CHAPTER 5

Effect of Hederagenin on the Type 2 Diabetes comorbidity
Non alcoholic fatty liver disease (NAFLD)

5.1 Introduction

5.1.1 Diabetes – NAFLD bondage

Type 2 Diabetes mellitus (T2DM) is a non-autoimmune, complex, heterogeneous and polygenic metabolic disease condition in which the body fails to produce enough insulin, characterized by abnormal glucose homeostasis. Its pathogenesis appears to involve complex interactions between genetic and environmental factors. T2DM occurs when impaired insulin effectiveness (insulin resistance) is accompanied by the failure to produce sufficient beta cell insulin. This may lead to death, if left untreated due to acute metabolic decompensation producing dehydration acidosis.

Recent reports suggest Type 2 diabetes mellitus, obesity and dyslipidemia often coexists with NAFLD. In particular, hyperlipidemia and insulin resistance importantly contribute to the initiation and progression of NAFLD. Recent clinical studies also shows that NAFLD is one of the main common liver diseases that lead to the liver cirrhosis and hepatocellular carcinoma. However, the molecular mechanisms responsible for progression of NAFLD have not been fully understood. Further, intestinal cholesterol absorption is elevated in those with Type 2 diabetic patients with coronary heart diseases and low cholesterol absorption associates with fewer recurrent cardiovascular events. Dyslipidemia including hypercholesterolemia is a major risk factor for coronary heart disease. The level of cholesterol is tightly regulated by endogenous synthesis in the liver and dietary absorption /biliary reabsorption in the small intestine. (Federico, 2008)
5.1.2 NAFLD links NASH

Non alcoholic steato hepatitis (NASH), a progressive metabolic liver disease, is one of the major consequences of the current obesity epidemic. It lies on a spectrum of non alcoholic fatty liver diseases that range from simple steatosis to cirrhosis whereas simple steatosis seems to be a benign and non progressive condition, NASH is recognized as a potentially progressive disease that is characterized by varying degrees of progressive steatosis, lobular inflammation and fibrosis of the liver may cause cirrhosis, an end-stage liver disease, and hepatocellular carcinoma (HCC). The pathogenesis of NASH is known to be a multifactorial process. The current concept in the pathogenesis of NASH involves a “two-hit” theory in which an initial metabolic disturbance, such as insulin resistance, causes steatosis, and a second pathogenic stimulus causes oxidative stress and reactive oxygen species, leading to steatohepatitis. It has been reported that hepatic thiobarbital acid reactive substances (TBARSs) and 8-hydroxy-deoxyguanosine (8-OHdG), as reliable markers of lipid peroxidation and oxidative DNA-damage, respectively, were significantly increased both in the animal models and human samples of NASH. However, it is not known whether reactive oxygen species can result in progression of liver fibrosis and hepatocarcinogenesis, because, like diabetes mellitus, NASH is almost certainly a polygenic disease affected by several factors, with disease pathogenesis related to multiple hits (Bradford and Gerald, 2000).

5.1.3 Prevalence

In United States, NASH is considered to be the 3rd most common liver disease after hepatitis C and alcoholic fatty liver. Global prevalence on NASH is 10-24% amongst general populace but increases to 25–75% in obese diabetic individuals.

Animal models of hepatic steatosis and steato hepatitis have improved the understanding of the pathogenesis of NASH. The different animal models of NASH have been extensively reviewed. Non alcoholic fatty liver disease (NAFLD) ranges in severity from steatosis to steatohepatitis to the above plus fibrosis leading to cirrhosis. Recent observational studies indicate a prevalence as high as 25% in the
United States (Farrell and Larter, 2006; Browning and Horton, 2004), where NAFLD may be a leading cause of cryptogenic cirrhosis. Although most individuals with NAFLD seem to either remain stable or improve over time, little is known about the progression of this disorder to steato hepatitis and cirrhosis.

Much of the increased prevalence of NAFLD is driven by obesity. However, high rates of NAFLD in relatively normal weight people (by Western standards) from the Indian subcontinent and Southeast Asia suggest that, even in the absence of obesity, insulin resistance leads to hepatic fat accumulation. Indeed, patients with total lipodystrophy, who have no adipose tissue, have severe insulin resistance with marked hepatic steatosis. Studies of the molecular basis of NAFLD have largely focused on triglyceride (TG), the major lipid stored in hepatocytes. Although much is known about the regulation of hepatic TG synthesis, secretion, and storage, much less is known about the role of TG and/or its precursors in stimulating the inflammatory changes needed for the progression of steatosis to steato hepatitis (Petta, 2009).

5.1.4 Mechanism of action

Mitochondrial free cholesterol is considered as a central molecule in the pathogenesis of steatohepatitis (Mari et al., 2006). Hepatic TG levels are determined by the availability of fatty acids (FA) from the circulation, \textit{de novo} lipogenesis of FA from glucose, oxidation of FA, and the secretion of TG on very low-density lipoproteins (VLDL) (Goldberg and Ginsberg, 2006). Each of these processes may be altered by insulin resistance in ways that predispose to steatosis. Thus, insulin resistance leads to increased lipolysis of adipocyte TG and more FA flux to the liver (Yu and Ginsberg, 2005). Insulin resistance may be associated with reduced lipoprotein lipase- mediated lipolysis of plasma chylomicron or VLDL TG, leading to hepatic uptake of remnant lipoproteins carrying more TG than normal. \textit{De novo} lipogenesis is increased in insulin resistance; insulin-mediated stimulation of SREBP-1c is a key contributor, although glucose mediated stimulation of ChREBP can also play a significant role (Browning and Horton, 2004). Aberrant expression of PPARg2 in insulin resistance livers can also stimulate \textit{de novo} lipogenesis.
Oxidation of hepatic FA is regulated at several points, but is likely to be limited in the face of adequate hepatic glycogen and increased lipogenesis with elevated levels of malonyl-CoA (Fig. 5.1).

Finally, insulin can target apoB for posttranslational degradation; the balance between systemic hyperinsulinemia and hepatic insulin resistance will determine how much apoB will be available to carry TG out of the hepatocyte. It has been shown that increased hepatic TG stimulates increased VLDL TG secretion by targeting apoB away from degradation and toward secretion and/or by increasing the amount of TG on each VLDL (Goldberg and Ginsberg, 2006). However, recent studies suggest greater complexity. For example, stimulation of hepatic lipogenesis by an LXR agonist results in increased TG secretion but has no effect on apoB secretion. In vivo over expression of mtGPAT, an enzyme that synthesizes diglycerides, increases both hepatic TG content and secretion (Linden et al., 2006), but over expression of either DGAT1 or DGAT2, enzymes that synthesize TG and increase hepatic TG, has had inconsistent effects on TG secretion.

On the other hand, there are demonstrations that increased delivery of FA to the liver can increase apoB secretion without increasing TG secretion. Together, these results indicate compartmentalization of hepatic TG into pools with tight or loose connections to TG secretion or differential effects of FA and TG on apoB and TG secretion. To further complicate matters, levels of lipid droplet proteins such as ADRP and perilipin, activities of hepatic lipases such as TGH, ATGL, and HSL, and the activity of MTP (which transfers endoplasmic reticulum TG and cholesterol onto apoB) may all confound the relationship between hepatic TG accumulation and the assembly and secretion of VLDL (Linden et al., 2006).
Fig. 5.1 Mechanism of progression from lipid homeostasis to steatohepatitis

A Forces Regulating Hepatic Lipid Homeostasis

B Dysregulation of Hepatic Lipid Homeostasis Leading to Steatosis

C Dysregulation of Hepatic Lipid Homeostasis Leading to Steatohepatitis
5.1.5 How steatosis progresses to steatohepatitis

This is under intense investigation. Inflammation, together with evidence of apoptosis and necrosis of hepatocytes, differentiates steatohepatitis from steatosis. Most investigators accept a “TWO HIT” hypothesis; steatosis appears to be the required background abnormality upon which inflammation, cellular dysfunction, and cell death can occur. Increased FA oxidation and ROS formation could lead to a state of oxidative stress, with sequelae that include lipid peroxidation, membrane damage, and mitochondrial dysfunction, the latter leading to more ROS formation. However, the evidence that there is increased FA oxidation in steatotic livers is limited. There are evidences supporting an alternative lipid-based mechanism for the progression of steatosis to steatohepatitis (Figure 5.1C) (Mari et al., 2006) present.

