CHAPTER 3

Effect of Hederagenin on the \textit{in vivo} pharmacological model for Type 2 Diabetes

3.1 Introduction

A number of alternative therapies for Type 2 diabetes are currently under development that takes advantage of the actions of the incretin hormones glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide on the pancreatic $\beta$ cell. One such approach is based on the inhibition of dipeptidyl peptidase IV (DPP IV), a serine aminopeptidase present in soluble form in the circulation and also expressed on a variety of cell types (Murielle Combettes \textit{et al.}, 2006) which is the major enzyme responsible for degrading the incretins \textit{in vivo}. Cleavage of GLP-1 and GIP by DPP IV yields N-terminally truncated forms of these peptides that are inactive or even antagonistic and is largely responsible for the short plasma half-lives of these hormones. In an effort to harness the therapeutic potential of the incretin hormones, DPP IV resistant GLP1 analogs (incretin mimetics) and DPP IV inhibitors (incretin enhancers) have emerged as new classes of anti-hyperglycemic agents for the treatment of patients with Type 2 diabetes.

DPP IV exhibits characteristics that have allowed the development of specific inhibitors with proven efficacy in improving glucose tolerance in animal models of diabetes. While enhancement of insulin secretion, resulting from blockade of incretin degradation, has been proposed to be the major mode of inhibitory action, there is also evidence that inhibition of gastric emptying, reduction in glucagon secretion and important effects on $\beta$ cell differentiation, mitogenesis and survival, by the incretins and other DPP IV sensitive peptides, can potentially preserve $\beta$ cell mass, and improve insulin secretory function and glucose handling in diabetics. (Christopher Mcinstosh \textit{et al.}, 2006)
Studies conducted with several orally administered DPP IV inhibitors have revealed that inhibition of DPP IV increases active (intact) GLP-1 and GIP plasma concentrations and improves glycemic control in both animal models of Type 2 diabetes (Francesco Giorgino et al., 2006) and patients with Type 2 diabetes. In addition, the dose-dependent effects of Hederagenin on plasma DPP IV activity, and oral glucose tolerance were determined in high fat diet/Streptozotocin (HFD/STZ) induced diabetic mice model.

**Objective**

To analyze the activity of Hederagenin in the glucose lowering effect streptozotocin induced murine models of diabetes.

### 3.2 Materials and Methods

#### 3.2.1 Reagent 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).

#### 3.2.2 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 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 standard pellet diet and water.

3.2.3 **Oral Glucose Tolerance Test (OGTT) in lean mice**

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

b. Blood basal samples ($T_0$) were taken.

c. Animals were randomized according to their baseline blood glucose level and grouped into five groups. The animals were also dosed according to the groups divided.

d. Group 1 Normal control - distilled water
   Group 2 Vehicle control - 0.25% w/v (10 ml/kg) carboxymethyl cellulose (CMC)
   Group 3 Metformin (500 mg/kg, p.o.),
   Group 4, 5 and 6 Hederagenin (200, 400 and 600 mg/kg, p.o.) respectively

After dosing the animals as specified, a glucose load of 2g/kg and either vehicle (water) or were simultaneously administered orally.

e. Blood glucose concentrations were measured at 15, 30, 60 and 120 min ($T_{15}$–$T_{120}$) post treatment by tail cut method using glucometer (Accu-Check, Roche, Germany)

f. The reduction in blood glucose produced by the compounds metformin and Hederagenin was calculated using the area under the curve method with basal value as the zero. (AUC$_{0-120\ min}$).

3.2.4 **Effects of Hederagenin on plasma DPP IV enzymatic activity in lean mice**

a. Animals were fasted overnight and divided into three groups (n = 6).

b. Following which they received different dose of Hederagenin (200, 400 and 600 mg/kg, per oral).
c. Blood samples were collected from retro-orbital plexus under anaesthesia at 0, 15, 30, 60, 120 min post treatment.

d. Plasma was separated and DPP IV enzyme activity was analyzed based on the following protocol

- Assay buffer was prepared by weighing 1 g of albumin (as 1%) in a 100ml bottle and initially dissolving it with 50 ml of ultra pure water. Then 1.0 ml of 25mM HEPES, 10ml of 140mM sodium chloride (NaCl) was added and dissolved. Finally, the volume was made to 100 ml after adjusting the pH of the buffer with NaOH (0.1 M). 50µl of assay buffer was added to the well labelled ‘BLANK’ and ‘SAMPLES’

- 10µl of sample was added to ‘SAMPLES’ well and 20 µl of 1:10 MgCl₂ to all the wells.

- 10 µl of substrate (1 mM H-Gly-Pro AMC-HBr) was added and the plate was covered with aluminum foil to prevent exposure to the light, as the substrate is light sensitive and incubated in dark for 20 minutes.

- After incubation for 20 minutes, the reaction was stopped by adding 10µl of 25% glacial acetic acid.

- The plate was read at excitation $\lambda = 360$ nm, emission $\lambda = 460$ nm, in a Spectra max GeminiXS Fluorescence Reader.

