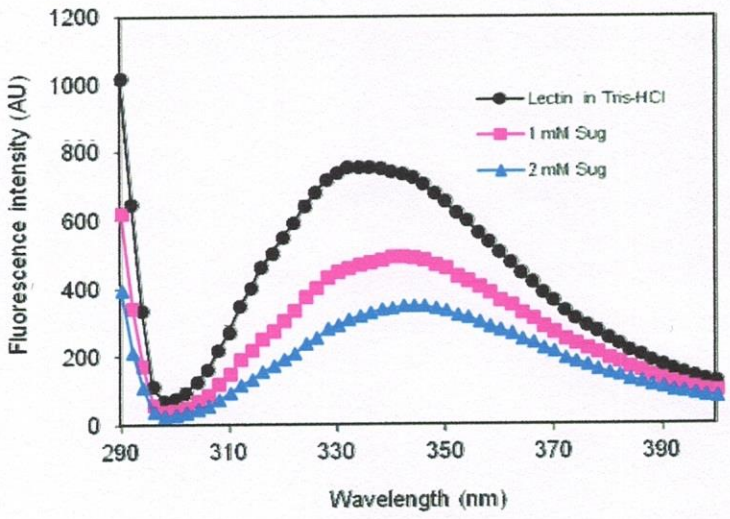


Fig: 2J. PgL showed standard emission spectra at 346 nm. When 1 mM sugar was added to the lectin, the fluorescence intensity decreased and after increasing the concentration to 2 mM the intensity decreased even more.

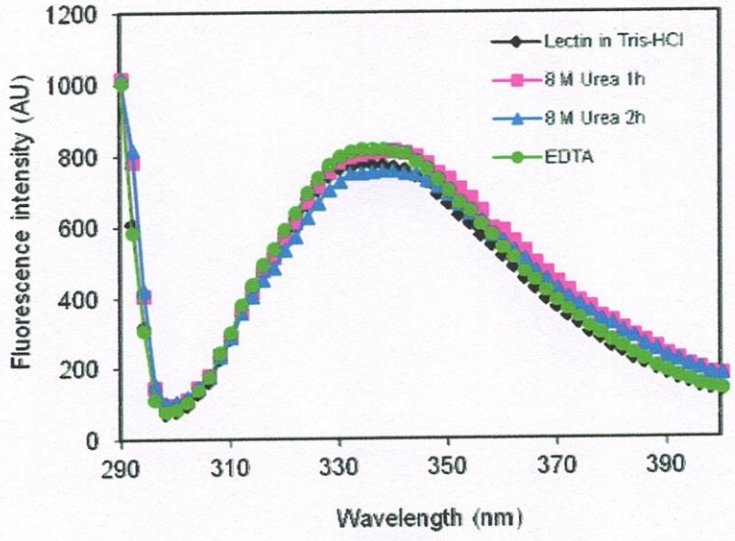


Fig: 2K. 8M urea treated PgL for 1 hour and 2 hour and also EDTA treated PgL for 1 hour did not show any significance difference against the standard PgL intensity.

2.15 Discussion

One lectin was purified first time from the juice of *Punica granatum* (pomegranate) cultivated in Bangladesh by using ion exchange column chromatography and designated as PgL. The lectin migrate with single band with the molecular weight of 28.0±1.0 kDa both in the presence and absence of β-marcaptoethanol. The lectin agglutinated human erythrocytes (O), rat red blood cells with the minimum concentration of 12.5 μg/ml as checked which is almost similar to *Arisaema jacquemontii* lectin (11.5 μg/ml) (Kaur et al., 2006). A different result was observed for HTTL (*Helianthus tuberosus* lectin) which could not agglutinate any human blood group (Saseelan et al., 2002). PgL did not show any hemagglutination activity against chicken erythrocytes.

Hemagglutination inhibition studies revealed that 2-nitrophenyl-β-D glucopyranoside and 4-nitrophenyl-α-D mannopyranoside were the inhibitor for PgL and the minimum inhibitory concentration was 0.37 mM for 4-nitrophenyl-α-D- mannopyranoside and 0.74 mM for 2-nitrophenyl-β-D glucopyranoside. No other carbohydrates showed similar inhibitory effect in this study. Similarly, NNTL (lectin purified from *Nymphaea nouchali* tuber) was also a complex sugar specific lectin. O-nitrophenyl-β-D galactopyranoside was the inhibitor for NNTL (Kabir et al., 2011).

