MATERIALS AND METHODS

3.1 Introduction to Diabetes mellitus

Diabetes mellitus is a complex metabolic disorder due to relative or absolute deficiency of insulin. It is characterized by hyperglycemia, often associated with glucosuria and a tendency to develop ketoacidosis. Neuropathy and vascular complications may evolve during the course of the disease.

3.1.1 Epidemiology of diabetes mellitus

It has become now a disease of major concern globally and is a leading cause of death in most countries. In 2013, the International Diabetes Federation (IDF) estimated that 382 million people would be diabetes worldwide, and by 2035, it was predicted to rise to 592 million. 80% of them live in underdeveloped and developing countries, and of the total, more than 60% live in Asia. According to Diabetes Atlas published by the International Diabetes Federation (IDF), there were estimated 40 million persons with diabetes in India in 2007 and this number was predicted to rise to almost 70 million people by 2025. The countries with the largest number of diabetic people would be India, China and USA by 2030.

3.1.2 Etymology and history of diabetes mellitus

Charak and Sushruta (600-400 B.C.) of ancient India recognized many of the currently known facts of the disease and described it as “Mudhumeha” (rain of honey), having noted the sweetness of the urine. In the first century A.D. the name diabetes was coined by Aretaeus and Celsus. Claude Bernard (1850) was the first to note hyperglycaemia as a feature of diabetes. A description of islet cells of the pancreas by Langerhans (1869) production of experimental diabetes though pancreatectomy by Von Mering and Minkowskey (1889) and detection of islet cell lesion in diabetes by Opie (1901) were followed by successful extraction of insulin by Banting and Best in 1921.

3.1.3 Classification of diabetes mellitus

The classification of diabetes adopted by the WHO Expert Committee on diabetes mellitus in 1980 has got general acceptance. Here three major clinical classes’
viz. insulin dependent diabetes mellitus (IDDM), non-insulin dependent diabetes mellitus (NIDDM) and malnutrition related diabetes mellitus (MRDM) are given below.

3.1.3.1 Type-1 diabetes mellitus

Individuals who develop insulin dependent diabetes mellitus (IDDM) or Type-I or juvenile onset Diabetes mellitus are thought to be born with normal islets beta cells in normal numbers. The typical lesion in the islets of Langerhans at the time of diagnosis is the lymphocytic infiltration and selective destruction of the beta cells (Gepts, 1984). At the time of acute clinical presentation, probably 85-90% of the beta cells have been destroyed. Shortly after the start of insulin therapy, improved beta cell function and probably increased beta-cell mass, is demonstrable in most patients. This remission period may last several months, but usually residual beta cell function is again in progressive decline 6-9 months after diagnosis of diabetes. The clinical onset of insulin-dependent diabetes mellitus is typically abrupt, although, Gorsuch et al. (1981) indicated that an autoimmune process may operate and lesser degrees of glucose intolerance may be present for months, or over years, before clinical onset. Complications of the eye, kidney and nervous system occur many years after onset of disease. Survival depends on the provision of daily injections of insulin. Patients with insulin dependent diabetes mellitus have a shortened life span. Recently, possibly because of improved management of the disease, increased longevity has been noted in some countries. The incidence of insulin dependent diabetes mellitus is rare. No male/female difference in incidence is evident.

3.1.3.2 Type-2 diabetes mellitus

Non-insulin-dependent diabetes mellitus (NIDDM) or Type-II or adult onset diabetes mellitus usually comes to light in the middle years of life. Individuals with non-insulin-dependent mellitus are characterized by a combination of inadequate insulin secretion and resistance of peripheral tissues to its actions (De Fronzo et al., 1983). The synthesis of an abnormal, biologically less active insulin molecule as a result of mutation of the insulin gene (mutant insulin) has been demonstrated to be the cause of non-insulin-dependent diabetes mellitus in a few patients (Shoelson et al., 1983). For unknown reasons, beta cell mass in patients with non-insulin-dependent diabetes
mellitus may be reduced at the time of diagnosis. Moreover, the insulin response to a glucose challenge is diminished in many individuals. Hyperglycemia is often present for several years before diagnosis. Some patients with NIDDM may require insulin injections to control blood sugar levels (Phillips et al., 1979). The life expectancy of NIDDM patients is less than that of non-diabetics. Obesity has long been accepted as a risk factor for NIDDM, and the risk is related to both the duration and the degree of obesity. Susceptibility to diabetes appears to be unmasked by a number of environmental factors such as sedentary life-style, dietary factors, stress, urbanization and acculturation. High and low rates of diabetes have been linked to a number of social factors including occupation, marital status, religion, economic status and level of education.

