4. MATERIAL AND METHOD

4.1 BLOOD SAMPLE COLLECTION

Blood samples were collected from voluntary donors with a history of type 1 and type 2 diabetes mellitus under treatment in Jolly Grant Hospital, Dehradun (U.K.). Blood samples from hyperlipidemic patients were collected from India in the year 2010 from laboratory of Pathology department, Jolly grant Hospital, Dehradun. Control serum samples were obtained from six healthy individuals of the same age group that of patients under following standard operating procedure (SOP)

Sera were collected from all the samples and were stored in aliquots at -4°C with sodium amide as preservation. The details of the study subjected are summarized in Table-13.

### TABLE 13: Biochemical details of study subjects

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (year)</th>
<th>Sex (M/F)</th>
<th>Blood glucose/Total Cholesterol (mg/dl)</th>
<th>HbA1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 diabetes</td>
<td>35</td>
<td>M</td>
<td>326</td>
<td>7.1</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>59</td>
<td>M</td>
<td>435</td>
<td>9.0</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>44</td>
<td>F</td>
<td>240</td>
<td>-</td>
</tr>
<tr>
<td>Normal human subjects</td>
<td>34 ± 21.3</td>
<td>4M/2F</td>
<td>105</td>
<td>4.5</td>
</tr>
</tbody>
</table>

HbA1c : Glycosylated hemoglobin
4.1.1 Materials used

Materials used for the blood samples collection is listed below:

a. Blood collection kit

- 23G butterfly needle
- Vacutainer needle holder
- Latex gloves
- Tourniquet
- Alcohol wipes
- Cotton wool
- Small plasters

b. Sample tubes

- 10ml Fluoride and plain test tube

4.1.2 Venipuncture

‘Venipuncture’ is the process of collecting blood sample from the patient vein. Blood collection was carried out by under mentioned protocol.

4.1.3 Procedure for vein selection

The median cubital and cephalic veins of the arm were most frequently selected, as shown in figure 24.
**FIGURE 24**: Median cubital and cephalic veins of the arm

### 4.1.4 Procedure of blood sample collection

Blood samples were collected under standard protocol mentioned in annexure 1.

**FIGURE 25**: Blood sample collection from cephalic veins of the arm
4.1.5 Separation of plasma from whole blood sample

Clotting begins when a blood sample is collected from a patient into serum separator tube (SST). The specimens were allowed to stand at ambient temperature until a clot has formed. (Approx. 30 minutes) Once a clot was formed, the specimens were centrifuged as mentioned in annexure 2.

**FIGURE 26**: Blood sample in serum separator tube (SST).

4.1.6 Estimation of blood glucose level

Blood glucose level of all diabetic sample and non diabetic sample was measured by GOD-POD end point kinetic method (Enzopak kit). The detailed procedure mentioned in annexure 3.

**FIGURE 27**: Centrifuge Machine  **FIGURE 28**: Cups inside Centrifuge Machine
4.1.7 Estimation of serum cholesterol level

Serum cholesterol level of all the diabetic sample and non diabetic sample was measured by Cell Biolabs’ Total Cholesterol Assay Kit. The detailed procedure mentioned in annexure 4.

**FIGURE 29:** Blood sample after Centrifugation

**FIGURE 30:** Serum in test tube
4.2 ISOLATION AND PURIFICATION OF IgG BY PROTEIN-A AGAROSE AFFINITY COLUMN

Serum IgG was isolated by affinity chromatography on Protein-A agarose CL-4B Affinity column, as it is simple, reliable method for purifying total IgG from crude protein mixtures such as serum/plasma.

Serum (0.3 ml) diluted with equal volume of PBS, pH 7.4 was applied to column equilibrated with the same buffer. The washing was done 2-3 times. Unbound IgG was removed by extensive washing with PBS, pH 7.4. The bound IgG was eluted with 0.58 % acetic acid in 0.85% sodium chloride (Goding, 1978) and neutralized with 1 ml of 1 M Tris-HCl, pH 2.3. 3 ml fractions were collected and read at 251 and 278 nm. The IgG concentration was determined considering 1.40 OD280 = 1.0 mg IgG/ml. The isolated IgG was then dialyzed against PBS, pH 7.4 and stored at –20°C with 0.1% sodium azide.

4.2.1 Preparation of buffer solution

Buffers necessary for this procedure was prepared prior to starting the procedure by following protocol.