The activity of the lectin was stable at 30-80°C. From the heat stability assay, it can be assumed, the PgL is a thermostable lectin. It was found that small glossy black soybean lectin (Peng Lin et al., 2008) and *Phaseolus vulgaris cv* lectin were preserved its 100% activity in the temperature range 0-70°C (Arishya Sharma et al., 2009). pH stability of PgL showed that its activity remains 100% at pH range 6 to 8.2 . Below and above the pH the hemagglutination activity decreased remarkably. Similarly, it was found that dietary garlic (*Allium sativum)* lectin ASA II retains 100% activity at pH 6 and 8 (Fatema Clement et al., 2010).

In the presence of 8M urea the lectin lost its activity by 75% compared with the control. Furthermore, EDTA treated PgL did not show any hemagglutination activity when divalent ions were absent in the hemagglutination buffer. But after addition of 10mM of Ca^{2+} or Mn^{2+} to the hemagglutination buffer PgL showed hemagglutination

activity. Similarly, *Nymphaea nouchali* tuber lectin (NNTL) is a divalent ion dependent lectin (Kabir et al., 2011).

The purified PgL was glycoprotein in nature as it gave yellow-orange colour in the presence of phenol-sulfuric acid (Dubois *et al*., 1956). The percentage of sugar present in the glycoprotein was calculated from the standard curve of glucose and it was found that the protein contained 40% of neutral sugar. On the other hand, potato lectin contains 50% sugar on a weight basis (Pramod et al., 2006). Different result was also observed for *Canavalia ensiformis* (Goldstain *et al*., 1986), *Cratylia floribunda* (oliveria *et al*., 1991), *Canavalia brasiliensis* (Moreira *et al.,* 1984), *Canavalia cathartica* (Suseelan *et al.,* 2007) and *Dioclea virgata* (Moreira *et al.,* 1991) did not contain any sugar .

CHAPTER THREE

BACTERIAL AGGLUTINATION AND TOXICITY STUDY OF *PUNICA GRANATUM*

3.1 Introduction

Constituents from natural resources as well as synthetic organic and inorganic compounds have been receiving much attention in biological systems. Many of these compounds are being used as chemotherapeutic agents. The pathogenic organisms are developing resistance heriditically towards some antibacterial agents. It is therefore, necessary to find out consistently new, safer, effective and inexpensive agents for the purpose. In this connection, it is very important to determine whether purified protein solutions are active against test organisms or not.

It is well known that a large number of human and animal diseases are caused by pathogenic microorganisms. Infections due to these micro organisms have been major cause of morbidity and mortality in both developed and developing countries. Historically many of the new antibiotics were isolated from natural sources like medicinal plants. Many more 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 bacterial agglutination is to determine the susceptibility of the microorganism to any agent.

Agglutination is defined as specific clamping of particulates such as cells or cell sized objects. Lectin can cause agglutination of bacteria. Bacterial agglutination occurs when lectin binds carbohydrates on bacterial cells, linking the bacterial cells together. Several different bacteria have been typed by their lectin agglutination patterns including *Lactobacillus* isolates (Annuk et al., 2001). Gorskia et al. showed that a high proportion of *Lactobacilli* bound to the mannose specific lectin *Concovalin A* (Con A) and that this was useful as an additional marker for strain characterization

(Gorskia et al., 1994). In future, it will be possible in clinical cases of infection caused by pathogens having specific attachment mechanisms, such as the mannose binding lectin, to interfere with bacterial adherence and colonization to mammalian cell surface by using specific inhibitor. Bacterial agglutination study may help to better understand the role of cell surface carbohydrates in pathogenesis of several pathogenic bacteria (Claudia et al., 2008). Endocytosed lectins act as power powerful exogenous growth factors for the small intestine, and they can induce dramatic shift in the bacterial flora. Bark et al., showed that extracts of the fungus *Sclerotinum rolfsii* produced a lectin in the media that agglutinate several gram negative bacteria (Bark et al., 1984). So the study of bacterial agglutination by lectin may provide us much information about bacterial pathogenesis.

3.1.1 Test organisms used for the study

The choice of test organisms will obviously depend greatly on the purpose of the investigation. The pure cultures were collected from the Institute of Biological Science,University of Rajshahi,B angladesh. The following pathogenic bacteria were used for the study of bacterial agglutination activity of PgL.