If antioxidant therapy is helpful in relieving symptoms and complications in a diabetic patient based on the present evidences, the physician will consider this aspect first to relieve and improve the patient. Presently, therapeutic strategies mostly try to relieve the clinical manifestation of diabetes and its complications. The major challenge in diabetes research is to define not only the cause–effect relationship between various risk factors and complications, but also to comprehend the effects of pharmaceutical agents that are beneficial in the management of diabetic complications (Ashok Tiwari and madhusudhana, 2002). Although exercise and diet improvement may reduce the overall magnanimity of this disease, development of novel dietary supplements or drugs that successfully prevent the onset of NASH is desired (Okamoto et al., 2002).

Recently, consumption of natural antioxidants and hepatoprotective plant products has gained popularity in last few decades mainly due to their cost effectiveness and minimal side effects. Recent studies have also shown effectiveness of herbal drugs with multifactoral therapeutic potential as a possible therapy against NASH (Haddad et al., 2009). With this fact, the following research work has been planned in NASH.
Objective

To analyze the effect of Hederagenin, in high-fat diet inducing non alcoholic fatty liver disease (NAFLD) murine model and also to test the antioxidant property in liver.

5.2 Materials and methods

5.2.1 Reagents and instruments required

The chemical and drugs used in the study were Hederagenin, Glucose, Hep G2 cell lines, trypsin, EDTA, carboxymethyl cellulose (CMC), metformin, isoflurane (anaesthetic agent) catalase, superoxide dismutase (SOD) hydrogen peroxide, formaldehyde eosin, hematoxylin was purchased from Sigma–Aldrich, St. Louis, MO, USA. TNF-α ELISA kit (Ray Biotech Inc., Norcross, 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, sodium chloride solution, Tris hydrochloride, thio barbituric acid.

5.2.2 Experimental animals

Male C57BL/6 mice (6–8 weeks of age) were purchased from Biogen Laboratory animal facility, Bangalore, India. They were housed and maintained in clean polypropylene cages and fed with either low fat diet or high fat diet and water ad libitum. This study was performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the animal ethical committee (Approval No. 971/BC/06/CPCSEA). 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 diet (as mentioned in 5.2.3) and water.
5.2.3 Composition of High fat diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard diet</td>
<td>87.7</td>
</tr>
<tr>
<td>Pork fat</td>
<td>10</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>2</td>
</tr>
<tr>
<td>Bile salts</td>
<td>0.3</td>
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</tbody>
</table>

5.2.4 Experimental Design - *In vivo* model of NAFLD

Male C57BL/6 mice were randomly divided into six groups; 6 mice each.

- Group 1 - Normal group and was maintained on normal mouse chow diet throughout the experiment (sixteen weeks).
- Group 2 - NAFLD group received distilled water (1 ml/kg/day, p.o.),
- Group 3 received Hederagenin (100 mg/kg/day, p.o.),
- Group 4 received Hederagenin (300 mg/kg/day, p.o.),
- Group 5 received Hederagenin (500 mg/kg/day, p.o.),
- Group 6 received Metformin (350mg/kg/day, p.o.).

The groups 2 to 6 were maintained on a HFD containing 87.7% standard diet (w/w), 10% pork fat (w/w), 2% cholesterol (w/w) and 0.3% bile salts (w/w) (Pan *et al*., 2006) for eight weeks. For an additional eight weeks, HFD was given in addition to the following treatment regimens.

At the end of the experimental period, the final body weight of overnight fasted each animal was recorded and were given mild Isofuran anesthesia and whole blood was collected by retro orbital sinus puncture in EDTA coated vials. Plasma was obtained by cold centrifugation (4°C) of the vials for 10 min at 3000 rpm. Later animals were sacrificed by cervical dislocation and, liver, heart, pancreas, kidney and epididymal
fat pad were excised and stored at -80°C (Cryo Scientific Ltd., India) for further evaluations.

5.2.5 Measurement of Plasma biochemical parameters

After the last drug treatment, animals were fasted overnight. Blood samples were collected by retro orbital method and blood samples were centrifuged for 5 min at 4000 rpm. The collected plasma were estimated for fasting blood glucose and insulin (ELIZA), alanine transaminase (ALT) and aspartic transaminase (AST). Serum total cholesterol and triglycerides were determined using (Flex reagent) by performing in Dimension Xpand Plus, Siemens India.

5.2.6 Estimation of Insulin

Description

The major function of insulin is to counter the concerted action of a number of hyperglycemia-generating hormones and to maintain low blood glucose levels. Because there are numerous hyperglycemic hormones, untreated disorders associated with insulin generally lead to severe hyperglycemia and shortened life span. Insulin is synthesized as a preprohormone in the β cells of the islets of langerhans. Its signal peptide is removed in the cisternae of the endoplasmic reticulum and it is packaged into secretory vesicles in the Golgi, folded to its native structure, and locked in this conformation by the formation of 2 disulfide bonds. Specific protease activity cleaves the center third of the molecule, which dissociates as C peptide, leaving the amino terminal B peptide disulfide bonded to the carboxy terminal A peptide. With respect to hepatic glucose homeostasis, the effects of insulin receptor activation are specific phosphorylation events that lead to an increase in the storage of glucose with a concomitant decrease in hepatic glucose release to the circulation. The assay was performed as ultra sensitive mouse insulin kit (catalog 90080)
Materials required

Antibody coated microplate, Microplate frame, Sample diluents, Mouse insulin standard, Wash buffer, Anti-insulin enzyme conjugate, Enzyme substrate, Stop solutions, Microplate reader, plasma sample.

Procedure

- The antibody-coated microplate was affixed to the frame and dispense 95µl of sample diluent per well.
- 5µl of the sample (or working mouse insulin standard) was pipetted per well.
- The microplate was incubated for 2 hours at 4°C
- Each well was washed five times with wash buffer and dispense 100µl of anti-insulin enzyme conjugate per well
- The microplate was incubated for 30 minutes at room temperature.
- Each well was washed seven times with wash buffer and dispense 100µl of enzyme substrate solution per well
- Microplate was incubated at room temperature while avoiding exposure to light ↓ ↓
  40 minutes – 10 minutes –
  low/wide range assay high range assay

- The enzyme reaction was stopped by adding 100µl of enzyme reaction stop solution per well
- A450 was measured and subtract A630 values within 30 minute.
- The insulin concentrations were calculated using the standard curve.
- Each well should be washed with 300µl of wash buffer. Aspirate the wells completely
5.2.7 HOMA-IR

Insulin resistance was determined using the homeostasis model assessment index for insulin resistance (HOMA-IR) (Mathews et al., 1985) using the following formula:

\[
\text{HOMA-IR index} = \frac{\text{fasting glucose (mmol/L)} \times \text{fasting insulin (\(\mu\text{U/ml}\))}}{22.5}
\]

5.2.8 Liver Processing

- Prior to dissection, the animals were perfused with a phosphate buffered saline (PBS) solution in order to rinse the tissue and to remove any red blood cells and clots.

- The liver was rapidly dissected and washed free of blood with ice-cold 0.9% NaCl solution.

- The liver was weighed and the liver index was calculated by the formula

\[
\text{Liver index} = \frac{\text{liver weight}}{\text{body weight}} \times 100
\]

- One part of the liver (0.3 g) was then blotted and finally kept at -80 °C.

- Then the liver tissues were homogenized either in phosphate buffer pH 7.4 (supernatant A) for determination of reduced glutathione or in Tris hydrochloride buffer pH 7.4 (supernatant B) for malondialdehyde assay, and then centrifuged at 3000×g for 15 min at 4 °C.

- Liver samples were taken 5 mm away from the edge of the largest hepatic lobe, fixed with 10% (v/v) formaldehyde, embedded in paraffin wax, stained with hematoxylin and eosin (H&E) and Masson's trichrome stain.
5.2.9 Hepatic triglycerides

Introduction

Triglycerides (TG) are water-insoluble lipids consisting of three fatty acids esterified to a glycerol backbone. TG is transported as core constituents of all lipoproteins, but is major components of TG rich chylomicrons and very low-density lipoproteins (VLDL). A major source of TG is dietary fat. Dietary fats are hydrolyzed in the gut into free fatty acids and mono- and diglycerides then transported through the intestinal villi. After absorption through the gut, they are resynthesized into new triglycerides and assembled into chylomicrons. TG is rapidly hydrolysed in the capillary beds by lipoprotein lipase, releasing glycerol and free fatty acids, which are absorbed by adipose tissue for storage. When required, lipases hydrolyze triglycerides from adipose tissue into fatty acids and glycerol, which enter the blood stream. Fatty acids are oxidised in the mitochondria and peroxisomes to produce energy. TG play an important role in metabolism by containing more than twice as much energy. The measurement of TG levels in conjunction with other lipid assays, are useful in the diagnosis of primary and secondary stages of diabetes mellitus associated hyperlipidemia, liver obstructions, lipid metabolism and other metabolic disorders.