4.2.1.1 Wash/Binding buffer

The wash/binding buffer was diluted 1:5 in distilled water (dH2O) in a clean container (i.e. 20 mL buffer concentrate + 80 mL dH2O). Enough diluted buffer was prepared based on the sample quantity to be dialyzed and ~30X the quantity of agarose used for purification. Prepared buffer can be stored up to 1 week at 2 - 8°C. Final 1X concentration of buffer was 0.1M Sodium Phosphate, 0.15M NaCl, pH 7.4.

4.2.1.2 Elution buffer

The elution buffer was diluted 1/10 in distilled water in a clean container (e.g. 10 mL buffer concentrate + 90 mL dH2O). Prepared buffer was stored at 2 - 8°C. Final 1X buffer concentration was 0.2M Glycine, pH 3 ± 1.85.
4.2.1.3 **Storage buffer**: Ready to use.

4.2.2 **Sample preparation**

To maintain proper ionic strength and pH for optimal binding, serum samples was diluted 1/1 with binding buffer.

4.2.3 **Column and resin preparation**

- 20% Ethanol was poured in the bottom of a petri dish. Frit was making floated in ethanol. By using the large round end of a 1 mL pipette tip, pressure was applied on frit firmly into the ethanol to force air out. The process was repeated until the frit was completely wet.
- The frit was pushed into the barrel of the column until it rests firmly on the bottom.
- The end of the column was clipped with the cap removed, to create a hole to allow liquid to flow through.
- The frit was washed with 5 column volumes of 1X Wash/Binding Buffer.
- A 1/1 suspension of resin was prepared in 1X Wash/ Binding buffer. The required amount of agarose per mg immunoglobulin to be purified was estimated by the binding capacity.
- Slurry was poured into column and column was allowed to flow by gravity to pack the column bed.
- The packed affinity resin was equilibrated with 10 column volumes (CV) of the wash/binding buffer. For example, if the packed bed is 1 mL, equilibrate with 10 mL Wash/Binding Buffer.

4.2.4 **Sample purification**

- Sample was applied gently to the column by layering onto the top of the resin.
- Column was washed with 10 CV of the 1X Wash/Binding buffer, or until the absorbance of eluate at 280 nm approaches the background level.
• Before beginning the elution step, enough tubes set up was prepared to collect
the entire elution volume as 1 mL fractions (4 CV will be used to elute the
antibody). To each collection tube 240 mL 5X Wash/ Binding Buffer was
added. To elute the antibody, gently 1 mL of 1X Elution Buffer was added to
the top of the resin, the eluate was collected in a prepared collection tube.
Repeated until the entire volume was collected, up to 4 column volumes.

4.2.5 Column regeneration

Once the sample was eluted, the affinity matrix was washed with 2 CV of
eletion buffer. The column was re-equilibrated with 10 CV of 1X Wash/Binding
Buffer.

4.3 PROTEIN ESTIMATION

Protein concentration was determined by the method of Lowry et al., (1951) or
Dye binding method (Bradford, 1976). The IgG concentration was determined
considering 1.38 OD_{278} =1.0 mg IgG / ml (Khan and Siddiqui, 2006) The detailed
procedure is mentained in annexure 5.

4.3.1 Protein estimation by dye-binding method

This assay is based on color change when coomassie Brilliant Blue G 250 in
acidic medium binds strongly to protein hydrophobically and at positively charged
groups (Bradford, 1976). In the environment of these positively charged groups,
protonation is suppressed and a blue color is observed ($\lambda_{\text{max}} = 595$ nm).

4.4 PREPARATION OF PLANT EXTRACTS

Extraction, as the term is used pharmaceutically, involves the separation of
medicinally active portions of plant from the inactive or inert components by using
selective solvents in standard extraction procedures. The products so obtained from
plants are relatively impure liquids, semisolids or powders intended only for oral or
external use.
4.4.1 Maceration process for preparation of salep (*Eulophia campestris*) extract

Samples of plant material i.e. Salep (*Eulophia campestris*) were collected, in the form of rhizomes and dried artificially by Warm-air drying method, which is the most widely used method for medicinal plants, uses a counter-current flow of warm air in plate chamber.