3.2.5 Effects of Hederagenin on fasting blood glucose levels in normoglycemic mice

a. The animals were fed with standard pellet diet and given water ad libitum

b. At 7 weeks of age, mice were divided into 5 groups (n = 6 per group) based on body weight and fasted overnight.

c. Each group was administered vehicle alone (0.25% w/v CMC or glibenclamide (10mg/kg, per oral) or highest tested dose of Hederagenin (600mg/kg, p.o.).
d. Blood samples were collected by tail cut method at different time points (T15–T120 min, 15, 30, 60 and 120 min) post treatment

e. Blood glucose was measured by using commercially available glucometer (Accu-Check, Roche, Germany). The results were expressed as area under the curve (AUC0–120 min)

3.3 Results

3.3.1 Oral glucose tolerance test (OGTT) in lean mice

The acute effect of Hederagenin was evaluated by OGTT on overnight fasted animals. Hederagenin significantly (p < 0.05) reduced blood glucose excursion by 35.4 % and 52.0 % at tested doses of 400 mg/kg and 600 mg/kg respectively as shown by (Fig. 3.1A AUC0–120 min). Results suggested that improvement in glucose tolerance. Metformin also (500 mg/kg) significantly (p < 0.05) lowered glucose excursion (AUC0–120 min) by 62.1 % as opposed to 52.0 % for Hederagenin (600 mg/kg) (Fig. 3.1B). However, Hederagenin at 200 mg/kg did not exhibit any significant blood glucose lowering when compared with vehicle control.

3.3.2 Effects of Hederagenin on plasma DPP IV enzymatic activity in lean mice

Plasma DPP IV was inhibited in a dose dependent manner at 15 min post treatment and continued up to 2 hours of study (Table 3.1; Fig. 3.2). The degree of improvement in glycemic control observed in the Hederagenin treated animals was dependent on the extent of plasma DPP IV inhibition.

3.3.3 Effects of Hederagenin on fasting blood glucose levels in normoglycemic mice

The effect of Hederagenin (600 mg/kg) on fasting blood glucose levels in normoglycemic mice was determined and found that Hederagenin did not affect fasting blood glucose level and maintained normoglycemic condition throughout the study (Fig. 3.3 A and B). On the other hand, glibenclamide (10 mg/kg) significantly (p < 0.05) reduced normal blood glucose levels.
3.1 Effect of Hederagenin on Oral Glucose Tolerance Test (OGTT) in lean mice

[A] Blood glucose levels

![Graph of OGTT showing blood glucose levels over time for different groups: Normal, vehicle control, Metformin (500mg/kg), Hederagenin (200mg/kg), Hederagenin (400mg/kg), and Hederagenin (600mg/kg).](image)

[B] Area Under the Curve (AUC 0-120min)

![Bar graph showing AUC for different groups: Normal, vehicle control, Metformin (500mg/kg), Hederagenin (200mg/kg), Hederagenin (400mg/kg), and Hederagenin (600mg/kg).](image)

Results are expressed as mean ± S.E.M. *p < 0.05 as compared to normal control and #p < 0.05 as compared to vehicle control.
Table 3.1 Acute inhibition by Hederagenin on plasma DPP IV in lean mice

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<tr>
<th>Time (mins)</th>
<th>% Inhibition of plasma DPP IV</th>
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<tr>
<td></td>
<td>Hederagenin (200mg/kg)</td>
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Fig 3.2 Acute inhibition by Hederagenin on plasma DPP IV in lean mice.
Fig. 3.3 Effect of Hederagenin on blood glucose levels in normoglycemic mice

[A] Blood glucose levels

Results are expressed as mean ± S.E.M. *p <0.05 as compared to vehicle control.
3.4 Discussion

The present studies investigated the antidiabetic effects of DPP IV inhibitors, in streptozotocin-induced mildly diabetic mice, which exhibit a mild decline in glucose tolerance due to loss of early-phase insulin secretion and have many pathological features resembling Type 2 diabetes. Drugs that inhibit DPP IV enzyme have been demonstrated to decrease hyperglycemia and improve impaired glucose metabolism and promote insulin secretion from pancreatic cells (Murielle Combettes et al., 2006). The results of in vitro studies showed that Hederagenin inhibited the DPP IV enzyme dose-dependently.

Oral glucose tolerance test (OGTT) in lean mouse provides an ideal model for investigating the reduction of glucose excursion in stipulated time period. Further, this model would provide convincing idea whether the particular compound works on diabetic disease or not. Hederagenin proved that at a dose concentration of 400 and 600 mg/kg, the glucose excursion was significantly reduced (AUC$_{0–120\text{ min}}$) in a dose dependent manner, implying DPP IV inhibition. The metformin (500mg/kg) and Hederagenin (600mg/kg) more or less showed similar reduction.

Hederagenin appears to inhibit DPP IV enzyme in both, time and dose-dependent manner, from 30 min post treatment and continued upto 2 hours which was confirmed by estimating DPP IV enzyme in plasma of Hederagenin-treated mice using fluorometric assay. The degree of improvement in glycemic control observed in the Hederagenin treated animals was depend on the extent of plasma DPP IV inhibition. However, Hederagenin, even at the highest dose (unlike glibenclamide) did not reduce fasting blood glucose, suggesting only a corrective role, which is in sharp contrast with glibenclamide. Hypoglycemia is the most frequent adverse drug reaction associated with sulfonylureas and the uniformly euglycemic effect of Hederagenin marks a significant advantage.