In tropical developing countries, young diabetics often present with a history of nutritional deficiency and a constellation of symptoms, signs and metabolic characteristics which fail to meet the criteria used to classify the two main clinical subclasses of diabetes IDDM and NIDDM. The distinctive clinical features and course, the uncertain etiology and pathophysiology, and the great number of such cases in some regions justify the creation of a 'new, major clinical class of diabetes, namely malnutrition-related diabetes mellitus (MRDM). This new category of diabetes includes the variety of types known in the past as tropical diabetes, pancreatic diabetes, pancreatogenic diabetes, endocrine pancreatic syndrome and ketosis-resistant diabetes of the young.

These types of diabetes have been extensively reviewed by Keen and Ekoe (1984), Bajaj and Agarwal (1984) and McMillan and Geevarghese (1979). A recent monograph has summarized the considerable body of epidemiological, clinical and biochemical information on MRDM and has suggested the existence of at least two subclasses: fibrocalculous pancreatic diabetes and protein-deficient pancreatic diabetes (Bajaj et al., 1984).

Although Steinberg (1961), Nilsson (1964) and Steinberg et al. (1970) reported that a simple autosomal recessive gene is responsible for the inheritance of this disorder, several other geneticists have opined that the mode of inheritance is polygenic or multifactorial (Lamy et al., 1961; Simpson, 1962; Mimura, 1962; Gordon, 1964;
Mimura et al., 1964; Neel et al., 1965; Thompson, 1965 and Falconer, 1967). Family studies have shown the existence of strong genetic components in IDDM as well as NIDDM (Pyke, 1979).

Diabetes is an ailment in which there is too much sugar in the system. Everybody has some sugar in the blood because sugar is needed for energy. Sugar comes from Carbohydrates, the commonest and the cheapest foods available. When we eat carbohydrate food, it is digested in the small intestine and converted to sugar. This sugar is then absorbed into the blood stream and becomes available for energy. The process of digestion is a slow one and so sugar is only gradually absorbed into a slow one and so sugar is only gradually absorbed into the blood. The liver stored the excess sugar and makes it available to the body when needed. If the sugar is not used up, then it is converted to fat. The amount of sugar present in the blood for immediate needs is carefully regulated. After a meal the sugar in the blood increases slightly but the excess is stored so quickly and efficiently in the liver that the blood is soon normal again. The efficiency of this mechanism depends on a hormone called insulin. It is synthesized in the β-cell of islets of langerhans and its function is to store away all the excess sugar as soon as it appears in the blood after a meal.

3.1.4 Pathophysiology of diabetes mellitus

3.1.4.1 Changes in the islets of langerhans cells of pancreas during diabetes mellitus

In the diabetes mellitus the beta cells of the pancreas are decreased in number or degranulated. In juvenile onset diabetes there are no beta cells and in adult onset diabetes only about one-half of the beta cells are present. In some cases the beta cells are infiltrated with lymphocytes, suggesting an auto-immune mechanism for diabetes. Obese people have hypertrophied adipose cells which because of their size are less sensitive to the action of insulin. For these reasons there is not enough insulin to cope and so excess sugar accumulates in the blood. This excess sugar clogs the whole energy system and the body makes strenuous efforts to get rid of it by excreting it through the kidneys. The extra sugar is passed out in the urine. This means that when diabetes mellitus occurs large quantities of urine have to be passed to get rid of the unwanted sugar.
3.1.5 Insulin

3.1.5.1 Discovery and biosynthesis of insulin

The discovery by Steiner et al. (1967) of proinsulin, a precursor of insulin and its subsequent structural analysis was a significant advance in diabetes research (Chance et al., 1968 and Steiner et al., 1969). It has been identified as a single chain molecule with the A and B chains linked by connecting peptide of variable length. β- Cells of pancreas are responsible for the synthesis of insulin.

![Biosynthesis of insulin](image)

**Figure 3.1 : Biosynthesis of insulin (cited from Le,2012).**

Insulin is synthesised (Fig.3.1) in the β-cells of pancreas only. Preproinsulin, which is a single chain precursor of insulin is translated from insulin mRNA. During post translational modification, this preproinsulin is inserted in the endoplasmic reticulum, where a signal peptide from preproinsulin is removed. After removal of signal peptide, this protein is called as proinsulin. Proinsulin has three domains: A
domain (carboxylic terminal), B domain (amino terminal) and C-peptide, which connects chain A and B domains. Because of the action of specific endopeptidases in the Endoplasmic reticulum, C-peptide cleaved generating mature insulin. It was thought that a possible cause of diabetes could be defective conversion of proinsulin to insulin, resulting in the production of biologically ineffective insulin.

3.5.1.2 Secretion of insulin

Though there are many biomolecules such as amino acids, nutrients, various peptides, ketone bodies are responsible for the secretion of insulin from β-cells of pancreas but glucose is the main regulator for its synthesis. Glucokinase is the enzyme responsible for the conversion of glucose to glucose-6-phosphate and the reaction is called as glucose phosphorylation, this is the rate limiting step for the glucose dependent insulin secretion (Fig. 3.2).