Table. 3A. List of the test pathogenic bacteria

Serial No.	Name of test organism
	Gram-positive
1	*Listeria monocytogenes*
2	*Bacillus subtilis*
	Gram-negative
3	*Escherichia coli*
4	*Salmonilla typhi*
5	*Salmonella enteritidis*

3.1.2 Conditions necessary for growth of pathogen

For optimum growth of bacteria, we must have the required nutrients, the permissive temperature, enough moisture in the medium, the proper gaseous atmosphere, proper salt concentration, appropriate pH and there must be no growth-inhibiting factor.

3.1.3 Preparation of media

The following media were used for the growth of the above test bacteria.

Table 3B. Composition of nutrient broth culture medium

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

Table: 3C. Characteristics of the ingredients used in media

Raw materials	Characteristic	Nutritional value
1. Peptone	The product resulting from the digestion of oteinaceous materials, e.g.; meat, casein and gelatin, digestion of the proteins materials is accomplished with acids or enzymes, many different peptones (depending upon the protein used and the method of digestion) are available for the use in bacteriological media, and peptones differ in their ability to support growth of bacteria.	Principal source of organic nitrogen may also contain some vitamins and sometimes carbohydrates depending upon the kind of proteinaceous material digested.
2. Agar	A complex carbohydrate obtained from certain marine algae: Processed to remove extraneous substances.	Used as a solidification agent for media, agar dissolved in aqueous solutions, gels when the temperature is reduced below 45°C: Agar is not considered as a source of nutrient to the bacteria.
3. Yeast extract	An aqueous extract of yeast cells, commercially available as powder	A very rich source of the vitamins B: also contains organic nitrogen and carbon compounds.

3.1.4 Sterilization procedure

Glassware's were sterilized in the autoclave at 121^0C temperatures and a pressure of 15 lb/sq. inch for 15 minutes. Micropipette tips, culture media, cotton, forceps, blank discs etc were also sterilized.

3.2 Bacterial agglutination study

3.2.1 Materials

(a) 20mM Tris-HCl buffer, pH 8.2, 1% NaCl & 10mM $CaCl_2$

(b) Protein solution

(c) Bacterial cells: (*Escherichia coli*, *Salmonilla typhi*, *Listeria monocytogenes*, *Bacillus subtilis* and *Salmonella encephalitis*) in 1% NaCl

3.2.2 Procedure

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

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

3.3 Toxicity study of PgL

3.3.1 Introduction

Brine shrimp lethality bioassay is a recently developed method for bioactive compound assessment. This bioassay indicates toxicity as well as a wide range of pharmacological activities (e.g. anticancer, antiviral, insecticidal, pesticidal, AIDS etc.) of the compounds. Extracts and isolated compounds from plant origin can be tested for their bioactivity by this method. 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 (Mc Laughlin *et al.,* 1990; Perssone *et al.,* 1980; Meyer *et al.,* 1982).

The method has advantages of being very simple, rapid (24 hours), inexpensive. It easily utilizes a large number of organisms for statistical validation, requires no special equipment and a relatively small amount of sample. In the present study, PgL was used for its cytotoxicity study using brine shrimp lethality test.

3.3.2 Principle

Brine shrimp eggs are hatched in simulated seawater to get nauplii. Test samples are prepared by the addition of calculated amount of distilled water for obtaining desired concentration of the test sample. The nauplii are counted by visual inspection and are taken in vials containing 2.5 ml of seawater. Then samples of different concentrations are added to the pre-marked vials through micropipette. The vials are then left for 24 h and then

the nauplii are counted again to find out the cytotoxicity of the test compound and compared to the results with positive control.

3.3.3 Experimental design

3.3.4 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 lamp to attract shrimp.

d) Pipettes

e) Micropipette (10µl- 100µl)

f) Vials, (4 ml).

g) Magnifying glass. (3X magnifying glass)

3.3.5 Procedure

a) Preparation of simulated seawater

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

b) Hatching of brine shrimp

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

Eighteen clean vials were taken for the sample in five 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 containing 2.5 ml of artificial sea water (prepared by dissolving 38.0 gm of NaCl in 1 liter DW) and then 1.0, 0.75, 0.5, 0.25, 0.125 ml PgL were added to each vial at the final concentration of 100, 75, 50, 25, and 12.5 µg/ml. The volume of each vial was adjusted to 3.5 ml by the addition of artificial sea water. The entire test was performed at around 30°C, under the continuous light regime. Three replicate were used for each experiment.

d) Counting of nauplii

After 24-hours of incubation, the vials were observed using a magnifying glass and the number of survivors in each vial were counted and noted. From these, the nauplii were counted averagely of each three vials, which contained same conc. of sample & the percent (%) mortality was calculated for each dilution. The concentration-mortality data were analyzed by using Probit analysis (Finney *et al.,* 1971).

