CHAPTER 4

Effect of Hederagenin on the Type 2 Diabetes comorbidity: Hyperlipidemia

4.1 Introduction

Diabetes mellitus is a common disorder of carbohydrate, fat, and protein metabolism in which the circulating glucose concentration is increased. The uncontrolled diabetes can cause serious microvascular (e.g. retinopathy, neuropathy, nephropathy, or peripheral limb amputation) and macrovascular complications (e.g. hyperlipidaemia leading to heart disease or stroke). As a consequence of the many complications associated with the disease, diabetes is estimated to reduce life expectancy by approximately 10 years, and is the fifth leading cause of death worldwide. Maintaining good glycaemic control has a risk reducing effect on microvascular complications and the associated adverse outcomes of retinopathy, neuropathy, and nephropathy. However, due to confounding factors, the relationship between controlling hyperglycaemia and reducing macrovascular risk has been more difficult to demonstrate. For eg, most Type 2 diabetes patients have comorbid conditions that also increase the risk of macrovascular events, such as excess weight or obesity (79–85%), hypertension (76–84%), or hyperlipidaemia (55–70%). Furthermore, the treatments used to reduce blood plasma glucose can have unwanted effects, such as weight gain, that may also increase a patient’s risk of macrovascular events. (Monographs, 2011)

These comorbid conditions disrupt the protective normal oxidative mechanisms and leads to increased generation of free radicals such as $\text{O}_2^-$ (superoxide anion), $\text{OH}$ (hydroxyl radical) and $^1\text{O}_2$ (singlet oxygen) (which are formed as a part of the normal metabolic process). However, our body systems protect themselves against free radical damage by antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), glutathione dependent enzymes such as glutathione peroxidise (GPx), glutathione-S-transferase (GST), etc., as well as compounds such as ascorbic
acid, α-tocopherol and glutathione. The antioxidants property along with any drug play a role in helping them to revive the normal oxidative property, hence an intensive research of the Hederagenin to check for the antioxidant property is attempted.

Objective

To analyze the effect of Hederagenin in the high-fat diet inducing hyperlipidemia murine model and also to test the antioxidant property.

4.2 Materials and Methods

4.2.1 Reagents and instruments required

The chemical and drugs used in the study were Hederagenin, Streptozotocin (STZ), Glucose, carboxymethyl cellulose (CMC), metformin, pioglitazone, isoflurane (anaesthetic agent) catalase, superoxide dismutase (SOD) hydrogen peroxide, formaldehyde, eosin, hematoxylin was purchased from Sigma–Aldrich, St. Louis, MO, USA. Animal restrainer (e.g., Broom restraint, Plas Labs), Micrometer, glucometer (Accu-Check, Roche, Germany) and Advia (Hematology analyzer), hematoxylin and eosin stains, semi automated microtome, Disterene phthalate xylene.

4.2.2 Animals

All the studies were conducted in compliance with guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and approved by the Institute of Animal Ethics Committee (IAEC-P.No.12/P.No-03/PPK/2008), (IAEC-P.No.10/P.No-07/PPK/2008). All the studies were conducted as per the norms of the committee for the purpose of supervision of experiments on animals. Male swiss albino mice weighing 28–30 g were purchased from Sasthra college of Pharmacy, Nellore, India. All animals were housed 2/cage and kept in the animal house for one week for proper acclimatization before starting the experiment under controlled conditions of illumination (12 h light/12 h darkness) and temperature ranging 20-25°C. They were housed under the
above laboratory conditions, maintained on high fat diet (mentioned below) and water.

4.2.3 Compostition of High fat diet (HFD)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>HFD(g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lard</td>
<td>270</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>30</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10</td>
</tr>
<tr>
<td>Nutrilab diet</td>
<td>400</td>
</tr>
<tr>
<td>Casein</td>
<td>230</td>
</tr>
<tr>
<td>Cellulose</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>5</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>5</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>1</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>2.5</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>2.5</td>
</tr>
</tbody>
</table>

4.2.4 Effects of Hederagenin on high fat diet/streptozotocin (HFD/STZ) induced diabetic mice

- Four week-old mice were fed with HFD (~50% kcal) comprising of components with different percentage of energy.
- After 4 weeks of HFD feeding, the mice were injected once with low-dose of STZ (125mg/kg,i.p.) to induce partial insulin deficiency.
- Two weeks after STZ injection, the majority of HFD/STZ-treated mice displayed hyperglycemia and glucose intolerance.
- At six weeks of HFD feeding, animals were with similar degrees of hyperglycemia.
- According to their body weights, animals were randomly divided to control or treatment groups.
The diabetic control and the normal control groups received the vehicle (0.25% CMC, 10ml/kg) and the treatment groups were given pioglitazone (10mg/kg, p.o.) and Hederagenin (200 mg/kg, p.o) as suspensions in 0.25% CMC (10ml/kg), once daily.