![Figure 3.2: Secretion of insulin (cited from Zhanxiang and Debbie, 2009)](image)

**Pathway of Insulin secretion (Fig.3.2):** Glucose enters the cells via the GLUT2 transporter (1) and undergoes glycolytic and mitochondrial metabolism (2), which ultimately has the effect of increasing the ATP: ADP ratio (3). An increased ATP: ADP ratio leads to the closure of ATP-sensitive KATP channels (4) and to membrane depolarization (5), which triggers the opening of voltage-dependent Ca$^{2+}$ channels (VDCCs) (6). The resulting influx of Ca$^{2+}$ (7) induces the fusion of insulin-containing
granules with the plasma membrane and insulin release from the cell (8). PM, plasma membrane.

3.1.5.3 Receptor of insulin

![Figure 3.3 Receptor of insulin (cited from Chhabra, 2012)](image)

Receptor of insulin (Fig. 3.3) is composed of two sub-units extracellular α-subunit (hormone binding domain) and intracellular β-subunit (ATP-binding and tyrosine kinase domain). Both these domains are connected by disulphide bonds.

3.1.5.4 Mechanism of action of insulin

After binding of insulin to its receptor, it stimulates intrinsic tyrosine kinase which prompting receptor autophosphorylation and recruits intracellular signaling molecules like insulin receptor substrate (IRS) and other adapter proteins which initiate the complex cascade of phosphorylation and dephosphorylation reaction.
Materials and Methods

Figure 3.4 Mechanism of action of insulin (cited from Chhabra, 2012)

In case of insulin transport system activation (Fig. 3.4) of phosphorylation-3 kinase (PI-3 kinase) pathway, this activates translocation of glucose transporters GLU-4 to the cell surface, important event for glucose uptake by muscles and fats. Other insulin signaling pathway induces effects on lipid metabolism, effects on growth and effects on protein metabolism.

3.1.5.5 Role of insulin diabetes mellitus

The pattern of insulin secretion in diabetes is subject of much controversy in diabetes research. Juvenile onset diabetes is absolutely deficient of insulin. In most maturity onset diabetes, the insulin response to glucose is found to be delayed, but many have appreciable levels of circulating serum insulin. Obesity is associated with a relatively high insulin response to glucose (Perley and Kipnis, 1967). In general, there is an inverse correlation between insulin response to glucose and the severity of carbohydrates intolerance (Mc Kiddie and Buchanan, 1969). Reaven et al. (1974) produced evidence that peripheral resistance to insulin rather than failure of pancreatic
insulin secretion is the primary fault in these diabetic subjects. Fujita et al. (1974) suggested that the majority of maturity onset diabetics show a delayed and impaired insulin response to glucose, and that this is the initiating factor in the biochemical disturbance.

Insulin operates through the action of cell receptors. The cause of the insulin resistance and hyperinsulinism in the obesity appears to be due at least in part to a reduction in the number of insulin receptors. In the experiments of Olefsky (1976) reduced numbers of insulin receptors were found in adipocytes, liver cells and muscle cells in hyperinsulinaemic experimentally obese mice. Reduced numbers of insulin receptors have been found in obese men also. It appears that insulin affects directly the concentration of its own receptors. A decrease in the receptor number is associated with hyperinsulinism, while an increase in receptor number is associated with insulin deficiency (Kahn, 1979). If weight reduction is achieved, blood glucose falls and insulin receptors are increased. In the adipocytes, there is also a defect in intracellular glucose metabolism. It may be more important than the reduction in receptor number in the causation of insulin resistance (Olefsky, 1976). It is not yet clears whether the reduction in receptor number precedes the hyperinsulinaemia or vice versa.

In the untreated state, diabetes mellitus is recognized by chronic elevation of the concentration of glucose in the blood (hyperglycemia). This is sometimes accompanied by symptoms of severe thirst, profuse urination, weight loss, and stupor, culminating in coma and death in the absence of effective treatment. More often, presenting symptoms are much less severe without disturbance of consciousness, occasionally symptoms are totally absent. The high concentration of blood glucose and other biochemical abnormalities result from deficient production or action of insulin. The severity of its symptoms is largely determined by the degree to which the insulin action is deficient.

**3.1.5.6 Diagnosis of diabetes mellitus**

The diagnosis of diabetes mellitus is based on the documentation of elevated fasting blood sugar, elevated blood glucose two hours postprandial or an abnormal glucose tolerance test. The accuracy of a glucose tolerance test is influenced by diet, physical activity, age, associated diseases and drugs. The diagnosis of diabetes becomes difficult in certain circumstances.
3.1.5.7 Complications of diabetes mellitus