3.4 Results

3.4.1 Bacterial agglutination

Five pathogenic bacterial strains, gram-positive *LIsteria monocytogenes, Bacillus subtilis* and gram-negative *Escherichia coli*, *Salmonella enteritidis, Salmonella typhi* were used for bacterial agglutination test. PgL did not able to agglutinate the above mentioned bacteria.

3.4.2 Brine-Shrimp lethality bio-assay

In brine-shrimp lethality bio-assay, the lectin did not show any toxic effect on brine shirimp nauplii, as compared with the control.

3.5 Discussion

PgL did not agglutinated the bacterial strains: gram-positive *Listeria monocytogenes, Bacillus subtilis* and gram-negative *Escherichia coli, Salmonella enteritidis*, *Salmonella typhi.* So it can be said that these bacterial strains are not sensitive to PgL. PgL is a 4-Nitropheneyl α-D-mannopyranoside specific lectin. Probably, these bacterial strains does not contain the above mentioned complex sugar in their cell wall lipopoly-saccharide (LPC) and hence did not agglutinated by PgL.

In brine-shrimp lethality bio-assay the *P. granatum* lectin did not show any toxic effect on brine-shrimp nauplii. Therefore, probably the lactin is not cytotoxic in nature.

CHAPTER FOUR

STUDY OF ANTIPROLIFERATIVE ACTIVITY OF PgL *IN VITRO* AND *IN VIVO* IN MICE

4.1. Introduction

An essential part of drug development is the testing of potential new purified compounds against animal tumors both *in vitro* and *in vivo*. *In vitro* test determines whether the compound has any effect against neoplasm or not and *in vivo* test determines dose response curves on animals bearing transplanted tumor. This gives an indication of the effects of the new drugs not only on the tumor but also on the host, thereby indicating its toxicity and therapeutic index.

4.2 Methods and materials

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

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

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

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

4.2.1 Instruments used:

i. 96-well flat bottom culture plate

ii. Titer plate reader

iii. CO_2 incubator

iv. Microscope

v. Haemocytometer

vi. Fluorescence Microscope (Olympus-iX71)

vii. Vortex shaker

viii. Centrifuge

4.2.2 Chemicals used:

i. Phosphate buffer saline (PBS)

ii. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

iii. Isopropanol

iv. EDTA

v. PBS (phosphate buffer saline)

vi. Sugar

vii. Urea

viii. Hoechst 33342 dye

ix. M Phosphate buffer saline (pH 7.0)

x. Propidium Iodide (PI) staining solution

4.2.3 Experimental Animal

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).

4.2.4 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°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 ICDDRB, Dhaka. The nutrient composition of the diet is given in the table:

Table 4A. The nutrient composition of the diet (per 100gms of diet)

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

4.2.5 Experimental Tumor Model

Transplantable tumor (Ehrlich ascites carcinoma) used in this thesis were obtained from Indian Institute of Chemical Biology(IICB), Kolkata-700032, West Bengle, India and were maintained in our laboratory in Swiss Albino mice by intraperitoneal transplantation.

4.2.6 Ehrlich ascites carcinoma (EAC) cell line

In 1907, Ehrlich located these tumors in the mammary gland of a white mouse and thus the tumor was named after him. The present form Ehrlich ascites carcinoma cell has been developed by Loewenthal and John from one of the several lines of carcinoma. The later arises from spontaneous epithelial tumors probably of mammary gland origin. External surface of EAC cell is covered with a thin cell membrane as revealed by the electron microscopic examination of ultrathin section. The membrane matrix of Ehrlich ascites tumor cells are specially strong. Extensive studies on the morphology of normal and cancer cells have shown that both the surface and intracellular membranes have 'unit membrane' structure, a bimolecular lipid leaflet lined on both sides of protein and or polysaccharide materials.

EAC cell being a cancer cell also possesses the same type of structure .Tumor can be grown subcutaneously as solid form, but the present ascitic form is produced by infecting tumor cell suspension into the mouse peritoneal cavity. The ascitic tumor develops as a milky white fluid containing rounded tumor cells. One million tumor cells multiply to yield about 25-100 million tumor cells /ml. Host survives for 14-30 days.

4.2.7 Transfusion of Ascitic Tumor

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 aspiration .The freshly drawn fluid was diluted with normal saline and the tumor cells number was adjusted to approximately 3×10^6 cells/ml by counting the number with haemocytometer. The viability of the tumor cells were checked by trypan blue dye (0.4%) exclusion assay. Cell sample 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.