BioAssay Systems' triglyceride assay kit uses a single working reagent that combines TG hydrolysis and glycerol determination in one step, in which a dye reagent is oxidized to form a colored product. The color intensity at 570nm is directly proportional to TG concentration in the sample

Sample preparation

- The liver was rapidly removed and hepatic triglycerides were measured from these tissues (50 mg) according to methods modified from (Folch et al., 1957).

- Briefly, snap frozen liver kept at −80 °C was homogenized and extracted with chloroform/methanol (2:1 v/v) solution.
• The lipid extraction was considered complete when the minced liver tissue settled on the bottom of the vial after vortexing.
• The lower organic phase was separated in a separate tube and dried using nitrogen evaporator. This can also be stored at -20°C until analysis.
• At the time of immediate analysis, the dried sample was resolubilized in 2-propanol containing 10% Triton X-100.
• Hepatic triglyceride levels were determined by Bioassay enzymatic kits

Materials required - Hepatic triglyceride kit assay

• Assay Buffer: 24 ml
• ATP: 250 μl
• Dye Reagent: 220 μl
• Enzyme Mix: 500 μl
• Lipase: 1000 μl
• Working reagent preparation: Working reagent was prepared for each well, by mixing 100 μl assay buffer, 2 μl enzyme mix, 5 μl Lipase, 1 μl ATP and 1 μl dye reagent in a clean tube
• Standard: 100 μl (equivalent to 100 mmol/L Triglyceride) Standard was diluted in distilled water and transferred

Note: All components will be equilibrated to room temperature. Keep thawed Lipase and Enzyme mix in a refrigerator or on ice.

Procedure:

• 10 μl diluted standards into wells of a clear 96-well plate. Note: Diluted standards can be used for future assays when stored at 4°C.
10 μl of samples are transferred into separate wells of the 96-well plate.

100 μl of working Reagent into standards and sample wells.

The plate was tapped to mix and incubated for 30 min at room temperature.

The plate was read at optical density of 570nm (550-585nm).

*Note: If the Sample OD is higher than the Standard OD at 1.0 mmole/L triglyceride, dilute sample in water and repeat the assay. Multiply by the dilution factor n.*

**Calculation**

OD Blank H<sub>2</sub>O (water, #4) was subtracted from the standard OD values and plot the OD against standard concentrations. The slope was determined using linear regression fitting. The triglyceride concentration of sample is calculated as

\[
[\text{Triglyceride}] = \frac{\text{OD}_{\text{SAMPLE}} - \text{OD}_{\text{H2O}}}{\text{Slope}} \times n (\text{mmol/L})
\]

OD<sub>SAMPLE</sub> and OD<sub>H2O</sub> are optical density values of the sample and the water blank (# 4), n is the dilution factor. For example if samples are diluted 5-fold prior to assay, n = 5.

**Conversions**

1 mmol/L triglyceride equals 88.5 mg/dL or 10 ppm.
5.2.10 Estimation of Tumour Necrosis Factor Alpha TNF-α mouse, Biotrak ELISA system (RPN2718)

Description

This assay employs a quantitative *in vitro* enzyme linked immunosorbent technique. An antibody specific for mouse TNFα has been coated on to the microplate provided in the kit. Samples are pipetted into the wells along with biotinylated antibody reagent. If present, the mouse TNFα is bound by both the immobilized and the biotinylated antibody. After washing away any unbound sample proteins and biotinylated antibody, a streptavidin HRP conjugate is added to the 8 wells. Any mouse TNFα which was bound by both the immobilized and the biotinylated antibody during the first incubation will be bound by the streptavidin conjugate. Following a wash to remove any unbound conjugate, a substrate solution is added to the wells and color develops in proportion to the amount of mouse TNFα bound in the initial step.

Materials Provided

Anti-mouse TNF-α precoated 96 well strip plate, Lyophilized recombinant mouse TNF-α standards, 2 vials, Biotinylated antibody reagent, 1 vial, 8 ml, containing 0.1% sodium azide. Standard diluent 1 vial, 20 ml, containing 0.1% sodium azide, wash buffer, 1 vial, 50 ml Streptavidin-HRP concentrate, 1 vial, 12-13 ml, stop solution, 1 vial, 13-15 ml, <1% sulfuric acid and adhesive plate covers.

Sample Dilution

Collected serum was diluted with standard diluent (1:5).

Wash Buffer

The entire contents of the 30 X wash buffer bottle (PBS, 50 ml) were diluted to a final volume of 1.5 liters with ultrapure water.
Preparation of Standard Curve

The standard vial provided with the kit were reconstituted with 1250μl of ultrapure water to get the initial concentration of 2450 pg/ml (m) TNF-α is the first point of the standard curve. Two tubes were labelled, one tube for each of the additional dilutions: 350 pg/ml and 50 pg/ml. 600μl of appropriate diluents was pipette into each tube. 100μl of reconstituted initial concentration of 2450 pg/ml (m) was transferred into the first tube labeled 350 pg/ml and 100μl of this dilution into the second tube labeled as 50 pg/ml. These concentrations, 2450pg/ml, 350pg/ml and 50 pg/ml provided were used for the standard curve points.

Procedure

- Diluted samples and standards (50μl) were added to each wells
- Biotinylated antibody reagent (50μl) was added to all wells, in duplicate.
- Plates were incubated for 2 hours at room temperature.
- Plates were washed for 5 times using the wash buffer
- Streptavidin-HRP conjugate (100μl) were added to each well and incubated for 30 minutes at room temperature.
- The aspiration/wash step was repeated as in step d.
- TMB substrate solution (100μl) were added to each well and incubated for 30 minutes at room temperature in the dark.
- Stop solution (100μl) were added to each well.
- Optical density of each well was noted using a spectrophotometer set to 450nm.

5.2.11 Hepatic Malondialdehyde assay (MDA)

Hepatic malondialdehyde (MDA) was determined by the reaction with thiobarbituric acid according to the method of 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 sample. 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 mmol/g tissue (Suresh Kumar et al., 2012)

5.2.12 Hepatic reduced glutathione (GSH)

Hepatic reduced glutathione (GSH) was determined using the spectrophotometric method of Ellman (1959). 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-nitrobenzoic 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/g tissue

5.2.13 Maintenance of HepG2 cells

Human hepatocellular carcinoma cells (HepG2) (obtained from National Centre for Cell Sciences, Pune, India) were seeded (1 × 10⁵ cells/25 mm T flask) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Himedia Pvt. Ltd., Mumbai, India) containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (10 X ; Himedia Pvt. Ltd., Mumbai, India) at 37°C with 5% CO₂ (Thermo scientific, forma II water jacketed CO₂ incubator). Cells were subsequently passage
every third day by trypsinization with 0.25% Trypsin-EDTA (Himedia Pvt. Ltd., Mumbai, India) solution.

### 5.2.14 Steatosis calorimetric assay

Steatosis, also known as fatty liver, is a pathological process characterised by abnormal accumulation of lipid within cells. There are two distinct patterns of steatosis: microvesicular and macrovesicular. The former is frequently seen in alcohol induced liver injury; as a complication of metabolic syndrome such as obesity and Type 2 diabetes and is a marker of the hepatotoxic side effect of any drug. Microvesicular steatosis is most commonly related to mitochondrial dysfunction and defects in β-oxidation responsible for fatty liver. Adverse from of steatosis will lead to significant impairment of liver function, extensive fat accumulation resulting in cirrhosis and liver failure. In the process of determining the hepatotoxicity of a drug candidate, steatosis play a major role and Hep G2 cell lines are used for mechanism based testing of hepatotoxic effects. Lipid accumulation was quantified using microplate reader after staining the plate with oil red O.

**Plate configuration**

A 96 well plate was used, in which the wells are configured as Blank (wells without cells), Normal control (wells with normal cells), positive control (wells with chloroquine induction), Vehicle control (wells with vehicle), experimental drug (wells with different concentrations of drug)

**Materials required**

- **Fixative (10X) – 10% formalin** - The working solution was prepared by diluting the stock solution to 1:10 in PBS.