The major threat to the health and life of Diabetics represent progressive damage to the eyes, kidneys, nerves and arteries. The leading cause of visual disability arising in middle-aged and elderly people in economically advanced societies is diabetes-associated damage to the retina of the eye (Kohner et al., 1982). Progressive impairment of renal function, accompanied by urinary protein loss and culminating in end-stage renal failure threatens the health and life of up to half those patients who develop IDDM in youth or adolescence (Andersen et al., 1983). Damage to nerve fibers conducting sensation and supplying muscles, blood vessels and viscera is the most common complication of diabetes. There are several distinct patterns of neurological diseases giving rise to a number of clinical syndromes and disabilities (Ward, 1985). Coronary heart disease occurs more frequently and has notably more serious consequences in diabetics than in nondiabetics. A considerable amount of disability in diabetics is caused by the peculiar susceptibility of the foot to severe tissue damage (Edmonds et al., 1982). In recent years, new possibilities have appeared for limiting the progression of these complications by improved control of the diabetic state no reducing the risk of blindness, end-stage renal failure, neuropathic tissue damage, ischaemic tissue damage, amputation, heart attack and stroke.

Diabetes is a disease for life. Laboratory tests are required to assess the metabolic state prevailing from time to time. In severe cases, the life of the recently diagnosed diabetic depends on regular injections of insulin, a regular pattern of meals and a suitably adjusted life-style. At the other extreme, a weight-reducing diet may suffice to correct the metabolic disturbance completely. There are four main objectives of the managements of diabetes: to preserve the life of the diabetic patient and relieve the symptoms of the disease, to enable the patients to have as normal a social life as possible, to establish and maintain good metabolic control and to avoid the complications of diabetes mellitus.

3.2 Introducing Amritsar and its People

The district of Amritsar lies in the north-western region of India. It is situated between 31.37 North latitude and 74.55 East longitudes, at an average altitude of 219
meters above the mean sea level. The district has an area of 114 sq. Km. Average temperature lies between 3.0 to 47.7 °C, encounters four season winter December to March (3-15°C), summer April to June (20-42°C), monsoon season July to September and post monsoon season September to November. According to 2011 census total population of the district was 2,490,891. The sex ratio was 884 females for every 1000 males, and child sex ratio 825 girls for every 1000 boys. Total literacy was 84.19%; male literacy was 86.90% and female literacy 81.16%. Hinduism and Sikhism were majority religion in Amritsar with 49.36% and 48.00% people following them. In Amritsar district, however Muslims 0.51% and Christians 0.23% and many more religions all including in other religions were also present.

3.3 Material

3.3.1 Patients and controls

A total of 263 blood samples from patients with diabetes mellitus (type-2) were obtained from Guru Nanak Dev Hospital and Puneet Diabetic Centre, Amritsar, Punjab, India. The samples represented adequately the Sikh, Hindu, Muslim and others, only patients with type-2 diabetes mellitus were studied. For comparison, 251 unrelated normal healthy individuals were sampled randomly from this district place. Since sex differences are known not to exist in the LH system (Sehajpal and Shrivastava, 1980 and 1981), the samples collected from both males and females were pooled for the various analyses. Table 3.1 showed the number of diabetic patients and controls tested for both the LH and the ABO systems in the present study.

Table 3.1: Number of diabetic patients and controls tested for the LH and the ABO systems in each studied group

<table>
<thead>
<tr>
<th>S.no</th>
<th>Populations</th>
<th>Sample size</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Patients</td>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Sikh</td>
<td>113</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Hindu</td>
<td>135</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Muslim</td>
<td>01</td>
<td>01</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Others</td>
<td>14</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>263</td>
<td>251</td>
<td></td>
</tr>
</tbody>
</table>
3.3.2 Chemicals and reagents

The chemicals and reagents used for the isolation and characterization of the anti-LH lectin from *Erythrina lithosperma* were analytical grade.

The chemical used for the present study were- sodium chloride and sodium azide, copper sulfate, sodium potassium tartarate, potassium iodide, sodium hydroxide, coomassie brilliant blue R-250, sodium carbonate, Folin-Ciocalteau reagent, D-glucose, phenol and sulphuric acid, Acrylamide, bisacrylamide, Tris-HCl, sodium dodecyl sulphate (SDS), N, N', N", N"'- tetramethylethylenediamine (TEMED), glycine, β-mercaptoethanol, bromophenol blue, acetic acid, ammonium persulphate, ethylenediaminetetra-acetic acid (EDTA), sodium acetate, sodium phosphate, Tris base, D-glucose, D-ribose, maltose, N-acetyl glucosamine (GlcNAc), salicin, sucrose, raffinose, lactose, D- galactose and D-melibiose, sodium hydroxide and HCl, Calcium chloride and magnesium chloride, bovine serum albumin purchased from Molychem and ABD monoclonal antibodies (J. Mitra & Co Pvt. Ltd).

The reagent used for the present study were- ethanol, o-phosphoric acid, sulfuric acid, hydrochloric acid, ethyl acetate, acetone, alcohol, isoamyl alcohol, chloroform, anthrone AR ,glycerol, benzene AR and phenol crystal were purchased from Finar Pvt. Ltd.