- Rhizome of Salep (*Eulophia campestris*) was placed in plate chamber dryer and drying air was maintained at 72°C for 6 hours.
- Dried rhizome of Salep (*Eulophia campestris*) was removed after hardening. After drying, samples was grounded to pass a 1.0-mm screen (20 mesh) using the appropriate Wiley Mill.
- After grinding, the sample was thoroughly mixed and a 5- to 8-g aliquot withdrawn for analysis and storage.
- Dried and ground salep plant (10 g) was extracted with 60 mL of 50% ethanol at 37 °C for 10 days with frequent agitation until the soluble matter was dissolved and then filtered and stored at 4 °C. Other method like Infusion and Digestion was also employed but this was simple and easy.

4.4.2 Sample recovery

Samples were recovered by evaporating the ethanol using Rotary Evaporator. Samples after drying were dissolved in 25 mL of phosphate buffer saline and stored at 4°C for further use.

4.4.3 Process for preparation of Whitton root (*Eulophia nuda*) extract

For preparation and recovery of whitton root (*Eulophia nuda*) extract same protocol as mentioned above was followed.
4.5 IN-VITRO GLYCATION INHIBITION WITH PLANT EXTRACTS.

To measure glycation inhibition with plant extracts, different concentrations of protein (BSA) and glucose (two of each) and three concentrations or volumes of plant extracts salep and whitton root were used separately as given in Table: 14

**TABLE : 14** Different concentrations of protein (BSA) and glucose (two of each) and three concentrations or volumes of plant extracts.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Components of reaction</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B : Buffer (PBS)</td>
<td>0.1M</td>
</tr>
<tr>
<td>2</td>
<td>P1 : Protein (BSA)</td>
<td>15 mg/mL</td>
</tr>
<tr>
<td>3</td>
<td>P2 : Protein (BSA)</td>
<td>7.5 mg/mL</td>
</tr>
<tr>
<td>4</td>
<td>G1 : Glucose</td>
<td>25.0 mM</td>
</tr>
<tr>
<td>5</td>
<td>G2 : Glucose</td>
<td>5.5 mM</td>
</tr>
<tr>
<td>6</td>
<td>PFA1: Plant Filtrate</td>
<td>300 μL</td>
</tr>
<tr>
<td>7</td>
<td>PFA2 : Plant Filtrate</td>
<td>150 μL</td>
</tr>
<tr>
<td>8</td>
<td>PFA3 : Plant Filtrate</td>
<td>75 μL</td>
</tr>
</tbody>
</table>

PFA : Extract of Salep (*Eulophia Campestris*) and Whitton root (*Eulophia nuda*)

Glucose, BSA with or without inhibitor (plant extracts in PBS pH 7.4) were prepared and their mixtures were incubated at 37°C and 50°C for 5 weeks. During this, samples were drawn for glycation inhibition activity after 1<sup>st</sup>, 3<sup>rd</sup> and 5<sup>th</sup> week of incubation. The samples were kept at 4°C until analysis.
4.5.1 **Trichloracetic acid (TCA) method for Maillard reaction inhibitory activity of plant extracts**

This method is also known as TCA treatment method described by Matsuura et al. (2002) was followed with some modification.

4.6 **MODIFICATION OF IMMUNOGLOBULIN**

Immunoglobulin G (IgG) was modified by a published procedure (Khan et al., 2007). IgG sample was modified with 50, 100 and 150 mM glucose concentrations incubated with varying time intervals at 37°C. The modified IgG was studied by UV absorption spectroscopy on Shimadzu-1700 spectrophotometer, Japan. All samples were extensively dialyzed against PBS, pH 7.4.

4.6.1 **Spectroscopic analysis**

The ultraviolet spectra of native and glycated IgG were recorded in the wavelength range of 200 to 400 nm on a Shimadzu UV spectrophotometer. Fluorescence emission spectroscopy of native and glycated IgG samples were performed on Hitachi-RF200 (Japan) fluorimeter.

4.6.2 **Determination of carbonyl formation**

Carbonyl contents of native and ROS-modified IgG were analyzed (Levine et al., 1990) with slight modification. The reaction mixture containing native and ROS-modified protein (6.7 μM), 0.5 ml of 10 mM 2, 4-dinitrophenyl hydrazine (DNPH)/2.5 M HCl was added and thoroughly mixed. After addition of 250 μL TCA (20%) and centrifugation, the pellet was collected and washed three times with 1 ml ethanol:ethyl acetate (1:1) mixture. The pellet was then dissolved in 1ml of 6M guanidine hydrochloride solution and incubated at 37°C for 15 minutes. After centrifugation, the supernatant was collected and carbonyl content was estimated from the absorbance at 370nm using a molar absorption coefficient 22,000 M⁻¹cm⁻¹. Samples were spectrophotometrically analyzed against a blank of 1ml of 6M guanidine hydrochloride solution. Protein concentration was determined in the samples by the
Lowry method (Lowry et al., 1951). Protein carbonyl content was expressed as n mol/mg. The detailed protocol is mentioned in annexure 6.