- **Oil red O solution** – 3mg/ml of stock was prepared in isopropanol solution. Then the working solution was prepared by diluting the stock in 60% in water (i.e in the ratio of 6:4 –stock:water) and filtered through 0.25-0.45µm syringe filter before use.
• Ice cold PBS
• 60% isopropanol
• Free Fatty Acid free BSA (1%): 10 mg of BSA in water (heat the solution at 55°C).
• Linoleic acid and Arachdonic acid (40 mM stock) in absolute ethanol.
• Potassium hydroxide (KOH) (1M)

Preparation of FFA-BSA complex

FFA-BSA complex was prepared by mixing the various concentrations of FFA with BSA. For instance 5 mM FFA/10% BSA stock solution was prepared by adding 50 µl of 100 mM FFA solution to 950 µl of 10% BSA solution at 55°C and then vortex the contents for 10 seconds.

Preparation/Dilution of 5 × 10³ cells

• The cells are trypsinised and centrifuged for 5 minutes at 4°C.
• Then resuspend and discard the media to remove or nullify the effect of Trypsin.
• 30 ml of fresh complete DMEM media was added to resuspend the cells.
• From this 10µl was taken for counting the cells using Neubaur chamber.

The cells were counted as 56 in number then the total number of cells is,

Number of cells (n) = 56*10⁴ cells /ml.

Thus 1 ml (1000µl) contains = 56*10⁴ cells /ml.

Then 1µl contains = 56*10⁴/10³ which is equal to 56*10.

Then 5000 cells will be present in = 5000/56*10 i.e 8.9µl whose volume is made up to 100µls with media which gives about 5000 cells/100µls/well.
Treatment of cells

A 96 well plate was seeded with $5 \times 10^3$ cells/well and was allowed to grow overnight. On day 2 the cells were treated with FFA-BSA complex (2mM), after 6 hours of induction the cells were treated with different concentrations of Hederagenin (20-200 µM) except in normal, vehicle control wells.

5.2.14 A Qualitative analysis of in vitro NASH - Staining of cells with oil red O

- The spent media was removed and washed with 3 times in PBS.
- 50 µl of 10% Formalin was added to each well for 10 minutes.
- Then the formalin was removed completely and fresh 10% formalin (50 µl) was added to fix the cells for 1 hr.
- The formalin was removed and the wells were washed with 50 µl of 60% Isopropanol and the wells were allowed to dry completely.
- 25 µl of Oil red O working stock solution was added and incubated for 1 hr. (should be added into the middle of the wells) Note: Add Oil red O working stock to the empty wells also.
- The wells are then washed with Milli Q water to remove the excess stain (4 times)
- Wells were dried, mounted in glycerine and examined under an inverted microscope (Lin et al., 2007) (Leica DMIL) and photographs were taken by canon Power Shot S 70 digital camera.

5.2.14 B Quantitative analysis of in vitro NASH

- HepG2 cells were maintained as described above and fixed in 10% formalin.
• After washing and drying completely, 100 µl of isopropenol (100%) was added to each well, incubated for 10 min and then transferred to another 96-well plate.

• The plate was read at 405 nm (Chi Li Lin et al., 2007) using ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc., Winooski, VT).

5.2.15 Cell viability assay – MTT assay

Principle

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann in 1983, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals which are solubilised. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a simple colorimetric assay. The results can be read on a multiwell scanning spectrophotometer (ELISA reader) (Menaka Thounaojam et al., 2012).

Material required

• MTT Reagent - A solution of 5 mg/ml MTT was prepared by dissolving in PBS. Then the solution was filter sterilized. Note: Always use freshly prepared reagent or use within two weeks time from the date of preparation. If frozen at –20 degree, it can be stored for 6 months.

• Hep G2 cells – 5 X10³ cells /well

• Phosphate buffered saline (PBS),

• Hemocytometer,

• 0.4% trypan blue

• Di methyl sulphoxide (DMSO)
Cell viability – Qualitative assay protocol

- The cell suspension was prepared at a high concentration (10^6 cells/ml)

- A hemocytometer slide was cleaned with 70% ethanol, was fixed with the coverslip in place by pressing it down over the grooves and semi-silvered counting area. *Note: Take care not to scratch the semi-silvered surface.*

- One drop of cell suspension was mixed with one drop of trypan blue and 20µl was pipette into the tip of a micropipette.

- Then the suspension was immediately transferred to the edge of the coverslip, and allowed to run into the edges of the grooves in counting chamber and left for 1-2minutes. *Note: Do not leave longer.*

- The slide was placed on a microscope under 10X objective and focus on grid lines in chamber.

- The slide was moved so that the largest area is seen bounded by three parallel lines (1mm²)

- The cells lying on the top and left-hand lines of each square are counted within this area and not those on the bottom or right-hand lines.

- Hundreds of cells per 1mm² area are indicated as good cell viability.

- If there are less than 100 cells, count one or more additional squares (each surrounded by three parallel lines) surrounding the central square

- The cell viability percentage was calculated by counting the number of stained cells and the total number of cells X 100. *Note: Dye exclusion viability tends to overestimate viability*

- Most viability tests rely on a breakdown in membrane integrity determined by the uptake of a dye to which the cell is normally impermeable (e.g., trypan blue)
Cell viability – Quantitative analysis - MTT assay

- For cell viability quantitative MTT assay, HepG2 cells culture (checked after qualitative analysis) were plated as $2 \times 10^3$ cells/well in 96-well plates as described earlier for 24 hours.

- Then a sub confluent monolayer culture was trypsinized and cells are collected in growth medium containing serum.

- Cell suspension was centrifuged at 1000 rpm for 5 min to pellet cells, and resuspend in growth medium and to count the cells.

- The cells were diluted at a concentration of $50 \times 10^3$ cells/ml.

- The cell suspension was transferred to a 9 cm petridish, and, with a multichannel pipette 100 µl of cell suspension was added to each well except for the blank wells giving 5000 cells/well

- The plate was incubated at 37°C in a humidified atmosphere for 24 hours.

- At the end of 24 hours, 100 µl of medium was added to blank wells (contains only 200 µl of medium without drug) and control wells (contains 100µl of cell suspension and 100µl of medium without drug) and 100µl of different concentrations (0.2µM, 2µM, 20µM, 200µM) of Hederagenin was added and diluted with medium to the desired test wells in duplicates.

- Then the plate was incubated for 48 hours

- 20µl of MTT was added to all the wells except for the blank wells and incubated for 4 hours at 37°C in a humidified atmosphere.

- Then the medium was aspirated and 150 µl of DMSO was added and kept it for shaking to dissolve the crystals.

- The Purple colour developed was read at 570 nm in an ELISA reader.
5.2.16 Cytotoxicity assay using LDH activity

Cytotoxicity was measured as the fraction of lactate dehydrogenase (LDH) released into the medium. HepG2 cells were maintained in 96-well plates for 24 h as described above. After the collection of supernatants, cells were washed with phosphate buffered saline (PBS) and lysed with 1% Triton X 100 in PBS. Cell lysates were collected, vortexed for 15 s and centrifuged at 7000 rpm for 5 min. LDH activity in supernatant and cell lysate was measured by a commercially available kit (Reckon Diagnostics Ltd., Baroda, India).

Principle

Lactate dehydrogenase is a cytoplasmic enzyme distributed very widely in the body. It is found in organs like heart, liver, kidney and skeletal muscle. Elevated LDH levels in serum are observed in several hemolytic, neoplastic, cardiac, skeletal muscle and renal diseases. These may also be found in destructive renal disease, progressive muscular dystrophy, megaloblastic anaemia, liver cirrhosis, hepatitis, hepatic metastasis, hepatoma and pulmonary embolism. Lactate to pyruvate in the presence of lactate dehydrogenase is a reversible reaction. However the L P reaction is favoured at a pH of 7.4 and this reaction utilises NAD as a coenzyme which is more stable compared to NADH. (Menaka Thounaojoma et al., 2012)

Reaction

LDH catalyzes the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. LDH activity in serum is proportional to the increase in absorbance due to the reduction of NAD.

\[
\text{LDH} \\
\text{Lactate} + \text{NAD} \rightarrow \text{Pyruvate} + \text{NADH}
\]

Materials required

LDH coenzyme, LDH Buffered substrate, Reconstitution vial

*Note: All reagents are stored at 2-8°C*
Procedure

- 5µl of sample was added to specific wells and 100µl of working reagent was added to all wells. Two wells were left only with reagent which is noted as “BLANK”.

- The mixture was mixed well and the absorbance was read exactly after one min and thereafter 30, 60 and 90 seconds at 340nm.