In the treatment schedule of 28 days, blood was collected on the 28th day by retro orbital puncture under mild anesthesia, centrifuged and examined for the biochemical, haematological and histopathological analysis. Total protein and antioxidant properties are also determined

Subsequently, OGTT was performed at the end of the experiment.

Body weight and daily food intakes were monitored.

Animals were sacrificed and livers were isolated to measure antioxidant status intake was monitored.

4.2.5 Oral Glucose Tolerance Test

The animals were fasted for 16-18 hours (overnight).

Blood basal samples (T₀) were taken.

Animals were randomized according to their baseline blood glucose level.

Distilled water (normal control) or glucose load of 2 g/kg, p.o. were administered immediately after treatment with 0.25% w/v (10 ml/kg) carboxymethyl cellulose (CMC) (vehicle control) or metformin (500 mg/kg, p.o.), Hederagenin (200, 400 and 600 mg/kg, p.o.) respectively after which a glucose load of 2g/kg and either vehicle (water) or simultaneously administered orally.

Blood glucose concentrations were measured at 15,30, 60 and 120 min (T₁₅–T₁₂₀) post treatment by tail cut method using glucometer (Accu-Check, Roche, Germany)
The reduction in blood glucose produced by the compounds metformin and Hederagenin were calculated using the area under the curve method with basal value as the zero. (AUC \(_{0–120\text{ min}}\)).

4.2.6 Histopathology of mouse pancreas and kidney

The isolated pancreas and kidney tissues fixed in 10% neutral-buffered formalin, dehydrated by passing through a graded series of alcohol, and embedded in paraffin blocks and 5\( \mu \text{m} \) sections were prepared using a semi-automated rotary microtome (model RM2245, Leica Microsystems, Wetzlar, Germany). The sections were stained in hematoxylin and eosin. The sections were mounted by Disterene Phthalate Xylene (D.P.X.)

4.2.7 Biochemical parameters

The metabolic markers associated mainly with diabetes Plasma glucose (PG), Total cholesterol (TC), Triglycerides (TG), Total protein (TP), Blood urea nitrogen (BUN) and Creatinine (CREA), glycosylated haemoglobin (HbA\(_1\)C) were analysed using automated analysers (Dimension Xpand Plus, Siemens)

4.2.8 Haematological parameters

The toxicological effect of chronic supplementation of Hederagenin,hematological parameters such as white blood cell (WBC), red blood cell (RBC), platelet count, hemoglobin (Hb) level, mean corpuscular hemoglobin(MCH),mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV) were determined by the use of automated hematological analyzer (ADVIA-120,Siemens,India).

4.2.9 Body weight

Body weight of individual animals will be recorded on the Day of randomization, Day 1 and on weeks 4 and 6 during the entire observation period.
Course of action: On the above days, the animals will be weighed non-fasted. For the calculation of organ to body weight ratios, a final, fasted body weight will be taken on the day of scheduled euthanasia.

4.2.10 Feed intake

Cage-wise feed intake will be recorded on day 1 and on week 4 and 6

4.2.11 Lipid peroxidation –Malondialdehyde estimation

Malondialdehyde (MDA), an index of free radical generation/lipid peroxidation, was determined as described by Ohkawa et al., (1979). Briefly, the reaction mixture consisted of 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid added to 0.2 ml of blood plasma. The mixture was made up to 4.0 ml with distilled water and heated at 95°C for 60 min. After cooling the contents under running tap water, 5.0 ml of n-butanol and pyridine (15:1 v/v) and 1.0 ml of distilled water was added. The contents were centrifuged at about 3000 rpm for 10 min. The organic layer was separated out and its absorbance was measured at 532 nm using double beam UV-Visible spectrophotometer against a blank. MDA values are calculated using the extinction coefficient of MDA-thiobarbituric acid complex 1.56 × 105 l/mol × cm and expressed as nmol/ml (Suresh kumar et al., 2012).