4.6.3 Determination of keto-amine formation

Ketoamine of native and glycated- IgG were analyzed by published methods with slight modification (Khan et al., 2007). The reaction mixture containing native and glycated protein (200µl), 100 µl of NBT 0.5 mM in 300 µl sodium carbonate buffer was thoroughly mixed and incubated at 37°C for 2 hours.

Ketoamine was estimated from the absorbance at 510 nm. Samples were spectrophotometrically analyzed against blank (NBT + sodium carbonate).

4.6.4 Time dependent effect on Immunoglobulin G modified by glucose

The normal IgG treated with glucose was incubated for various time intervals ranging from 7-60 days. The time intervals taken were 7, 20, 30, 40, 50 and 60 days. After incubation at 37°C normal and modified samples were quantified on UV spectrophotometer. IgG modification with varied concentration of glucose normal IgG was taken and incubated with 300 µl of various concentrations from 50, 100 and 150 mM and incubation was done for 60 days at 37°C. Dialysis of normal and modified IgG was performed against PBS, pH 7.4 after incubation completes.

4.6.5 Determination of melting temperature

Thermal denaturation of IgG was done in order to access the degree of modification in the IgG. The normal and modified samples were subjected to heat denaturation. The samples were heated at 25 to 100°C and absorbance at various temperatures was recorded at 280 nm. The melting temperature was evaluated with the help of curve plotted for the % denaturation against the temperature.
% denaturation was calculated as follows:

\[
\text{% denaturation} = \frac{A_T - A_{25}}{A_{\text{max}} - A_{25}} \times 100
\]

Where,

\( A_T = \) Absorbance at a temperature \( T^0C. \)

\( A_{\text{max}} = \) Final maximum absorbance on completion of denaturation.

\( A_{25} = \) Initial absorbance at \( 25^0C. \)

**4.6.6 Purification of normal Immunoglobulin G**

Immunoglobulin G was isolated from normal human serum by affinity chromatography on Protein-A agarose CL-4B affinity column. The purified IgG from normal human sera was found to elute in a single symmetrical peak (Figure: 31). The purified IgG migrated as single band (Fig. 31 Inset), as shown by SDS-polyacrylamide gel electrophoresis under non-reducing conditions.
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4.6.7 Characterization of glycated IgG

Glycated IgG was characterized by UV absorption spectroscopy (Figure. 32). Shows UV absorption spectra of native and glycated IgG incubated with 50 mM 100 mM and 150 mM glucose concentrations for 7 days at 37°C. After 7 days incubation glycated IgG showed hyperchromicity at 280 nm.

FIGURE: 31 Elution profile of normal human IgG on Protein-A agarose CL-4B affinity column.

Inset: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.
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**FIGURE: 32** UV absorption spectra of IgG incubated with 50 mM (—), 100 mM (—) and 150 mM (—) glucose for 7 days. IgG native (—) without glucose under identical experimental conditions.