- The mean change absorbance per minute was calculated as

\[
LDH \text{ activity} = \Delta A/\text{min} \times F
\]

\[
= \frac{1}{6.22} \times \frac{T.V.}{S.V.} \times 1000 = 3376
\]

\[
\text{T.V.} = \text{Total Volume} = 0.105\text{ml}
\]

\[
\text{S.V.} = \text{Sample Volume} = 0.005 \text{ml}
\]

\[
6.22 = \text{Millimolar Extinction Coefficient of NADH at 340 nm.}
\]

5.2.17 Protein estimation – Hepatic tissue homogenate

Hepatic tissue was homogenized with ice-cold lysis buffer (pH 7.4) containing 137 mmol/l of Sodium chloride (NaCl), 20mmol/l of Tris–Hydrochloride, 1% Tween 20, 10% glycerol, 1mmol/l of phenyl methyl sulfonyl fluoride (PMSF), and a protease inhibitor mixture in DMSO solution. The tissue homogenates was then centrifuged at 2,000g for 10 minutes at 4°C. The protein concentrations of each fraction were determined by Pierce BCA (Bicinchoninic Acid) Protein Assay Reagent Kit (cat.23225)

Principle

The BCA Protein Assay combines the well-known reduction of Cu$^{2+}$ to Cu$^{1+}$ by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu$^{1+}$) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a light blue
complex. This reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a coloured chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate.

In the second step, colour development reaction - bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-coloured reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The BCA reagent is approximately 100 times more sensitive (lower limit of detection) than the pale blue color of the first reaction.

**Preparation of the BCA Working Reagent (WR)**

- Working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B, Reagent A: B (50:1)).
- Standard working range = (20- 2,000μg/ml) and unknown samples in duplicates were added to a 96 plate well.
- 200 μl of the working reagent were added and incubate at 37°C for 30 minutes.
- The plate was read at 562nm using ELIZA plate reader.

**5.2.18 A Isolation of Protein - SDS-PAGE**

Appropriate volumes of chemicals were added in the same order mentioned and along with (50μg) protein of interest and rainbow marker were loaded on SDS-PAGE.
<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Separating gel (10ml)</th>
<th>Stack Gel (5ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide concentration</td>
<td>10%</td>
<td>4%</td>
</tr>
<tr>
<td>MW Range (kDal):</td>
<td>16 - 70</td>
<td>-</td>
</tr>
<tr>
<td>40% Acrylamide</td>
<td>2.5 ml</td>
<td>500 µl</td>
</tr>
<tr>
<td>Bis-acrylamide</td>
<td>1.33 ml</td>
<td>225 µl</td>
</tr>
<tr>
<td>1.5M Tris pH 8.8</td>
<td>2.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>1M Tris pH 6.8</td>
<td>-</td>
<td>1.26 ml</td>
</tr>
<tr>
<td>H2O</td>
<td>3.47 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>100 µl</td>
<td>35 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
<td>4 µl</td>
</tr>
</tbody>
</table>

After electrophoresis, the proteins were electro transferred to nitrocellulose membranes.

**Transfer**

SDS gel from electrophoresis apparatus was rinsed in transfer buffer (0.192M glycine, 25mM Tris base, 20% methanol). Using Whatman filter (3mm) gel was placed on a sheet of paper pre-wetted in transfer buffer. A layer of Nitrocellulose membrane (first wetted in methanol followed by transfer buffer) was applied to the gel and bubbles were removed by smoothing with gloved fingers, being careful to keep the membrane wet. The membrane was covered with another layer of pre-wetted Whitman filter paper (3mm) and placed in a blotting sandwich assembly. The assembly was immersed in a blot cell filled with transfer buffer, making certain that the membrane side faced the positive electrode and the gel faced the negative electrode. The power was applied at a constant current of 350 mA and allowed two hours for the transfer.

**Blocking the membrane**

The blot was removed from transfer apparatus and placed immediately into blocking solution with 5% BSA to prevent non-specific background binding of the primary
antibody or secondary antibody to the membrane and incubated at room temperature for one hour with agitation.

**Incubation with primary antibody**

The Anti-mouse antibodies are

- **acetyl-CoA carboxylase (ACC)** (Santa Cruz Biotechnology, Inc., CA, USA; Cat. No. 3662), phospho-ACC at Ser 79 (pACC; Cell Signaling Technology, MA, USA),
- **AMPKα** (Cell Signaling Technology; Cat. No. 2352),
- **phospho-AMPKα at Thr 172** (pAMPKα; Cell Signaling Technology; Cat. No. 2531),

These were diluted in blocking buffer at the optimized dilution of 1:1000 and incubated overnight at 4°C in a gel rocker. After incubation with primary antibody, the nitrocellulose membrane is washed with 5 ml of Tris buffer saline Tween20 solution for 5 times with agitation.

**Incubation with secondary antibody**

Anti-mouse IgG, respective HRP-linked Antibody (Cat.No.7076 cell signaling) was diluted in blocking buffer at the optimized dilution of 1:3000 and incubated at room temperature for one hour with agitation. After incubation with secondary primary antibody, the nitrocellulose membrane is washed with 5 ml of Tris buffer saline tween20 solution for 5 times with agitation.

**5.2.18 B Enhanced chemiluminescence (ECL) Detection**

- Detection solutions A and B in a ratio of 40:1 (for example, 2 ml solution A + 50μl Solution B) were mixed to a final volume of 0.1 ml/cm².
- The excess wash buffer was drained from the washed membranes and place protein side up on a sheet of saran wrap or other suitable clean surface. Then
the mixed detection was added on to the membrane and incubated for 5 minutes at room temperature.

- The excess detection reagent was drained by holding the membrane off gently in forceps and touching the edge against a tissue.

- Then the blot was placed facing the protein side down on to a fresh piece of saran wrap, the blots were wrapped smoothly without any air bubbles. X-ray film cassettes were placed on the blot.

- The white lights were switched off and the safe light was switched on for developing X-ray film.

- A sheet of autoradiography film was placed (for example, x-ray film) on top of the membrane and the cassette was exposed for 15 seconds.

- After exposure, X-ray film were dipped into developing solution for a minute rinsed in distilled water before immersing again into fixing solution.

- The film was removed from the fixing solution and again rinsed in distilled water

- The X-ray film was left for air dry.

5.2.19 Histopathological examination

All histological examinations were performed by an experienced pathologist who was blinded to the experiment groups. Histopathological changes were assessed by a semi quantitative method according to standards proposed by Dixon et al., (2004).

5.2.20 Statistical analysis

The values are expressed as Mean ± SE. The Graphs were generated using GraphPad Prism® (Version 5). Statistical analysis was undertaken using One-Way ANOVA with Dunnett’s post-test using GraphPad Prism®. The results were considered significant when P ≤ 0.05.
5.3 Results

5.3.1 Metabolic parameters

Liver enzyme activities

Feeding with a HFD for sixteen weeks induced a significant increase in serum activities of AST and ALT in mice as compared to the normal group (P≤0.05). All the treatment regimens significantly decreased the elevated activities of AST and ALT as compared to NAFLD group (P≤0.05), (Table 5.1).

Serum total cholesterol, Triglycerides and hepatic triglycerides

Table 5.1 shows a significant elevation in serum total cholesterol and triglyceride levels in HFD-fed mice as compared to normal mice (P≤0.05). The elevation in serum total cholesterol was significantly ameliorated only by treatment with Hederagenin. The elevation in serum triglycerides level was markedly attenuated by treatment with Hederagenin. In addition, hepatic triglyceride level was significantly higher in NAFLD group as compared to normal group (P≤0.05). Treatment with Hederagenin significantly reduced the high hepatic triglyceride level as compared to NAFLD group.

5.3.2 Fasting blood glucose, insulin and HOMR-IR index

A significant increase in fasting blood glucose, fasting insulin and HOMA-IR index was observed in mice with NAFLD as compared to normal mice (P≤0.05, Table 5.2). Hederagenin was the sole treatment that could decrease the elevated fasting blood glucose significantly as compared to the NAFLD group. However, fasting insulin was reduced significantly as compared to NAFLD group. All the implemented pharmacological agents significantly reduced the elevated HOMA-IR index as compared to NAFLD group (P≤0.05), (Table 5.2)

5.3.3 Effect of Treatment on Body Weight and Feed Intake and liver index

The animals fed with HFD diet for 6 weeks showed significant increase in body weight compared with diet fed animals (P < 0.05). Following treatment, there was
significant reduction (P < 0.01) in body weight gain and cumulative feed intake (P < 0.05) in Group V compared with Group II and no changes in body weight gain and feed intake was observed in other treatment groups [Table. 5.3]. A significant increase in the body weight and the liver index was observed in the HFD-fed mice as compared to normal mice (P≤0.05). The % increase in the body weight was significantly reduced in Metformin 350mg/kg group, Hederagenin (300 mg/kg/day, p.o.) group and Hederagenin (200 mg/kg/day, p.o.) as compared to NAFLD group (P≤0.05). Liver index was also improved in Metformin 350mg/kg group and in the Hederagenin (300 mg/kg/day and 200 mg/kg/day, p.o.) as compared to NAFLD group (P≤0.05). No significant differences in daily food and water intake were observed among the groups over the experimental period.