4.2.12 Estimation of glutathione level

The tissue sample (Liver 200 mg) was homogenized in 8.0 ml of 0.02 M EDTA in an ice bath. The homogenates were kept in the ice bath until used. Aliquots of 5.0 ml of the homogenates were mixed in 15.0 ml test tubes with 4.0 ml distilled water and 1.0 ml of 50% trichloroacetic acid (TCA). The tubes were centrifuged for 15 min at approximately 3000 g. 2.0 ml of supernatant was mixed with 4.0 ml of 0.4 M Tris buffer pH 8.9, 0.1 ml Ellman’s reagent [5,5-dithiobis-(2-nitro-benzoic acid)] (DTNB) added and the sample shaken. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank with no homogenate. Results were expressed as μmol GSH/mg tissue (Lindsay, 1968).
4.2.13 Catalase test

Catalase is an antioxidant enzyme omnipresent in mammalian and non-mammalian cells that destroys hydrogen peroxide by dismutation. Hydrogen peroxide is an ROS that is a toxic product of normal aerobic metabolism and pathogenic ROS production involving oxidase and superoxide dismutase reactions. Hydrogen peroxide is poisonous to eukaryotic cells and in high doses can initiate oxidation of DNA, lipids, and proteins, which can lead to mutagenesis and cell death. Eukaryotic catalases are heme enzymes found in the liver, kidney, and erythrocytes in high concentrations while the lowest concentrations are in the connective tissues. The enzyme is concentrated in the peroxisome subcellular organelles. This catalase assay was carried out by OxiSelect Catalase Activity assay kit.

**Principle**

Catalase Activity Assay involves two reactions. The first reaction is the catalase induced decomposition of hydrogen peroxide $H_2O_2$ into water and oxygen. The rate of disintegration of hydrogen peroxide into water and oxygen is proportional to the concentration of catalase (Reaction 1). A catalase containing sample can be incubated in a known amount of hydrogen peroxide. The reaction proceeds for exactly one minute, at which time the catalase is quenched with sodium azide. The remaining hydrogen peroxide in the reaction mixture facilitates the coupling reaction of DHBS and AAP in conjunction with an HRP catalyst (Reaction 2). The quinoneimine dye coupling product is measured at 520nm, which correlates to the amount of hydrogen peroxide remaining in the reaction mixture.

**CATALASE**

Reaction 1: $2H_2O_2$ $\longrightarrow$ $2H_2O + O_2$

HRP

Reaction 2: $2H_2O_2 (Left\ over) + DHBS + AAP$ $\longrightarrow$ Quinoneimine Dye
Materials Required

Catalase Standard (600,000 Units/ml), Hydrogen Peroxide (8.82 M solution), Chromogenic Reagent A (100 μl), Chromogenic Reagent B (6 ml) bottle, HRP Catalyzer (10 μl), Assay Diluent (10X), Sample Buffer (5X), Catalase Quencher (1 ml), distilled water, 96 well plate, micropipettes with tips, Spectrophotometer.

Reagent preparation

*Note: All reagents were brought to room temperature prior to use.*

- **1X Assay Diluent:** 10X Assay Diluent stock was diluted to a 1X solution with distilled water. Mixed and stored 1X diluents at room temperature.

- **1X Sample Buffer:** 5X Sample Buffer stock was diluted to a 1X solution with distilled water. Mixed and stored 1X diluents at room temperature. This was used for all sample and standard dilutions.

- **Hydrogen Peroxide Working Solution:** 12 mM Hydrogen Peroxide Working Solution was prepared by diluting the stock 8.82 M H₂O₂ solution in Assay Diluent (eg. 5 μl of H₂O₂ stock was added to 3.67 ml Assay Diluent). Mixed and stored at 2-8°C

- **Chromogenic Working Solution:** Chromogenic Working Solution was prepared by diluting Chromogenic Reagent A 1:100 with Chromogenic Reagent B. (Example: Add 0.10 ml of Chromogenic Reagent A to 9.9 ml of Chromogenic Reagent B) Mixed thoroughly. Then, add 1 μl of HRP Catalyzer per 1 ml of Chromogenic Working Solution. (Example: Add 10 μL to 10 mL of Chromogenic Working Solution) mixed to homogeneity. This reagent would be stable for 2 weeks when stored at 4°C and protected from light.

Sample preparation – Liver tissue homogenate

Prior to dissection, the animal was perfused with a phosphate buffered saline (PBS) solution in order to rinse the tissue and to remove any red blood cells and clots. Then the livers were collected, weighed and homogenized on ice in 5-10 ml cold
PBS with 1mM EDTA per gram of tissue. The sample was centrifuged at 10,000 x g for 15 minutes at 4°C to remove the supernatant and stored on at -70°C until processed.