4.2.8 Collection of EAC cells

The EAC cells collected from donor mice (Swiss albino) of 20-25 g body weight were suspended in sterile isotonic saline. A fixed number of viable cells (usually 3×10^6 cells/ml) were implanted into the peritoneal cavity of each recipient mouse, as described in the above experimental method as control. Mice were sacrificed on the sixth day and the intraperitoneal tumor cells were harvested by normal saline.

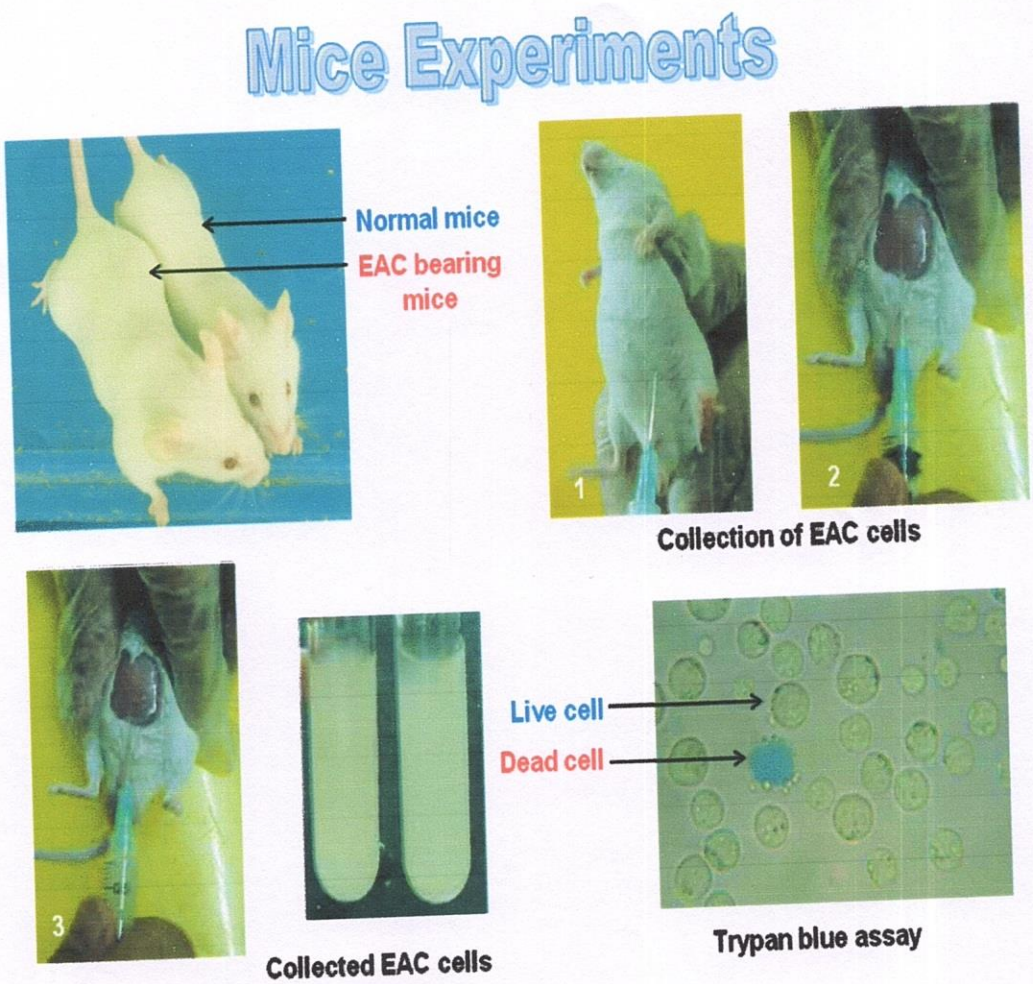


Figure 4A. Collection of EAC cell and trypan blue assay.

4.3 MTT colorimetric assay

It is now well-documented that apoptosis or programmed cell death is the key mechanism by which Chemotherapeutic agents exert their cytotoxicity. These colorimetric assays (MTT) are mainly useful in determination of cellular proliferation, viability and activation.

4.3.1 Method used for MTT colorimetric assay

MTT colorimetric assay was used to determine EAC cells proliferation. Cells (3×10^5 in 200 µl RPMI 1640 media) were plated in the 96-well flat bottom culture plate in the presence and absence of different concentrations (12.5–100.0 µg/ml) of *Punica granatum* lectin (PgL)

↓

Incubated for 24 h at 37°C in CO_2 incubator.