3.3.3 Instrumentation

The instruments used were-centrifuge (Khera Research Centrifuge KI 199), spectrophotometer (Khera UV Spectro), automatic blood analyzer cobas c 111, shaker, hot air oven and other instruments were used for isolation and characterization of lectin.

3.3.4 Human blood sample for hemagglutination

To characterize the anti-LH lectin, hemagglutination assay was performed by using human erythrocyte suspension (A+, B+, AB+ and O+).

3.4 LH specificity in Type-2 Diabetes Mellitus

3.4.1 Collection of samples

Seeds from *Erythrina lithosperma* were collected from Botanical Survey of India, Kolkata and Victoria Memorial Hall, Kolkata. The taxonomic position of *Erythrina lithosperma* plant is given below:
Materials and Methods

Class: Dicotyledons
Sub-class: Polypetalae
Series: Calyciflorae
Order: Rosales
Family: Leguminosae
Sub-family: Papilionatae
Tribe: Phaseoleae
Genus: Erythrina
Species: lithosperma

3.4.2 Isolation of the anti-LH lectin from *Erythrina lithosperma*

The anti-LH lectin from seed extracts of *Erythrina lithosperma* was prepared in normal saline. In the preparation of the anti-LH lectin, all essential steps were the same as described by Dunsfold and Bowley (1967) for other lectins. The seeds were ground to a fine powder and mixed with normal saline in the ratio of 1:9. The mixture was then allowed to stand at ambient temperature for four hours, with occasional stirring. After this period the slurry was centrifuged at 3000-4000 rpm, for 30 minutes. The clear supernatant was subsequently separated and stored under refrigeration with sodium azide added to it, in the ratio of 1:10000 parts, as preservative. Other lectins were also prepared in the same manner.

3.4.3 Collection and processing of human blood

Diabetic and normal blood samples were collected intravenously in E.D.T.A. (Ethylene Diamine Tetra Acetic acid disodium salt) and adequately in physiological saline, using the finger-prick technique. All blood samples were washed thrice in physiological saline and resuspended at a concentration of 2% in normal saline. For ABO typing, standard serological procedure (Race and Sanger, 1970) were followed.

3.4.4 Collection and preservation of serum samples

All human serum samples were collected from Guru Nanak Dev Hospital, Puneet Diabetic Centre and Health Centre, Guru Nanak Dev University. Whole blood
was collected intravenously and allowed to clot overnight at 4°C. Serum was obtained by breaking the clot, centrifuging the whole at 2500-3000 rpm, for 15 minutes. Add 0.01% sodium azide (2%) solution was added to the serum samples before storing them under refrigeration.

### 3.4.5 ABO typing

The finger was sterilized and pricked with sterilized lancet. The blood was collected in 0.85% NaCl (physiological saline) in test tube. RBCs pellet was washed thrice with physiological saline by centrifuging it at 2500-3000 rpm. 2% RBCs suspension prepared in physiological saline. ABO typing was done by normal serological method using 2% RBCs suspension and with anti-A, anti-B and anti-D.

### 3.4.6 Hemagglutination technique

The frozen seed extracts were thawed at room temperature just before the beginning of the experiments. Blood grouping slides with 12 cavities were used for the hemagglutination tests. 25μl red blood was added to an equal amount of seed extract. After 20-25 minutes results were recorded (Bowley et al., 1971) as noted below

- **C** – Complete agglutination, ++++ 12.
- **V** – Visual agglutination, several agglutinates being clearly visible without using microscope, +++ 10, ++ 9.
- **++** – Very large agglutinates visible under the microscope, ++ 8, + 7.
- **+** – Large agglutinates visible under the microscope, +5.
- **(+)** – Small agglutinates visible under the microscope, ±3.
- **W** – Small, but definite agglutinates, -2.
- **O** – No agglutination

### 3.4.7 Technique of the LH typing

For the LH typing, a single drop of anti-LH lectin *Erythrina lithosperma* from a pasteur pipet was added to four drops of 2% suspension of red blood cells in normal saline in a serological test tube measuring 15mm x 75mm. The mixture was then centrifuged for 20 seconds at 2500 rpm. A cell button was formed in all cases but in
some brisk manual shaking of the tube resulted in dispersal of the button into a large number of minor agglutinates where as in others, similar shaking left the cell button intact. The former type of reaction was called LH-negative and the latter type as LH-positive. The result of the tube test checked by the method of titration and full agreement was found. With LH-positive cells the titre ranged between 32-64 and with LH-negative cells between 8-16.