Method

- Catalase standard stock was prepared in the dilution of 1:60 in sample buffer to make a concentration of 10,000 units/ml solution (i.e 5µl stock in 295µl sample buffer)

- 10µl of stock was mixed with 990µl of sample buffer to make a working stock concentration of 100U/ml. From this, serial dilution would be performed by transferring 500µl of stock to successive tubes serially making a concentration of 50, 25, 12.5, 6.25, 3.125, 1.562Units/ml, one time only, later from all concentrations 20µl was pipette out in wells.

- 20 µl of the diluted catalase standards or unknown samples were added to a 96-well microtiter plate with one of the well maintained as blank by adding 20µl of the sample buffer.

- 50 µl of the hydrogen peroxide working solution (12mM) was added to each well, then mixed thoroughly and incubated exactly for 1 minute.

- The reaction was stopped by adding 50 µl of the catalase quencher into each well and mixed thoroughly.

- 5 µl of each reaction was transferred well to a fresh well.

- 250 µl of the chromogenic working solution was added to each well and the plates were incubated for 40-60 minutes with vigorous mixing.

- The plate was read at absorbance at 520 nm.

- The standard curve was drawn as catalase U/ml vs OD at 520 nm and unknown sample value was calculated
4.2.14 Superoxide dismutase (SOD)

Superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. SOD enzymes are classified into three groups: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular Ec-SOD.

Assay Principle

Superoxide anions (O2-) are generated by a Xanthine/Xanthine Oxidase (XOD) system, and then detected with a Chromagen Solution. However, in the presence of SOD, these superoxide anion concentrations are reduced, yielding less colorimetric signal. The activity of SOD is determined as the inhibition of chromagen reduction.

Materials Required

SOD Standard, Xanthine Solution (125 μl), Xanthine Oxidase Solution: 150X: (10 μl), Chromagen Solution (125 μl), SOD Assay Buffer 10X (1.5 ml) tube, distilled water, 96 well plate, micro-pipettes with tips, Spectrophotometer.

Reagents Preparation

- **SOD Standard**: One 20 μl tube provided at 5 Units/μl. Unit Definition: One unit will inhibit the rate of reduction of cytochrome c by 50% in a coupled system, using xanthine and xanthine oxidase, at pH 7.8 at 25°C in a 3.0 ml reaction volume.
• **IX SOD Assay Buffer**: Diluted one vial of 10X SOD Assay Buffer to 1X with deionized water. Mixed to homogeneity. The second vial was kept for 10X SOD Assay Buffer undiluted.

• **IX Xanthine Oxidase Solution**: Just prior to use, the 150X Xanthine Oxidase Solution was diluted to 1X with 1X SOD Assay Buffer and mixed to homogeneity

**Sample preparation – Liver tissue homogenate**

The tissue sample was homogenized in 5-10 ml of cold 1X lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA) per gram tissue. The cells are lysed with sonication or homogenation and centrifuged at 12000 x g for 10 minutes and the tissue lysate supernatant was collected and stored at -80°C until processed.

**Method**

• SOD Standard was thawed at 4°C.

• A freshly prepared a dilution series (1:4) of SOD Standard in the concentration range of 5 Units/µl – 1.2 mU/µl by diluting the SOD Standard in 1X Assay Buffer (see Preparation of Reagents).

• 10 µl of each dilution is transferred to a 96-well microtiter plate, including a 1X Assay Buffer blank.

• The following master mixture was prepared, adjusting for the required number of wells.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine Solution</td>
<td>5 µl</td>
</tr>
<tr>
<td>ChromagenSolution</td>
<td>5 µl</td>
</tr>
<tr>
<td>10X SOD Assay Buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>DI Water</td>
<td>60 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>80 µl</strong></td>
</tr>
</tbody>
</table>
• 80 µl of the above master mixture was transferred to each well.

• Finally, added 10 µl of pre-diluted 1X xanthine oxidase solution (see Preparation of Reagents) to each well, mixed well and incubated for 1 hour at 37°C.

• The plate was read at absorbance at 490 nm on a microplate reader.

4.2.15 Statistical analysis

Statistical comparisons were performed using Graph Pad Prism 5.02 by one-way analysis of variance (ANOVA), followed by posthoc Tukey’s test. Results were expressed as mean ± S.E.M.

4.3 Results

In the present hyperlipidemic study, the high fat diet was fed to diabetic animals resulting in change of metabolic parameters of the mice. But, when the mice was treated with Hederagenin then hyperlipidemic condition was recovering and the experiments on anti-oxidant assays proved that the drug is showing an anti-oxidant property. These indicated a good sign of the Hederagenin on comorbid conditions of Type 2 diabetes mellitus.