5.3.4 The inflammatory cytokine, TNF-α

Serum TNF-α level was significantly increased in NAFLD mice as compared to normal mice (1351±121 versus 3214±321, P≤0.05, (Fig. 5.2. A and B). Hederagenin could significantly attenuate this increase (P≤0.05,) and lower down the inflammatory cytokine levels. (Table 5.4; Fig. 5.2)

5.3.5 Oxidative stress markers

Lipid peroxidation

NAFLD group showed a significant increase in MDA content in the liver homogenate of mice as compared to normal mice (185±20 versus 526±50, P≤0.05). All treatment regimens significantly prevented this increase. Further, Hederagenin group showed statistically lower MDA level in comparison with metformin group (P≤0.05). (Fig. 5.3)

Hepatic reduced glutathione content

Feeding with a HFD for sixteen weeks induced a significantly decrease in reduced GSH content in liver homogenate of mice as compared to mice fed a normal diet
(62±6.2 versus 19±0.21, P<0.05). All pharmacological treatments significantly increased the level of 253 GSH as compared to NAFLD group (P<0.05) (Fig. 5.4)

### 5.3.6 Cell line studies – Oil red O assay and MTT assay

This assay summarizes the data on cell viability, cytotoxicity and LPO assessment of control and treated HepG2 cells. There was a significant decrement in the cell viability along with higher cytotoxicity and lipid peroxidation in FFA treated HepG2 cells compared to untreated cells. Changes in the cell viability and cytotoxicity were significantly minimized in co-presence of Hederagenin along with FFA (Fig. 5.5, 5.6, 5.7)

### 5.3.7 LDH Activity

The LDH activity showed that there was dose dependent decrease indicating the decrease in cytotoxicity. (Fig. 5.8)

### 5.3.8 Immunoblot analysis

In order to examine whether Hederagenin activates the AMPK pathway, we examined its effect on phosphorylation of AMPK in liver and muscle protein. Phosphorylation of AMPK was significantly decreased in the HFD group but significantly increased in the Hederagenin groups compared to that of the HFD group. Thus, Hederagenin may have a beneficial effect on obesity induced by HFD. However, further experiments in this area should be performed in the future. whether Hederagenin activates the increase AMP-activated protein kinase (AMPK) and acetyl-coenzyme A carboxylase (ACC) phosphorylation. Cells were coexposed to FAs (1mM) and various polyphenols (50mM) for 24 h. A: AMPK phosphorylation (pThr172-AMPK) and its substrate ACC (pSer79-ACC) phosphorylation are detected by Western blot analysis. The FAS protein levels were also examined under the same conditions. The numbers below the panels represent quantification of the immunoblot by densitometry. Representative immunoblot and densitometric quantification of AMPK threonine 172 phosphorylation shows a dosedependent effect by Hederagenin treatment in the presence of 1 mM FAs. C: De
novo lipogenesis is decreased by Hederagenin treatment. HepG2 cells were incubated with acetate for 2 h cotreated with Hederagenin or 5-amino-imidazole-4-carboxamide riboside (AICAR) in the presence of 1 mM FAs, and the radioactivity in the saponifiable fatty acid fractions was measured. This result demonstrated that the reduced acetate incorporation rates may be attributable to the increased ACC phosphorylation by Hederagenin treatments. Fatty acid oxidation rate was determined by cotreatment with Hederagenin and AICAR in the presence of 1mM FAs. Data depict means ± SEM of at least three experiments. Asterisks represent statistically significant differences from the FA-treated control group (* P ≤0.05, ** P ≤0.01) (Fig.5.9).

5.3.9 Histopathological examination

In the present study, the NAFLD group showed significant changes in the liver histology. Liver samples from the NAFLD group stained with Hematoxylin & Eosin or Masson's trichrome stain showed diffuse macrovesicular steatosis and multifocal portal inflammation as well as hepatocellular fibrosis. Liver samples from the metformin showed moderate portal fibrosis. The liver in the Hederagenin group showed only mild fatty change with few inflammatory cell infiltrations along with mild portal fibrosis.

The degree of steatosis, lobular inflammation and fibrosis in the NAFLD group was significantly higher than in the normal group. All the treatment regimens, with the exception of Hederagenin, significantly reduced the degree of steatosis; the steatosis score in Hederagenin was significantly lower than that observed in metformin group. However, the Hederagenin (500mg/kg) was the sole group that showed a significant decrease in the liver lobular inflammation. Finally, fibrosis was attenuated in all treatment groups, except the Hederagenin (100mg/kg) group. The fibrosis score in Hederagenin (500mg/kg) group was significantly lower than that recorded in metformin group (350mg/kg) (Fig.5.10, 5.11 and 5.12)
Table 5.1 Effect of Hederagenin (100, 300 and 500 mg/kg, p.o.), Metformin (350 mg/kg, p.o.), and their combinations on serum AST, ALT, TC TG and as well hepatic triglycerides level in the experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>TC (mg/dl)</th>
<th>Serum TG (mg/dl)</th>
<th>Hepatic TG (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>41±4</td>
<td>39±5</td>
<td>64±5</td>
<td>162±10</td>
<td>11±0.3</td>
</tr>
<tr>
<td>NAFLD control</td>
<td>93±7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74±5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>120±8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>231±10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hederagenin (100mg/kg)</td>
<td>72±8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69±3</td>
<td>100±4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>195±10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hederagenin (300mg/kg)</td>
<td>67±8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58±3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>97±4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>170±10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hederagenin (500mg/kg)</td>
<td>55±8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48±3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86±4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>163±10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Metformin (350mg/kg)</td>
<td>49±8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44±3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>70±4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>156±10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NAFLD: nonalcoholic fatty liver disease, HFD: high-fat diet, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, TC: total cholesterol. TG: triglycerides. Results are expressed as Mean±S.E.M. and analyzed using one-way ANOVA followed by Bonferroni's test for multiple comparisons. <sup>a</sup> P≤0.05 versus normal group. <sup>b</sup> P≤0.05 versus NAFLD group, n=6.

Table 5.2 Effect of Hederagenin (100, 300 and 500 mg/kg, p.o.), Metformin (350 mg/kg, p.o.) on fasting blood glucose, fasting insulin and HOMA-IR index in the experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>fasting blood Glucose (mg/dl)</th>
<th>fasting insulin (µU/ml)</th>
<th>Homo-IR index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>71±2</td>
<td>13±1</td>
<td>2.2±0.17</td>
</tr>
<tr>
<td>NAFLD control</td>
<td>89±3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24±4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.9±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hederagenin (100mg/kg)</td>
<td>80±4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21±3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.7±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hederagenin (300mg/kg)</td>
<td>79±4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20±8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.3±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hederagenin (500mg/kg)</td>
<td>77±4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16±5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Metformin (350mg/kg)</td>
<td>70±7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14±6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NAFLD: nonalcoholic fatty liver disease, HFD: high-fat diet, HOMA-IR index: Homeostatic Model Assessment–Insulin Resistance index. HOMA-IR index = [fasting glucose (mMol/L)×fasting insulin (µU/ml)] / 22.5. Results are expressed as Mean±S.E.M. and analyzed using one way ANOVA followed by Bonferroni's test for multiple Comparisons. <sup>a</sup> P≤0.05 versus normal group, <sup>b</sup> P≤0.05 versus NAFLD group, n=6.
Table 5.3 Effect of Hederagenin (100, 300 and 500 mg/kg, p.o.), Metformin (350 mg/kg, p.o.), on body weight, feed intake and liver index in the experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Increase in body weight (g)</th>
<th>Final body weight (g)</th>
<th>Cumulative feed intake (g)</th>
<th>Liver index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>33.3 ± 1.1</td>
<td>31.28±1.21</td>
<td>84.91±4.68</td>
<td>4.16±0.06</td>
</tr>
<tr>
<td>NAFLD</td>
<td>44.1 ± 1.0</td>
<td>44.1±3.16 ss</td>
<td>46.23±2.40</td>
<td>7.26±0.27 a</td>
</tr>
<tr>
<td>Hederagenin (100 mg/kg/day)</td>
<td>40.1 ± 0.9</td>
<td>39.92±3.21</td>
<td>45.75±2.68</td>
<td>6.04±0.31 b</td>
</tr>
<tr>
<td>Hederagenin (300 mg/kg/day)</td>
<td>39.2 ± 1.4</td>
<td>36.92±1.48</td>
<td>42.27±2.48</td>
<td>5.70±0.19 b</td>
</tr>
<tr>
<td>Hederagenin (500 mg/kg/day)</td>
<td>38.2 ± 1.2</td>
<td>34.60±1.34*</td>
<td>23.18±1.32*</td>
<td>5.12±0.19 b</td>
</tr>
<tr>
<td>Metformin (350 mg/kg/day)</td>
<td>36.3 ± 1.3</td>
<td>36.96±1.04*</td>
<td>24.99±1.33*</td>
<td>4.81±0.19 b</td>
</tr>
</tbody>
</table>