After 20 days incubation with same glucose concentration, glycated IgG showed extensive damage as evident by marked hypochromicity at 280 nm (Figure. 33) Similar results were obtained after 30 days, 40 days and 50 days incubation of IgG with same glucose concentration (Figure. 34, 35, 36). After 60 days glycation on the IgG became saturated, as same hypochromicity was observed (Figure. 37). Characterization of glycated IgG has been summarized in Table. 15.
FIGURE: 33 UV absorption spectra of IgG incubated with 50 mM (—), 100 mM (—) and 150 mM (—) glucose for 20 days. IgG native (—) without glucose under identical experimental conditions.
FIGURE: 34 UV absorption spectra of IgG incubated with 50 mM (—), 100 mM (—) and 150 mM (—) glucose for 30 days. IgG native (—) without glucose under identical experimental conditions.
FIGURE: 35  UV absorption spectra of IgG incubated with 50 mM (—), 100 mM (—) and 150 mM (—) glucose for 40 days. IgG native (—) without glucose under identical experimental conditions.
FIGURE: 36 UV absorption spectra of IgG incubated with 50 mM (—), 100 mM (—) and 150 mM (—) glucose for 50 days. IgG native (—) without glucose under identical experimental conditions.
FIGURE: 37 UV absorption spectra of IgG incubated with 50 mM (—), 100 mM (—) and 150 mM (—) glucose for 60 days. IgG native (—) without glucose under identical experimental conditions.
**TABLE 15:** Characterization of IgG incubated with 50, 100 and 150 mM glucose for 20 days and IgG without glucose under identical experimental conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Native IgG</th>
<th>Glycated IgG</th>
<th>% modification (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 mM</td>
<td>100 mM</td>
</tr>
<tr>
<td>U.V. absorbance at 280 nm (20 days)</td>
<td>0.83</td>
<td>0.75</td>
<td>0.50</td>
</tr>
<tr>
<td>Fluorescence intensity (Ext. 295 nm) (Em. 330 nm)</td>
<td>54.49</td>
<td>43.17</td>
<td>37.70</td>
</tr>
<tr>
<td>Fluorescence intensity (Ext. 280 nm) (Em. 330 nm)</td>
<td>42.86</td>
<td>41.32</td>
<td>36.1</td>
</tr>
<tr>
<td>Ketoamine estimation (nmole/mg protein)</td>
<td>4.20</td>
<td>8.02</td>
<td>8.35</td>
</tr>
<tr>
<td>Carbonyl content (nmole/mg protein)</td>
<td>9.67</td>
<td>13.25</td>
<td>20.6</td>
</tr>
<tr>
<td>Melting temperature (T_m) °C</td>
<td>58 °C</td>
<td>-</td>
<td>70 °C</td>
</tr>
</tbody>
</table>

**4.6.8 Fluorescence spectroscopy**

The fluorescence emissions were measured under identical condition. Tryptophan specific fluorescence analysis was conducted, when both native and glycated samples of IgG were excited at 295 nm. After 20 days incubation of IgG with 50 mM, 100 mM, and 150 mM glucose concentration, Tryptophan residues of glycated IgG showed significant loss of fluorescence intensity at 330 nm (Figure. 38).
The damage of tryptophan residue of glycated IgG samples were confirmed by the loss of fluorescence intensity at 330 nm using an excitation wavelength of 280 nm (Figure 39).

**FIGURE: 38** Tryptophan fluorescence emission spectra of IgG incubated with 50 mM (—), 100 mM (—) and 150 mM (—) glucose for 20 days. Native IgG (—) without glucose under identical experimental conditions. All samples were in PBS, pH 7.4. The excitation wavelength was 295 nm.
FIGURE: 39 Fluorescence emission spectra of IgG incubated with 50 mM(—), 100 mM (—) and 150 mM (—) glucose, for 20 days. Native IgG (—) without glucose under identical experimental conditions. All samples were in PBS, pH 7.4. The excitation wavelength was 280 nm.
4.6.9 Ketoamines analysis during advanced glycosylation end products formation

The ketoamine moieties formed by the glycation of IgG were measured calorimetrically by using nitro blue tetrazolium (NBT). After 7 days incubation of IgG with 50, 100, and 150 mM glucose concentrations, ketoamine formation was formed to be 5.36, 5.71 and 6 mole/mg protein respectively after 20 days incubation, ketoamine formation was 8.02, 8.35 and 9.46 nmole/mg protein, with their respective glucose concentrations. Formations of ketoamine became constant after 20 days incubation, as the results were similar after 30, 40, 50 and 60 days incubation of IgG with glucose under identical experimental conditions. The control non-glycated IgG gave a negligible ketoamine concentration of 4.2 n mole/mg protein (Figure 40).

**FIGURE: 40** Formation of ketoamine during incubation of IgG (1mg/ml) with glucose 50, 100 and 150 mM for 7 days (red), 20 days (green), 30 days (blue), 40 days (brown), 50 days (dark green), 60 days (sky blue) at 37°C. IgG (black) without glucose under identical experimental conditions.
4.7 Estimation of carbonyl content in advanced glycated end products.