4.3.1 Effect of Hederagenin on OGTT in HFD/STZ mice.

The chronic effect of Hederagenin was evaluated by OGTT on overnight fasted animals. Out of four groups, normal animals maintained the normal glycemic condition of 100-120mg/dl throughout 0-120 min, whereas the diabetic control fed with high fed diet showed an elevation in their glucose level which reached the highest peak of 350mg/dl at 30min and then slowly lowers at 60min and then it maintains at 200mg/dl. This is shown in the fig. 4.1 A the area under the curve was calculated individually and their mean±S.E.M was expressed as bar diagram in the fig. 4.1 B. Pioglitazone (10mg/kg) and Hederagenin (200mg/kg) significantly
lowered the glucose excursion in comparison with the diabetic control. Hederagenin showed better activity than pioglitazone.

4.3.2 Histopathological examination

The liver histopathological examination using hematoxylin and eosin (H&E) staining is shown in Fig. 4.2 figure 1 & 2. The control mice had a normal hepatic architecture with normal hepatocyte morphology and orderly arranged hepatic cell cords. The hepatocytes had abundant cytoplasm, distinct cell borders, and a round central nucleus. The STZ treatment led to severe pathological changes, such as mussy hepatic cords, focal necrosis, congestion in central vein, and infiltration of lymphocytes. The Hederagenin treatment did not have an effect on the STZ-induced pathological changes. However, the treatment group markedly alleviated these histopathological changes, and the hepatic architecture was similar to normal hepatic architecture. The renal histology is compared to the normal renal architecture of the normal mice (Fig. 4.2 figure 2), STZ appeared to induce severe pathological damages, such as mesangial expansion, glomerular hypertrophy, infiltration of inflammatory cell into the renal tubule-interstitium, narrow lumen, and sclerosis in part of glomeruli. The Hederagenin treatment changed morphology and led to a decreased extent of the expansion in glomerular and mesangial matrix. Further, there were no clearly histopathological abnormalities found in renal histology in the Hederagenin treated mice as compared to normal mice.

4.3.3 Biochemical parameters

Terminal blood samples were collected before euthanasia, and centrifuged at 2500rpm (revolutions per minute) for 15 minutes to separate the plasma. This plasma was analysed using automated analysers for total protein, glucose, triglycerides, cholesterol, BUN and creatinine. There was a rise in all parameters in diabetic control and after the treatment of Hederagenin (200mg/kg) and pioglitazone(10mg/kg) there was significant p <0.05 reduction in comparison to diabetic control. Percentage of HBA1C for diabetic animals was 8.1% and after the
treatment with pioglitazone and Hederagenin, the level reduced to 4.20 and 3.72 % respectively. This indicated the reduction of glucose level with respect to treatment (Table 4.1)

4.3.4 Body weight and Feed intake

The body weight and feed intake of Hederagenin treated animals, significantly (p<0.05) lowered than the diabetic control. However, pioglitazone (10mg/kg) did not produce any significant change in body weight or feed intake. (Table 4.1)

4.3.5 Haematological Parameters

The haematological markers in HFD/STZ mice were studied. The treatment or controls does not show any alteration in the values of RBC, WBC, PLT, Hb, HCT, MCV, MCH, and MCHC in comparison to the normal ranges. This is shown in the Table 4.2

4.3.6 Liver Anti-oxidant Activity

The continuous feeding of High fat diet to diabetic animals, resulted in oxidative stress which when in normal condition will be repaired by normal anti-oxidant enzymes present in our body. This was clearly indicated by the low values of the assay in comparison to normal control in GSH, CAT, and SOD. Chronic supplementation with Hederagenin and pioglitazone for 28 days resulted in significant (p < 0.05) elevation in liver antioxidants (GSH, CAT, and SOD) when compared to diabetic control. Both Hederagenin and pioglitazone significantly (p < 0.05) reduced oxidative stress resulting from insulin resistance, hyperlipidemia and diabetic conditions. MDA lipid peroxidase enzyme was seen in higher levels (0.59±0.01nmole/mg of tissue) in diabetic control group indicating membrane damage and the statistically significant reversal has occurred when the animals were treated with Hederagenin and pioglitazone (0.37±0.02 and 0.33±0.03 respectively). Tables and figures 4.3, 4.4, 4.5 and 4.6.
Fig. 4.1 Effect of Hederagenin on OGTT in HFD/STZ mice

[A] Blood glucose levels

[B] Area Under Curve (AUC(0–120min))

Results are expressed as mean ± S.E.M. *p < 0.05 as compared to normal control and # p<0.05 as compared to diabetic control.
Fig. 4.2. Figure 1: Effect of Hederagenin on histological changes of pancreatic islets at magnification of x200

Group I [Normal control ;A], Group II [Diabetic control; B], Group III [Pioglitazone 10 mg/kg,p.o.; C]; and Group IV [Hederegenin 200mg/kg ;D]
Fig.4.2 Figure 2: Effect of Hederagenin on histological changes of Kidney at magnification of x200.