NAFLD: nonalcoholic fatty liver disease, HFD: high-fat diet. Results are expressed as Mean±S.E.M. and analyzed using one-way ANOVA followed by Bonferroni’s test for multiple comparisons. a P≤0.05 versus normal group. b P≤0.05 versus NAFLD group, n=6.
Table 5.4 Estimation of Tumour Necrosis Factor Alpha TNF-α mouse, Biotrak ELISA system (RPN2718)

<table>
<thead>
<tr>
<th>Groups</th>
<th>mTNF α (pg/ml) (Mean± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>1351±121</td>
</tr>
<tr>
<td>NAFLD control</td>
<td>3214±321</td>
</tr>
<tr>
<td>Hederagenin (100mg/kg)</td>
<td>2988±231</td>
</tr>
<tr>
<td>Hederagenin (300mg/kg)</td>
<td>2765±265</td>
</tr>
<tr>
<td>Hederagenin (500mg/kg)</td>
<td>1980±198</td>
</tr>
<tr>
<td>Metformin (350mg/kg)</td>
<td>1456±143.10</td>
</tr>
</tbody>
</table>

Results are expressed as mean±S.E.M. and analyzed using one-way ANOVA followed by Bonferroni's test for multiple comparisons. $$P \leq 0.05$$ versus normal group, *P \leq 0.05 versus NAFLD group.
Fig. 5.3 Hepatic Malondialdehyde assay (MDA)

Fig. 5.4 Hepatic reduced glutathione (GSH)

Fig. 5.3 and 5.4 Results are expressed as mean±S.E.M. and analyzed using one-way ANOVA followed by Bonferroni’s test for multiple comparisons. $$P\leq0.05$$ versus normal group, *$$P\leq0.05$$ versus NAFLD group **$$P\leq0.05$$ versus treatment groups and NAFLD
Fig. 5.5 Qualitative analysis of *in vitro* NASH - Staining of cells with oil red O

Photomicrographs showing (A) untreated HepG2 cells, (B) FFA – BSA complex (2 mM) treated HepG2 cells showing cytoplasmic lipid accumulation and (C & D) FFA– BSA complex (2mM) along with Hederagenin extract (200 µg/ml) treated HepG2 cells showing lesser degree of cytoplasmic lipid accumulation (40×).
Fig. 5.6 Quantitative analysis of *in vitro* NASH

*Quantitative analysis - Oil red O assay*

![Graph showing absorbance levels with absorbance values and significance levels indicated.

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<tr>
<th>FFA(2MmM)</th>
<th>-</th>
<th>+</th>
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<tr>
<td>Hederagenin(µM)</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>50</td>
<td>100</td>
<td>200</td>
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Fig. 5.7 Quantitative analysis of *in vitro* MTT Assay

*MTT Assay*

![Graph showing % cell viability with % cell viability levels and significance levels indicated.

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<th>Hederagenin(µM)</th>
<th>-</th>
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Fig. 5.8 Quantitative analysis of *in vitro* Percentage LDH Activity

Effect of Hederagenin on (Fig. 5.6) oil red O staining, (Fig. 5.7) MTT assay, (Fig. 5.8) LDH release in Fatty acid treated HepG2 cells. Results are expressed as means ± S.E.M., n = 3. #P < 0.05, ##P < 0.01 and ###P < 0.001 compared with untreated, *P < 0.05, **P < 0.01 and ***P < 0.001 compared with FA and as non-significant.
Effect of Hederegenin on AMPK signaling pathway. Hederegenin activated AMPK signaling from liver. Total protein was collected from liver and muscle tissue (10–20 mg). Western blotting bands were analyzed with Image J software.
Fig. 5.10 Macroscopic examination – Isolated liver

(a) Normal Livers

(b) NAFLD - Diseased Livers – Fat accumulation leading to Decoloration of liver
Morphological evaluation of fatty liver in NASH mice. (A) Liver of Normal mice showing red coloured appearance, (B) Fat laden liver of NASH mice showing pale coloured appearance and (C) liver of NASH mice Hedergenin 100mg/kg showing red coloured appearance of liver that is similar to the liver of control rat. (D & E) Hedergenin 500mg/kg shows reduced liver vacuoles and less fatty deposit similar like Metformin 350mg/kg.
Fig. 5.11 Effect of Hederagenin on histological changes in liver at magnification of x200.

Group I [Normal control; A], Group II [NAFLD control; B]

Group III [Hederagenin 100mg/kg; C], Group IV [Hederagenin 300mg/kg; D]
Histology of the liver sections stained with hematoxylin and eosin (H&E) stain. Microscopic picture of sections of liver from normal group showing normal liver tissues with normal hepatic cords, portal area and hepatic lobules. Liver sections of NAFLD group show diffuse marked macrovesicular steatosis, multifocal portal inflammation and marked fibrosis. Liver sections from Metformin group show moderate local extensive steatosis with mild ballooning degeneration. Liver sections from Hederagenin 500mg/kg group show moderate fatty change, few inflammatory cells infiltrations together with mild portal fibrosis. Liver sections from Hederagenin 300mg/kg group group show mild steatosis, moderate steatohepatitis (H&E stain X100).
5.4 Discussion

The study focused on the effect of Hederagenin and metformin in high fat diet-induced NAFLD in C57BL/6 mice. The NAFLD animals developed marked obesity, hyperglycemia, and fatty liver. Histopathological examination of the livers of NAFLD control mice revealed moderate to severe steatosis, lobular inflammation and developed typical histopathologic non-alcoholic steatohepatitis lesions. There was severe hepatic fat accumulation along with increased liver weight; however, this was accompanied with only mild elevation of liver specific enzymes. A lack of correlation between the degree of NAFLD and levels of liver enzymes is not surprising, since in a clinical situation the liver enzyme levels do not readily correlate with severity of hepatic steatosis. These observed features are similar to the pathological features of human NAFLD. In the NAFLD control group, the animals showed significant reduction in plasma TG with corresponding increase in liver TG levels as compared with normal controls. Though such a reduction seems contrary with human metabolic syndrome condition, it has already been reported by other researchers.

In response to sudden excessive fat ingestion when the plasma lipid level exceeds oxidative capacity of energy requiring tissues like skeletal muscle, the liver acts as an effective buffer organ to avoid accumulation of circulating lipid and starts taking up lipid from plasma to store as TG. Excessive stored TG in hepatocytes is the hallmark of NAFLD which is strongly associated with hyperinsulinemia and hyperglycemia. (Shuqin Zheng et al., 2008)

In addition, significant elevations in total cholesterol and triglycerides in the sera of mice were observed in the current study. In agreement, a significant elevation in serum triglycerides or both serum total cholesterol and triglycerides was observed after HFD feeding. Further, the current study revealed that NAFLD mice showed high hepatic triglycerides level and this extends findings of others. Further, a significant increase in HOMA-IR index was observed in NAFLD group as compared to the normal group. These results are supported by previous studies. This
indicates that mice with NAFLD suffer from high insulin resistance and thus, insulin resistance plays an important role in the development of fatty liver.

In the current study, serum level of TNF-α was significantly increased by HFD feeding and this finding came on line with those obtained previously (Yalniz et al., 2007). Moreover, an elevation in tissue MDA and a suppression of tissue GSH were detected in NAFLD group; this seems to be consistent with previous reports (Venkatesh et al., 1999). These results highlight that inflammation and oxidative stress play important roles in NAFLD pathogenesis.