3.5 Properties of the Anti-LH Lectin

3.5.1 Estimation of total proteins in the crude extracts of the anti-LH lectin

3.5.1.1 Estimation of total proteins via Biuret method

The total protein concentration from the crude extracts of the anti-LH lectin was determined by Biuret method (Datta et al., 1959). Stock solution of standard bovine serum albumin (BSA) of concentration 2mg/ml, 0.2 N NaOH and Biuret reagent (3g of CuSO$_4$.5H$_2$O, 9g sodium potassium tartarate in 0.2 N NaOH to that add 5g of potassium iodide and made the final volume 1000 ml with 0.2 N NaOH) were prepared. Different concentration of the standard BSA (0-2 ml) were prepared and made the final volume 2 ml with distilled water and took 2ml of unknown sample (the anti-LH lectin). Add 3ml of Biuret reagent in all the samples and unknown sample, incubated these samples at 37°C for 10 minutes and cooled. The absorbance at 540nm was measured using UV-Visible spectrophotometer. For the determination of total protein concentration in the crude extracts of the anti-LH lectin a graph between concentration and absorbance was plotted.

3.5.1.2 Estimation of total proteins via Bradford method

The total protein concentration from the crude extracts of the anti-LH lectin was determined by Bradford method (Bradford, 1976). Stock solution of standard bovine serum albumin (BSA) of concentration 50μg/ml and Bradford reagent (100μg/ml of comassie Brilliant blue dye in 50 ml of 95% ethanol to that 100 ml of O-phosphoric acid was added and made the final volume 1000 ml with distilled water ) were prepared. Different concentration of the standard BSA (0-1 ml) were prepared and made the final volume 1 ml with distilled water and took 1ml of unknown sample (the anti-LH lectin), Add 5ml of Bradford reagent in all the different concentrated samples and unknown
sample, incubated these samples at 37°C for 10 minutes and cooled. The absorbance at 595nm was measured using UV-Visible spectrophotometer. For the determination of total protein concentration in the crude extracts of the anti-LH lectin a graph between concentration and absorbance was plotted.

### 3.5.1.3 Estimation of total proteins via Lowry method

The total protein concentration from the crude extracts of the anti-LH lectin was determined by Lowry method (Lowry et al., 1951). Stock solution of standard bovine serum albumin (BSA) of concentration 200 μg/ml and Lowry reagent (solution A-50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH (0.4 gm in 100ml of distilled water), solution B- 10 ml of 1.56% copper sulphate solution mixed with 10 ml 2.37% sodium potassium tartarate solution, solution C-mixed 2ml of solution B and 100 ml of Solution A and diluted folin-ciocalteau reagent (fc) (2:1) with distilled water ) were prepared. Different concentration of the standard BSA (0-1 ml) were prepared and made the final volume 1 ml with distilled water and took 1ml of unknown sample (the anti-LH lectin), Add 5ml of solution-C in all the different concentrated samples and unknown sample, incubated these samples at 37°C for 10 minutes and cooled. After 0.5 ml of fc-reagent was added, again incubated at 37°C for 30 minutes. The absorbance at 750nm was measured using UV-Visible spectrophotometer. For the determination of total protein concentration in the crude extracts of the anti-LH lectin a graph between concentration and absorbance was plotted.

### 3.5.1.4 Estimation of total proteins via UV method

The total protein concentration from the crude extracts of the anti-LH lectin was determined by UV-method. Stock solution of standard bovine serum albumin (BSA) of concentration 100 μg/ml was prepared. Different concentration of the standard BSA (0-2 ml) were prepared and made the final volume 2 ml with distilled water and took 2ml of unknown sample (the anti-LH lectin), incubated these samples at 37°C for 10 minutes Lectins are proteins and because of aromatic amino acid, these proteins are maximally absorbed at 280 nm. The absorbance at 280nm was measured using UV-Visible spectrophotometer. For the determination of total protein concentration in the crude extracts of the anti-LH lectin a graph between concentration and absorbance was plotted.
3.5.2 Estimation of total carbohydrates in the crude extracts of the anti-LH lectin

The total carbohydrate concentration from the crude extracts of the anti-LH lectin was determined by phenol sulphuric method (Dubois et al., 1956). Stock solution of D-glucose of concentration 1mg/ml and phenol-sulphuric reagent (phenol 5% and sulphuric acid 96%) were prepared. Different concentration of the standard D-glucose (0-1 ml) were prepared and made the final volume 1 ml with distilled water and took 1ml of unknown sample (the anti-LH lectin), to that 1ml of phenol (5%) and 5ml of sulphuric acid (96%) were added, incubated for 10 minutes in boiling water bath at 30-40°C for 20 minutes and cooled. The absorbance at 490 nm was measured using UV-Visible spectrophotometer. For the determination of total carbohydrate concentration in the crude extracts of the anti-LH lectin a graph between concentration and absorbance was plotted.

3.5.3 Molecular characterization of proteins

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

**Acrylamide (30% stock) 100ml**

Acrylamide = 29.2 g  
Bis-acrylamide = 0.8g  
Dissolve in the distilled water and make the final volume 100ml. Store under dark in amber color bottle at 4°C (can use Upto 3 month)

**Resolving gel/ separating gel buffer pH 8.8, 1.5M Tris-HCl (100ml)**

Tris-HCl = 18.17g  
Dissolve in 70 ml distilled water, adjust the pH 8.8 with 6N HCl and make the final volume 100ml with distilled water and store at 4°C.