↓

Aliquot was removed carefully

↓

180 µl of PBS and 20 µl of MTT were added and further incubated for 8h at 37°C.

↓

Aliquot was removed again and 200 µl of acidic isopropanol was added into each well and incubated again at 37°C for 1 h.

↓

Subsequently absorbance was read at 570nm using titer plate reader. The follow-ing equation was used to calculate the cell proliferation inhibition ratio:

$$\text{Proliferation inhibition ratio (\%)} = \frac{(A - B) \times 100}{A}$$

where A is the OD_{570nm} of the cellular homogenate (control) without PgL and B is the $OD_{570\ nm}$ of the cellular homogenate with PgL.

4.4 Cell growth inhibition of PgL *in vivo* in mice

EAC cells were collected from a donor Swiss albino mouse bearing 6-7 days old ascites tumors and the viability was observed by trypan exclusion assay. EAC cells (1×10^6) in 0.1 ml normal saline showing about 99% viability were injected intraperitonealy to each Swiss albino mouse. After 24 h of tumor inoculation, the mice were randomly distributed into four groups (six mice per group). Three groups of mice were treated intraperitonealy with PgL at the doses of 1.5, 3.0 and 4.5 mg/kg/day, respectively for five consecutive days. The remaining group was used as the control. Mice in each group were sacrificed on the 6^{th} day and the total intraperitoneal tumor cells were harvested by normal saline and counted by light microscope. Then the total numbers of viable cells in every mouse of the treated groups were compared with those of control group (EAC treated only). Finally percentage of inhibition was determined by trypan blue exclusion assay. Exactly 20 µl of cell suspensions were stained with equal volume of trypan blue (0.4% in 10 mM phosphate buffer saline) for 2 min. Then the numbers of viable cells were counted by light microscope. Cells those retained blue color were considered as dead cells. The proliferation inhibition ratio was calculated by the following equation:

Percentage of inhibition = 100 – {(cells from PgL treated EAC bearing mice / cells from EAC bearing control mice) ×100}.

4.5 Method for observation of cell morphological changes and nuclear damage

Harvested EAC cells were collected and washed three times by 10 mM cold phosphate-buffered saline, pH- 7.0. Finally the cells were suspended in 1 ml of PBS and the cell density was adjusted to 1×10^6 cells/ml. For this assay a control and a PgL treated sample of each 1 ml of cell suspension were taken, to which 10µl of Hoechst 33342 dye was added and mixed thoroughly. The mixtures were incubated separately at 37° C for 15 minutes and then centrifuged at 1,000 rpm for five minutes at 4°C. The precipitated cells were resuspended in 1,000µl of 10mM PBS.

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

4.6 Results:

4.6.1 Antiproliferative activity of PgL *in vitro* :

Effect of PgL on EAC cells was investigated by MTT assay. The lectin induced EAC cell death in a dose dependent manner (Fig. 4B). The lectin was found to have 19.8% inhibitory effect at a concentration 100 µg/ml. The effect decreased with the reduction in lectin concentration and it reached to 6.9% at 12.5 µg/ml.

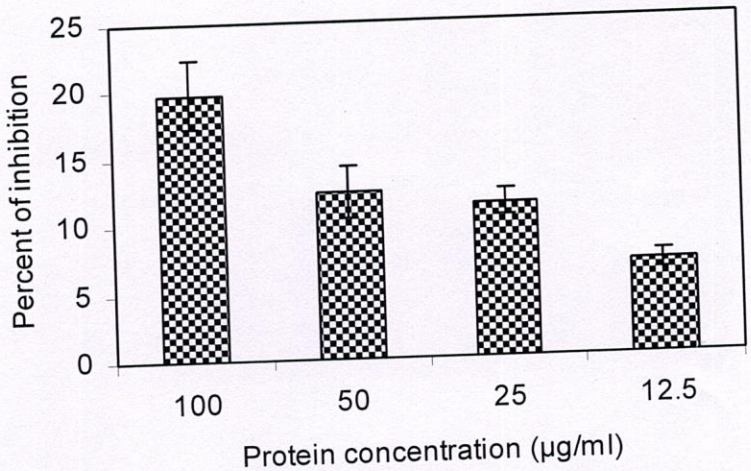


Fig.4B. *Punica granatum* lectin (PgL) inhibits EAC cells growth. The EAC cells were treated with various doses of PgL for 24 h. The inhibition ratios were measured by the MTT assay.