Data in this study showed that Hederagenin, induced a significant reduction in liver index and activities of AST and ALT. Consistently, ALT activity was reduced by Hederagenin in mice maintained on a diet-induced non-alcoholic steatohepatitis (Fujita et al., 2007) and HFD-fed rats. Hederagenin significantly lowered HFD-induced hypertriglyceridemia; whereas, the Hederagenin significantly lessened the hypercholesterolemia. These results agreed with those reported by Xuc et al., 2008. Metformin or Hederagenin, significantly decreased the HOMA-IR index. Consistently, the HOMA-IR index decreased significantly in mice with HFD-induced NAFLD and in obese rats after treatment with metformin. Recently, metformin was shown to significantly reduce plasma insulin and the HOMA-IR and diabetic mice.

Hederagenin and metformin decreased the elevated hepatic MDA and increased the GSH content. In a previous study, HFD-induced oxidative DNA damage in mice was attenuated by Hederagenin. The authors highlighted that the hepatoprotective mechanisms of Hederagenin was mediated by retrieving oxidative DNA repair, which in turn blocked the vicious cycle of reactive oxygen species production, improved insulin sensitivity and halted proinflammatory signalling transduction (Hsiao et al., 2008). In agreement, Hederagenin attenuated MDA concentrations in white adipose tissue in db/db mice (Sugimoto et al., 2009), in liver and kidney in alloxan-diabetic rats and in streptozotocin-diabetic rats (Majithiya et al., 2005).
In the present study, Hederagenin significantly decreased the elevated serum TNF-α, however, In contrast, other research groups found that the level of TNF-α in mice treated with Hederagenin was significantly lower than in HFD-fed mice with NAFLD (Xuc et al., 2008), Diet-induced mice (Fujita et al., 2007) and in ethanol and lipopolysaccharide-induced acute liver injury in mice (Ohata et al., 2004). The authors suggested that the therapeutic effect of Hederagenin on fatty liver may be associated with the regulation of TNF-α. Hederagenin induced a moderate improvement in the liver histology. Consistently, an improvement of hepatic steatosis, inflammation and liver cell ballooning was produced by Hederagenin (Fujita et al., 2007; Hsiao et al., 2008; Xu et al., 2006).

Increased hepatic lipid load, leading to augmented mitochondrial fatty acid oxidation and generation of ROS are attributed to the onset of “second hit” in NASH, further compounded by the increased quantum of mitochondrial ROS and LPO due to oxidation of mitochondrial poly unsaturated fatty acids (PUFAs). These sequences of events snowball into a chain reaction resulting in damage to mitochondrial membrane. The results obtained from the present study clearly demonstrates the efficacy of Hederagenin in contending NASH related increase in mitochondrial ROS load and can be easily related with the earlier reported potent free radical scavenging of the compound. (Thounaojam et al., 2010b). Compromised endogenous antioxidant status along with increased ROS generation can account for heightened mitochondrial oxidative stress and as such NASH animals in the present study are characterized by significantly depleted hepatic mitochondrial levels of both non-enzymatic and enzymatic antioxidants. Interestingly, co-supplementation with Hederagenin extract prevents the decrement in endogenous mitochondrial antioxidants characteristic of HFD induced NASH. Precipitous fall in mitochondrial non-enzymatic and enzymatic antioxidants by NASH and its prevention by concurrent supplementation with Hederagenin may be related with our earlier reported antioxidant potential of SR extract (Thounaojam et al., 2010 b,c).

Hepatic steatosis caused due to fat overload in experimentally induced NASH is marked by histopathological lesions such as hepatocyte ballooning, lobular inflammation and Mallory’s hyalinization. Fat accumulation and increased ROS
generation lead to lipid peroxidation and damage of mitochondrial membrane. Further, higher MDA generated due to elevated LPO induces the formation of neo-antigens by interacting with hepatic proteins resulting in inflammation and as such, the present histopathological observations are marked by significantly higher score of infiltration by inflammatory cells, as also reported by others (Bose et al., 2008; Haddad et al., 2009). Favourable effect of Hederagenin in preventing lipid accumulation, ROS formation and LPO, is also well reflected in the histologically observed minimal score of inflammation. However, a potential role of Hederagenin in combating inflammation cannot be overlooked in the light of the reported anti-inflammatory role of Hederagenin (Venkatesh et al., 1999). Swelling and/or enlargement of hepatocytes seen in the present study of NASH mice is a characteristic form of degeneration visible as hepatocyte ballooning, as also reported in cases of NASH. Mallory’s hyalinization also known as Mallory’s body is histoarchitectural aberrations of hepatocytes caused due to cross linking of cytokeratin, a feature reported in NASH (Albano et al., 2005). Both, hepatocyte ballooning and Mallory’s bodies, clearly visible in liver sections of NASH mice, were however not observable in Hederagenin supplemented mice, suggesting the protective effect of principles in Hederagenin against NASH associated histopathological changes.

Human hepatocarcinoma cells (HepG2) has been reported to develop morphological and biochemical transformations due to lipid accumulation when treated with FA. These set of changes are comparable to formation of fatty liver in humans (Okamoto et al., 2002) and hence is an ideal model for studying and quantifying experimentally induced NASH (Cui et al., 2010). In vitro studies on HepG2 cells undertaken essentially to draw confirmatory evidence in favour of the in vivo observations on NASH has revealed significant lipid accumulation in FA treated HepG2 cells. Cytotoxicity of FA on HepG2 cells is characterized by augmented LDH release and lipid peroxidation along with reduced cell viability. However, presence of Hederagenin along with FA significantly prevented lipid accumulation and LDH release, lipid peroxidation and cytotoxicity as assessed by cell viability
AMPK is one of the best-known target proteins regulating the metabolic pathway and functioning as an energy sensor. Activation of AMPK occurs upon depletion of ATP and blocks energy-consuming processes such as glucose uptake and β-oxidation in fat, muscle, and liver. Hepatic AMPK is activated by adipocyte-derivative hormones, such as adiponectin, resistin, and leptin, under normal conditions (Matteoni et al., 1999).

In liver and muscle tissue, there is an inverse relationship between AMPK and elevation of sterol regulatory element binding protein-1c (SREBP-1c) activity. SREBP-1c leads to increases in fatty acid synthesis as a result of the induction of fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). AMPK decreases FAS and ACC by suppressing SREBP-1c. However, the mechanism underlying AMPK regulation of SREBP-1c activity in the control of lipid homeostasis, especially insulin resistance, is still unknown. Stimulation of lipoprotein secretion by these hormones may protect the liver from intracellular lipid accumulation. Muscle tissue is a primary site for insulin resistance and is the tissue responsible for glucose disposal stimulated by insulin. Therefore, activation of AMPK may contribute to the reduction of lipid accumulation in liver, glucose tolerance, and insulin resistance induced by HFD. Our data show that phosphorylation of AMPK in the HFD group was significantly suppressed and supplementation with Hederagenin greatly recovered the phosphorylation of AMPK in liver tissues. Moreover, our data show that DLE-stimulated glucose uptake is accompanied by the activation of AMPK and ACC in cultured muscle cells. Hederagenin treatment recovered significantly HFD-induced inhibition of AMPK phosphorylation in muscle tissues. These results suggest that DLE may have a suppressive effect on HFD-induced lipid accumulation in the liver and may ameliorate insulin-stimulated glucose and insulin resistance in the muscle via an AMPK/ACC-mediated pathway.
Numerous scientific reports cite the identification of several flavonoids and phytochemicals, including coumaric acid, chicoric acid, monocaffeoyltartaric acid, cinnamic acid, caffeic acid, chlorogenic acid, and luteolin, in dandelion leaf. Among these compounds, caffeic acid has been shown to improve glucose disposal in insulin-resistant mice chlorogenic acid has been reported to have antiobesity properties in HFD-induced obesity, and luteolin has been shown to have a therapeutic effect on CCl4-induced liver fibrosis (Devries seimon et al., 2005). Therefore, we hypothesize that the suppressive effect of Hederagenin on HFD-induced hepatic lipid accumulation is due to these trepenoids.

In conclusion, Hederagenin significantly suppressed lipid accumulation in the liver, reduced biochemical, insulin resistance, and lipid in HFD-fed C57BL/6 mice via the AMPK pathway. These findings may provide molecular evidence for the use of Hederagenin as a therapy in the management of fatty liver and obesity-relative disorders.