Group I [Normal control ;A], Group II [Diabetic control; B], Group III [Pioglitazone 10 mg/kg,p.o.; C], and Group IV [Hederegenin 200mg/kg ;D]
Table 4.1 Effect of Hederagenin on metabolic parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal control</th>
<th>Diabetic control</th>
<th>Pioglitazone (10mg/kg)</th>
<th>Hederagenin (200mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP (g/dl)</td>
<td>5.0±0.4</td>
<td>6.3±0.36</td>
<td>5.3±0.29</td>
<td>5.5±0.12</td>
</tr>
<tr>
<td>Glucose(mg/dl)</td>
<td>100.3±4.41</td>
<td>241.7±6.28</td>
<td>137.7±7.74</td>
<td>90.7±4.41</td>
</tr>
<tr>
<td>TGL (mg/dl)</td>
<td>132.3±19.72</td>
<td>229.7±8.82</td>
<td>143.5±5.54</td>
<td>117.0±11.73</td>
</tr>
<tr>
<td>CHOL(mg/dl)</td>
<td>134.2±3.78</td>
<td>203.2±13.78</td>
<td>197.2±7.84</td>
<td>133.1±5.53</td>
</tr>
<tr>
<td>BUN(mg/dl)</td>
<td>30.1±3.43</td>
<td>231.8±11.56</td>
<td>94.2±3.62</td>
<td>30.1±3.43</td>
</tr>
<tr>
<td>CREA(mg/dl)</td>
<td>0.54±0.05</td>
<td>3.24±0.36</td>
<td>1.10±0.13</td>
<td>2.22±0.41</td>
</tr>
<tr>
<td>HBA1C(%)</td>
<td>3.72±0.3</td>
<td>8.1±0.3</td>
<td>4.20±0.23</td>
<td>3.72±0.3</td>
</tr>
<tr>
<td>Bodyweight (g)</td>
<td>27.76±0.16</td>
<td>38.84±0.74</td>
<td>36.46±0.75</td>
<td>31.18±1.90</td>
</tr>
<tr>
<td>Feed intake (g)</td>
<td>64.6±6.5</td>
<td>93.5±2.0</td>
<td>70.3±1.8</td>
<td>81.3±1.8</td>
</tr>
</tbody>
</table>

(TP- Total Protein; TGL- Triglycerides; CHOL- cholesterol; BUN- Blood urea nitrogen; CREA- creatinine, HBA1C- Glycosylated haemoglobin; ) Results are expressed as Mean±S.D *p<0.05 as compared to normal control #p<0.05 as compared with diabetic group
Table 4.2 Effect of Hederagenin on Haematological parameters.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal control</th>
<th>Diabetic control</th>
<th>Pioglitazone (10mg/kg)</th>
<th>Hederagenin (200mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10^6/µl)</td>
<td>7.9±0.4</td>
<td>10.2±0.9</td>
<td>7.2±0.4</td>
<td>8.3±0.2</td>
</tr>
<tr>
<td>WBC (10^3/µl)</td>
<td>8.6±0.7</td>
<td>14.1±1.2</td>
<td>11.2±0.9</td>
<td>12.1±0.9</td>
</tr>
<tr>
<td>PLT (10^3/µl)</td>
<td>817.0±27.1</td>
<td>940±21.1</td>
<td>867±0.2</td>
<td>882.3±22.0</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>14.9±0.5</td>
<td>15.4±0.4</td>
<td>14.3±0.2</td>
<td>14.9± 0.5</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>24.3±1.7</td>
<td>22.1±1.5</td>
<td>23.1±1.1</td>
<td>24.3±1.7</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>41.3±2.6</td>
<td>43.6±0.9</td>
<td>41.2±0.8</td>
<td>43.0±3.7</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>20.8±1.6</td>
<td>21.2±1.2</td>
<td>20.3±1.1</td>
<td>21.0±2.4</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>29.0±5.3</td>
<td>28.2±1.1</td>
<td>24.2±1.2</td>
<td>29.7±5.5</td>
</tr>
</tbody>
</table>