The oxidation of protein typically results in an increase in carbonyl contents. This is due to the oxidation of Lysine, Arginine, and Protein etc residues. Therefore, oxidative involvement in advanced glycated end product (AGEs) can be measured by estimation of carbonyl contents. After 7 days incubation of IgG with increased glucose concentration, gave negligible formation of carbonyl contents. After 40 days, there was a tremendous increase in carbonyl contents, similar results were obtained after 50 and 60 days incubation (Figure. 41). Carbonyl contents measurements of native and glycated IgG have been summarized in Table.1

**FIGURE: 41** Formation of carbonyl content during incubation of IgG (1mg/ml) with glucose 50, 100 and 150 mM for 7 days (red), 20 days (green), 30 days (blue), 40 days (brown), 50 days (dark green), 60 days (sky blue) at 37°C. IgG (black) without glucose under identical experimental conditions.
4.7.1 Absorption – Temperature Scan

During temperature-induced denaturation, the absorbance changes at 280 nm with the increase of temperature until the process of unfolding is completed. Thermal melting temperature (Tm) of pool samples of glycated IgG was found to be 58°C, whereas Tm of non-glycated IgG was 70°C. The complete unfolding of non-glycated and glycated IgG occurred at 73°C and 90°C respectively. After these temperatures both protein samples showed precipitation or aggregation as abnormal increase in their absorbance was noticed (Figure. 42).

![Figure: 42 Thermal denaturation profile of native IgG (---) and pool sample of IgG glycated (...) with 50, 100, 150 mM glucose for 20 days at 37°C.](image)
4.8 ISOLATION OF IgG FROM TYPE 1 DIABETES, TYPE 2 DIABETES AND HYPERLIPIDEMIC PATIENTS SERA

**FIGURE: 43** Elution profile of purified IgG from normal human (—), type 1 diabetes (—), type 2 diabetes (—) and Hyperlipidemic (—) patients, serum samples on protein-A agarose affinity column.

**Inset:** SDS – PAGE of purified samples on 7.5% gel. Lane one contains eluted IgG from normal human serum (fraction number 5), Lane 2, Lane 3 and Lane 4 contain purified IgG from type 1 diabetes (fraction number 5), type 2 diabetes (fraction number 5) and from hyperlipidemic (fraction number 5) serum, respectively. The amount of protein in all case was 35 μg.

IgG was isolated from patients and also from normal human subjects as described above in methodology. The preparation gives a single and almost symmetrical peak on Protein- A agarose CL-4B column (Figure. 43). All purified IgG samples were eluted with identical volume of Tris buffer. The preparation was also
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found to be pure with respect to charge to mass ratio, as evident by the electrophoretic pattern of purified IgGs under reducing conditions in which they gave a single band (Figure. 43 inset).

4.8.1 Detection of carbonylated serum protein and Immunoglobulin G in Type 1 diabetes, Type 2 diabetes and Hyperlipidemic patients.

![FIGURE : 44 Comparison of carbonyl content in normal human serum proteins (bar 1), type 1 diabetes serum proteins (bar 2), type 2 diabetes serum proteins (bar 3), Hyperlipidemic serum proteins (bar 4), IgG from normal human subject (bar 5), IgG from type 1 diabetes (bar 6), IgG from type 2 diabetes (bar 7), IgG from Hyperlipidemic patient (bar 8).](image)

Oxidative stress plays a central role in the formation of advanced glycated end products (AGEs) and protein carbonyl groups are the biomarkers of oxidative stress. The oxidation of protein typically results in an increase in carbonyl contents. The data showed that the serum protein carbonyl contents were increased in type 1 and type 2 diabetes patients and as well as in Hyperlipidemic patients as compared to the normal
subjects. The carbonyl contents of type 1 diabetes, type 2 diabetes, Hyperlipidemia and normal human serum protein were 1.68, 1.75, 1.72 and 1.57 nmol/mg protein, respectively (Figure. 44).

Further, to investigate the extent of alterations in the biological properties of IgG in type 1, type 2 diabetes and Hyperlipidemic patients IgG was isolated from type1 diabetes patients (DM-1-IgG), from type 2 diabetes patients (DM-2-IgG) and Hyperlipidemic patient (H-IgG) and also from the normal subjects (NH-IgG) and their carbonyl contents were compared. The carbonyl contents of DM-1-IgG, DM-2-IgG, H-IgG and NH-IgG were 0.43, 0.45, 0.43 and 0.33 nmol/mg proteins, respectively. The results were similar to those obtained with serum (Figure. 44)