4.6.2 Antiproliferative activity of PgL *in vivo*

EAC cells proliferation in mice was effectively inhibited with PgL treatment in a dose dependent manner. At 1.5 mg/Kg/day, growth inhibition of EAC cells was 18% and the inhibition increased to 32 and 33% at 3.0 and 4.5 mg/Kg/day PgL concentrations, respectively.

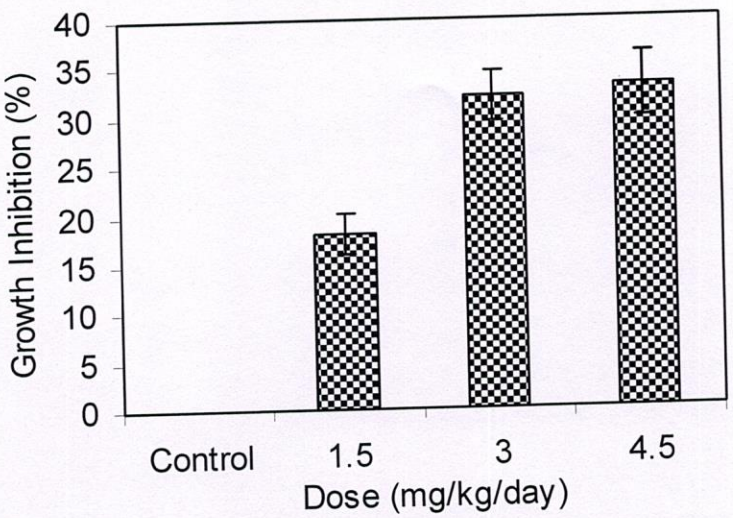


Fig.4C: Growth inhibition of PgL treated and PgL untreated EAC cells.

4.6.3 Observation of cell morphologic changes and nuclear damage

The effect of PgL on apoptotic morphological changes of EAC cells *in vivo* in mice were detected by fluorescence and optical microscopy after staining with Hoechst 33342. In the control group, the nuclei in which DNA resides were round and homogeneously stained (Fig. 4D (a)). Although no such morphological change and nuclear damage were observed in the cells treated with PgL, but in PgL treated sample the cells become cluster like form as compared to those were observed as disperse from in the control (Fig. 4D (b)). Cell morphology was also observed by optical microscopy (Fig. 4D(c) and 4D(d))