(RBC- Red Blood cells; WBC- White blood corpuscles; PLT-Platelet; Hb-Haemoglobin; HCT- Haematocrit; MCV-Mean Corpuscular volume; MCH-Mean Corpuscular Haemoglobin; MCHC – Mean corpuscular Haemoglobin Concentration). Results are expressed as Mean±S.D
Table 4.3  Estimation of Lipid Peroxidation - Malondialdehyde

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (U/mg of protein) (Mean± S.E.M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.29±0.02</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>0.59±0.01*</td>
</tr>
<tr>
<td>Pioglitazone (10mg/kg)</td>
<td>0.33±0.03#</td>
</tr>
<tr>
<td>Hederagenin (200mg/kg)</td>
<td>0.37±0.02#</td>
</tr>
</tbody>
</table>

Results are expressed as Mean±S.E.M *p<0.05 as compared to normal control #p<0.05 as compared within diabetic group
Figure 4.4 Anti-oxidant assay Estimation of Glutathione level

![Graph showing Estimation of Glutathione level](image)

Table 4.4 Anti-oxidant assay Estimation of Glutathione level

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glutathione (U/mg of protein) (Mean± S.E.M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>29.2±3.0</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>18.4±1.1*</td>
</tr>
<tr>
<td>Pioglitazone(10mg/kg)</td>
<td>25.0±1.4#</td>
</tr>
<tr>
<td>Hederagenin(200mg/kg)</td>
<td>27.7±3.6#</td>
</tr>
</tbody>
</table>

Results are expressed as Mean±S.E.M *p<0.05 as compared to normal control
#p<0.05 as compared within diabetic group
Table: 4.5 Anti oxidant Assay - Catalase Test

<table>
<thead>
<tr>
<th>Groups</th>
<th>Catalase (U/mg of protein) (Mean± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>128.3±7.4</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>71.2±5.6*</td>
</tr>
<tr>
<td>Pioglitazone(10mg/kg)</td>
<td>113.2±1.2#</td>
</tr>
<tr>
<td>Hederagenin(200mg/kg)</td>
<td>131.3±2.9#</td>
</tr>
</tbody>
</table>

Results are expressed as Mean±S.D *p<0.05 as compared to normal control
#p<0.05 as compared within diabetic group
Figure: 4.6 Anti-oxidant assay Estimation of Superoxide dismutase level

Table 4.6  Anti-oxidant assay Estimation of Superoxide dismutase level

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/mg of protein) (Mean± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>213.2±4.30</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>69.50±9.30*</td>
</tr>
<tr>
<td>Pioglitazone(10mg/kg)</td>
<td>155.20±6.30*#</td>
</tr>
<tr>
<td>Hederagenin(200mg/kg)</td>
<td>179.20±12.30*#</td>
</tr>
</tbody>
</table>

Results are expressed as Mean±S.D *p<0.05 as compared to normal control #p<0.05 as compared within diabetic group
4.4 Discussion

Diabetes Mellitus (DM) is increasing at an alarming rate worldwide, in developing countries, which can be mainly attributed to the sedentary lifestyle and calorie-rich diet. This in turn has been one of the major reasons for the increase in the incidence and prevalence of hyperlipidemia. This is often linked with abnormal lipid metabolism and is considered a major risk factor for (Saltiel and Kahn, 2001).

Lifestyle-related diseases, such as obesity, hyperlipidemia, atherosclerosis, Type 2 diabetes and hypertension, are widespread and increasingly prevalent in industrialized countries. This is accompanied by the rapid increase in the number of elderly people, this becomes a medical and a socioeconomic issue. A clustering of metabolic disorders (in particular abdominal obesity, hypertriglyceridemia, a low level of high-density-lipoprotein (HDL)-cholesterol, hypertension and high fasting-glucose level) in an individual, defined as metabolic syndrome, is known to increase cardiovascular morbidity and mortality. Although the pathogenesis of metabolic syndrome is complicated and precise details of the underlying mechanisms are not known, it has been suggested that the quality of dietary lipids may be an important modulator in terms of the risks associated with this syndrome. Animal studies and clinical trials have revealed different effects of herbal compounds significantly reduce lipid levels (Monograph 2011).