**Stacking gel buffer, pH-6.8, and 1.0M Tris-HCl (50ml)**

Tris-HCl = 3g  
Dissolve in 40 ml distilled water, adjust to pH 6.8 with 6N HCl and make the total volume 50ml with distilled water and store at 4°C.
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10% SDS (100ml)

SDS = 10g

Dissolve in 100 ml of distilled water.

Ammonium per sulphate-initiator (APS) 10% (1ml)

Dissolve 0.1 g APS in 1 ml distilled water.

TEMED (N N’ N’’ N’’’ Tetramethylenediamine):- catalyst.

Electrophoresis buffer pH 8.3 (100ml)

Tris-HCl = 3g and 1g SDS in 100ml of distilled water. Store at 4°C.

Glycine = 14.4 g

SDS = 1g

Dissolve in 100 ml of distilled water. Store at 4°C.

Sample buffer

Distilled water = 7.25 ml

Stacking gel buffer = 1.25 ml

Glycerol = 1 ml

β- mercaptoethanol = 0.5 ml

SDS = 150 mg and a pinch of bromophenol blue.

Staining solution:

Coomassie brilliant blue R 250 = 200mg

Methanol/ethanol = 50ml

Acetic acid = 7ml

Adjust the volume with distilled water and make it 100ml and filter it.

Destaining solution: Add 7ml acetic acid to 30ml methanol/ethanol and 63ml distilled water

Acetic acid = 7ml

Methanol/ethanol = 30ml

Adjust the volume with distilled water and make it 100ml.
Table 3.2: Composition of separating and stacking gels:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Separating Gel 10% (25ml)</th>
<th>Stacking Gel 5% (5ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (DW)</td>
<td>9.9</td>
<td>3.04</td>
</tr>
<tr>
<td>1.5 M Tris HCl</td>
<td>6.3</td>
<td>-----</td>
</tr>
<tr>
<td>0.5 M Tris HCl</td>
<td>-----</td>
<td>.63</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.25</td>
<td>0.05</td>
</tr>
<tr>
<td>Acrylamide-Bisacrylamide</td>
<td>8.3</td>
<td>0.83</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
<td>0.005</td>
</tr>
<tr>
<td>APS 10%</td>
<td>0.25</td>
<td>0.05</td>
</tr>
</tbody>
</table>

3.5.3.1 Casting of separating gel

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

3.5.3.2 Casting of stacking gel

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

3.5.3.3 Preparation and loading of samples

50 μl of aqueous solution of lectin (1 mg/10ml) was mixed with 100 μl sample buffer and mixed well.

3.5.3.4 Loading and running the gel

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

The lower tank of the electrophoretic unit was filled with the running buffer. The gel was clamped to the electrophoretic chamber. Then the upper tank was filled with the same buffer. Each sample was loaded to separate wells. After loading the samples to appropriate wells, the unit was connected to the power supply (1 50 V for 1.5 - 2 h) and the gel was run.

3.5.3.5 Staining and destaining the gels

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

3.5.4 Biochemical Characterization

3.5.4.1 Sugar inhibition test

To test the sugar specificity of lectins hemagglutination inhibition assay was performed by the method of Anderson and Mclure (1973). Various sugars, their derivatives and related compounds that are given below, were tested to determine the agglutination inhibition. The amount of each lectin was kept constant at twice the lowest concentration, which causes hemagglutination of the test cells as determined through serial double dilution technique. To 50 μl of 100 mM sugar solution, an equal volume of the extract was added and incubated at 37 "C for 1 h. To this mixture, 100 μl of 2% RBCs suspension was added and again incubated for 30 min. The restoration of red button formation in the presence of a sugar indicates inhibition of hemagglutination by that specific sugar. The list of sugars and derivatives tested in agglutination inhibition assay were D-glucose, D-ribose, maltose, N-acetyl glucosamine (GlcNAc), salicin, sucrose, raffinose, lactose, D- galactose and D-melibiose.

3.5.5 Physical Stability

3.5.5.1 Effect of change in pH on hemagglutination reactivity of anti-LH lectin or pH stability

The pH stability of the anti-LH lectin was studied by the method of Yamada and Akela (1982). Hemagglutination titre of the anti-LH lectin was carried out at different pH from 1, with an increment of one up to 9 using HCl and NaOH. 0.5 m1 of anti-LH
lectin (1mg/10ml), was mixed with an equal volume of the anti-LH lectin at different pH. After incubation for 1 h, 50 μl of the sample was taken and hemagglutination titre was determined against A, B, AB and O blood group cells, as described earlier in section 3.4.6.