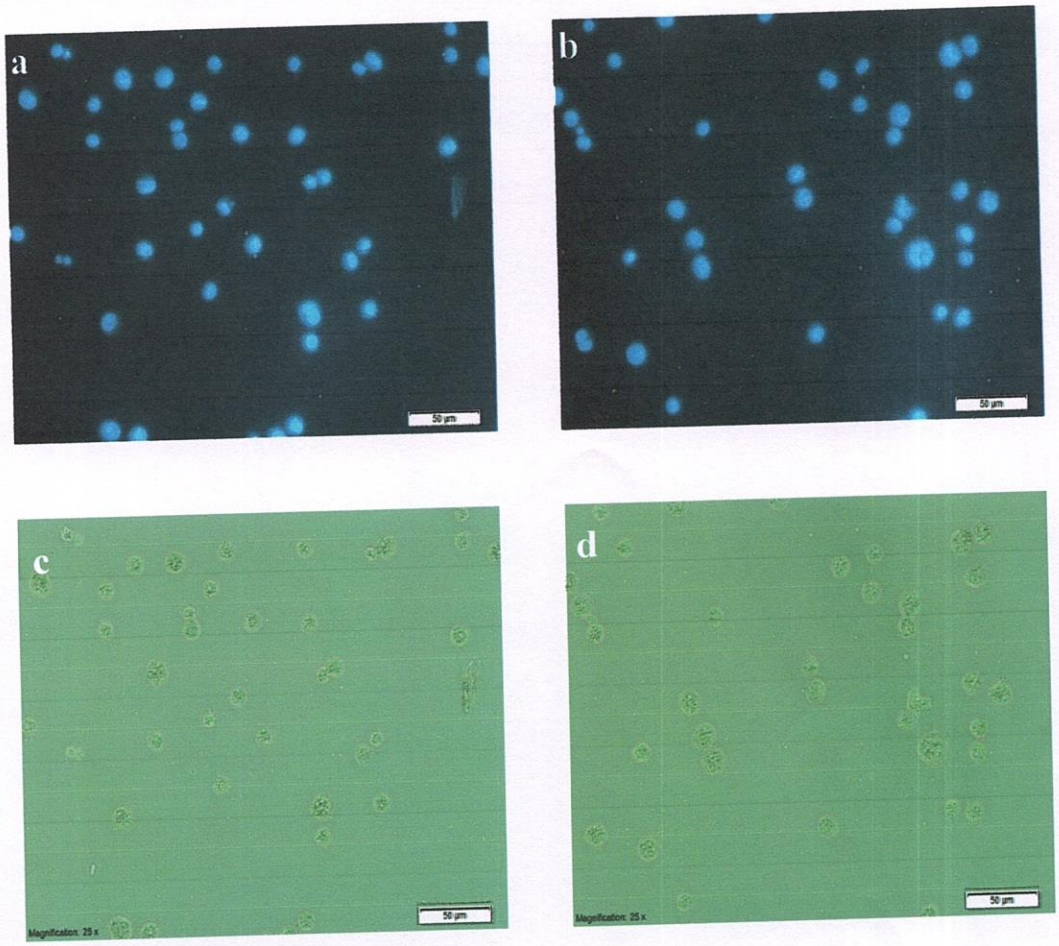


Fig. 4D. Morphological observation of PgL induced apoptosis in Hoechst 33342 stained EAC cells by optical and fluorescence microscopy (Olympus iX71). Cells were collected from untreated EAC bearing mice a (fluorescence) and c (optical) and PgL treated EAC bearing mice b (fluorescence) and d (optical).

4.7 Discussion

Multiplication of cancer cells were stopped by several kinds of plant lectins (Liu *et al*., 2010 and Kheeree *et al.,* Appl. 2010) and due to the differences in their sugar specificity, each lectin exhibits differences in their antiproliferative effect against tumor cell lines (Yan *et al*., 2010).

Apoptosis is intrinsic cell suicidal mechanisms that is regulated by numerous cellular signaling pathways and characterized morphologically by cell shrinkage, apoptotic body formation and condensation of chromatin. These morphological changes and death of apoptotic cells are occured by a series of proteases termed caspases, such as caspase-3, -6, -7, -8 and -9. In our previous study it was showed that KRL and Pea lectin (Kabir et al., 2011 and 2013) remarkable changed the EAC cell shape and nuclear morphology. In the present study such type of no change observed. The said results suggested that PgL induced cell growth inhibition is not the effect of an apoptotic cell death.

Various kinds of plant lectins have been reported to stop multiplication of cancer cells (Liu et al., 2010 and Kheeree et al., 2010) and due to their differences in their sugar specificity, each lectin shows differences in their antiproliferative effect against tumor cell lines (Yan et al., 2010). For example, lectin from *Pleurotus ostreatus* (Wang et al., 2000) can inhibit tumor growth in vivo, while wheat germ lectin acts on lung cancer (Timoshenko et al., 2001) and Concanavalin A could be used as an anti-hepatoma therapeutic agent(Lei et al., 2009). The present study was carried out to evaluate the antitumor effect of PgL on EAC bearing mice. The result showed that PgL decreased the EAC cell growth 18, 32 and 33% at dose 1.5, 3.0 and 4.5 mg/Kg/day respectively. This result was very significant when compared with EAC growth inhibition results of jacfruit lectin (Ahmed et al., 1988), *Kaempferia rotunda* lectin (KRL) (Kabir et al., 2011), *Nymphaea nouchali* tuber lectin (NNTL) (Kabir et al., 2011), Snake guard seed lectin (TCSL) (Kabir et al., 2012) and Pea lectin (Kabir et al., 2013). The information obtained from the present paper is not sufficient to use PgL as an anticancer agent, but the lectin might be a good material in cancer research due to their potent antiproliferative activity against EAC cells.

An intrinsic cell suicidal mechanism that can be regulated by numerous cellular signaling pathways and characterized morphologically by cell shrinkage, condensation of chromatin, and apoptotic body formation is known as apoptosis .The antitumor effect of PgL was also studied to assess the apoptotic nuclear morphology of the EAC cells by Hoechst 33342 staining. From the morphological observation under fluorescent microscope, there was no so much nuclear damage remarkably found in PgL treated cells except some agglutination as compared with the control group.

It was reported that some lectins induced apoptosis in various cancer cells such as KRL-induced apoptosis in EAC cells and it was confirmed by the observation of the changes in cell shape and nuclear morphology as compared to that of the control EAC cells (Kabir *et al*., 2014). Apoptotic morphological changes in EAC cells were also observed in the presence of pea lectin (Kabir *et al*., 2013).

CHAPTER FIVE

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