Most natural product and pharmacology groups still tend to search for new actives in poorly studied species instead of undertaking further research on species with proven activity. This could be due in part to researchers finding it difficult to find funds to support work on plants with proven activity and commercial companies not wanting to invest funds into developing a product in which they will not be able to protect the intellectual property. These reasons are justified, but more research need to be undertaken on species containing compounds with proven activity in order to turn a “potential” into an “actual” lead. Very few examples of plant-based leads, such as metformin, abiguanide derived from two linked guanidine units (guanidine derivatives are reported to be the active constituents of Galega officinalis (fabaceae), are currently prescribed in mainstream western medicine. The challenge will be to
see whether any of these species or the active compounds in the species progress through the validation system. This is unlikely unless they attract financial resources. In last 5 years, the species that received the most citations for their antidiabetic activity include species of *Panax* and *Phyllanthus*, *Momordica charantia*, *Allium cepa* and *A. sativum*. A growing body of evidence indicates that the traditional uses of these species can be supported by scientific evidence. Many of the active compounds in these species have been identified, but more research needs to be completed if products are going to be developed from these species. Currently, *Panax ginseng* is included in some of the products currently prescribed in China for treating diabetes and the same is true in India for the use of products containing *Momordica charantia*.

Implicit in this study, HFD-fed mice which are already mildly hyperglycemic, become more susceptible to develop significant hyperglycemia and hyperlipidemia with the diabetogenic effect of STZ (Erasto *et al*., 2005; Philip Wenzel *et al*., 2008), which are similar to human Type 2 diabetes. Increased triglycerides and total cholesterol are known markers of hyperlipidemia in HFD/STZ-induced diabetic mice. Hederagenin significantly reduced bodyweight and attenuated plasma glucose, triglycerides and total cholesterol better than pioglitazone suggesting a salutary effect on cardiovascular risks.

The rate of glycation is proportional to the blood glucose concentration glycated haemoglobin (HbA1C) was found to increase in the patients with uncontrolled or poorly controlled diabetes mellitus and amount of increase is directly proportional to the hyperglycemic state. Evidence showed that glycation itself may induce the formation of oxygen-derived free radicals in diabetic condition, and the level of HbA1C is considered as one of the markers of degree of oxidative stress in diabetes mellitus. Therefore, the measurement of HbA1C is supposed to be a very sensitive index for glycemic control. Hederagenin also reduced the levels of glycated haemoglobin indicating long sustained action on the body. (Jagdish Kakadiya *et al*., 2010) Histopathological study of kidney showed that both Hederagenin and pioglitazone mesangial cell number was slightly higher in STZ. The degree of
tubulointerstitial damage was modest. There were only few widened tubuli with incipient atrophy of the epithelial cells. In addition, slight focal interstitial fibrosis was observed. Intrarenal arterial vessel showed modest thickening of the walls. The effects of streptozotocin on glucose homeostasis reflect the toxin induced abnormalities in β-cell function (Tremblay and Doucet, 2000). The therapeutic advantage of Hederagenin was also reflected in the pancreatic histology of mice supplemented with Hederagenin where the damage induced on the islet and β-cells was minimal and preserved the islet architecture of the pancreas.

Hederagenin for a period of 28 days did not alter hematological parameters (RBC, WBC, PLT, hemoglobin, percentage hematocrit, MCV, MCH and MCHC) which were within the normal physiological ranges. There was no detectable hematological toxicity at therapeutic dose.

Similarly the relation between increased insulin resistance activity and oxidative stress deserves the most thorough consideration because, in the last several years, the continuing debate about a “primary mechanism” of diabetic complications has centred on oxidative stress and its relation with other hyperglycemia-initiated factors. Hyperglycemia is a well-known cause for elevated free radical levels, followed by production of reactive oxygen species (ROS), which can lead to increased lipid peroxidation, alter antioxidant defense and further impair glucose metabolism in biological system (Ryosuke et al., 2006).

In the present study the elevated levels of anti oxidant enzymes such as catalase, superoxide dismutase and glutathione and reduction of lipid peroxidise enzyme malondialdehyde of the diabetic animals is indicative of the fact that due to oxidative stress there occurs membrane damage. Chronic treatment with Hederagenin significantly improved the levels of endogenous antioxidant enzymes (GSH, CAT, and SOD) and reverted back the membrane damage by decreasing lipid peroxidation MDA level compared to diabetic control.
Thus, in the present study, Hederagenin displays antioxidant and hypolipidemic activity and protects the liver and kidney, from the diabetes induced injuries in STZ treated mice. Thus, there is a potential for development of plant based drug like Hederagenin as an important standard in the treatment of diabetes and alleviation of its complications.