3.5.5.2 Effect of metal ions on the activity of the anti-LH lectin

Effect of metal ion on activity of the anti-LH lectin was determined by Kawagishi et al. (1990) method. For this crude extracts of the anti-LH lectin was treated with different metal ions. The anti-LH lectin sample was mixed with 1 mM of CaCl₂, MgCl₂ and CaCl₂+MgCl₂ solution and incubated for 2 hrs, after that 0.2 ml of human erythrocyes suspension was added and incubated for 30 minutes. Hemagglutination titre was determined against the A, B, AB and O erythrocyte suspension, as described earlier in section 3.4.6.

3.5.5.3 Effect of temperature or thermal stability

The thermal stability of the crude lectin was done by the method of Chowdhary et al. (1987). 0.5 ml of the anti-LH lectin (1mg/10ml), in NaCl (pH 7.4) was incubated separately for 30 minutes in a range of temperatures between 30-100°C with an increment of 10°C. Aliquots of 100 μl were withdrawn, cooled and hemagglutination titre was determined against the A, B, AB and O blood group cells, as described earlier in section 3.4.6.

3.6 Association of HbA1c with Lipid Profiles in Patients with Type-2 Diabetes Mellitus

3.6.1 Study participants

A total of 52 confirmed cases of type-2 diabetes mellitus were obtained from Dr. Puneet Arora Diabetic and Research Centre, Green Avenue, Amritsar, Punjab. The samples comprised of both males and females. All diabetic patients were aged 40 years and above. The informed consent form was voluntarily taken from each diabetic patient who participated in the study.

3.6.2 Measurement of biochemical parameters

After an overnight fasting, approximately 5 ml venous blood was collected .The serum was separated immediately after collecting the blood sample via centrifugation at
3000 rpm for 10 minutes. Fasting blood glucose concentrations were measured within 30 minutes to 1 hour of sample collection. Lipid profile (total cholesterol, triglycerides, high density lipoprotein) was measured using automatic blood analyzer cobas c 111 (according to the manufacturer’s instructions). Low density lipoprotein and very low density lipoproteins were measured using the Friedewald’s formula (given below)

Low density lipoprotein = Total cholesterol – (High density lipoprotein – Triglycerides/5) VLDL= Triglycerides/5 the Glycated hemoglobin (HbAlc) were measured with a radioimmunoassay (HbAlc LD-500 Analyzer)

3.7 Statistical Analysis

We have made use of the following statistical tests to determine the various gene frequencies and to test the significance of the distribution patterns of the LH-specificity in populations we have studied.

3.7.1 ABO gene frequencies

We followed the method used by Bernstein (1930) for the calculation of p,q and r gene frequencies, the various formulae being

\[
p = 1 - \sqrt{O^- B^-}
\]
\[
q = 1 - \sqrt{O^- A^-}
\]
\[
r = \sqrt{O^-}
\]

Where O, A and B represent the present frequencies of O, A and B individuals. Theoretically the sum of p, q and r should be equal to unity, but in practice it is rarely so and therefore for a precise estimation of these gene frequencies, Bernstein’s (1930) correction factor (D) is applied.

\[
D = 1 - (p + q + r).
\]

And corrected estimates are given by:

\[
p' = (1+\frac{1}{2}D) (1 - \sqrt{O^- B^-})
\]
\[ q' = (1 + \frac{1}{2}D) (1 - \sqrt{O - A -') \]

\[ r' = (1 + \frac{1}{2}D) (\sqrt{O + \frac{1}{2}D}) \]

For the ABO system the formulae used for calculating expected frequencies were:

**Phenotype Expected frequencies**

\[ O = N (r')^2 \]

\[ A = N (p'^2 + 2p' r') \]

\[ B = N (q'^2 + 2q' r') \]

\[ AB = 2xN p' q' \]

Where \( N \) is the total number of individuals and \( p' \), \( q' \) and \( r' \) the gene frequencies of A, B and O phenotypes respectively.

### 3.7.2 LH-specificity:

The LH gene frequencies will be calculated as follows:

The LH gene frequencies were calculated thus,

\[ lh = \sqrt{lh^-} \]

\[ LH = 1 - lh \]

Where \( lh \) and \( LH \) represent the gene frequency of \( LH^+ \) and \( LH^- \) individuals were obtained as given below:

**Phenotype Expected frequencies**

\[ LH^+ = N (lh)^2 \]

\[ LH^- = N (LH)^2 + N X 2 (LH X lh) \]

Where \( N \) represents the total sample and the LH and lh, the frequencies of \( LH^- \) and \( LH^+ \) alleles respectively. The expected and observed frequencies will be compared by using the

### 3.7.3 Chi squared test.

\[ X^2 df = (O-E)^2 / E \]
Where $O =$ Observed frequency, $E =$ Expected frequency, $df =$ degree of freedom. The
degree of freedom was obtained by using the following formula

$$df = (\text{No. of columns} - 1) (\text{No. of rows} - 1).$$