Chapter 4

The potential of *Symlocos cochinchinensis* against high fructose saturated fat diet induced insulin resistance in rodent model and the molecular mechanisms of its activity

4.1 Introduction

The prevalence of obesity is escalating worldwide and is associated with an increased risk of developing insulin resistance and T2DM\(^1\). Many reports propose that obesity may be responsible for high incidence of T2DM due to the typical association of diabetes with obesity\(^\text{2}\). In the present clinical settings, only the chronic cases of obesity have received much attention\(^3\). But moderate obesity especially with visceral or central adiposity can still elicit chronic metabolic abnormalities characteristic of the insulin resistance syndrome, such as dyslipidemia, hypertension, insulin resistance, and glucose intolerance\(^4, 5\). The general urbanization of the world’s population with diminished physical activity resulted in the increased prevalence of obesity\(^4\). In addition, change in eating habit involving elevated consumption of refined sugar diets and animal fat is one of the amplifying factors for the development of obesity, hyperinsulinemia, insulin resistance, hypertension, hypertriacylglycerolaemia and chronic metabolic disorders\(^6, 7\). The subsequent imbalance in the carbohydrate metabolism and the attempts of the physiological system to nullify the metabolic derangements leads to an endocrine system overload, which further induce defects in lipid metabolism\(^8\).

The marked increase in the amount of dietary fructose consumption either in the form of sucrose or high fructose corn syrup, a common sweetener used in the food industry is the major dietary change in the last decades\(^9\). The increased flux of fructose to the liver results in rapid stimulation of *de novo* lipogenesis and triglyceride accumulation, which in turn contributes to reduced insulin sensitivity, hepatic insulin resistance and glucose intolerance\(^9\). Fructose accelerates the development of obesity and metabolic syndrome rapidly both in animal models and humans in comparison with glucose, as fructose is able
to bypass the main regulatory steps of glycolysis\textsuperscript{9, 10}. Besides this property, fructose is more lipogenic and do not induce thermogenesis\textsuperscript{9, 11}. Further, the diet rich in fructose together with saturated fat leads to an oversupply of lipid which can upset the interaction between lipid and carbohydrate metabolism necessary for the maintenance of fuel homeostasis and may result in the condition of insulin resistance and its related complications\textsuperscript{12, 13}.

Since the long term persistence of insulin resistance and metabolic syndrome induce deleterious complications like T2DM and cardiovascular disorders, the development of pharmacological interventions for these early symptoms may be extremely fruitful. Nowadays, natural phytotherapeutics receives more attention for healthcare due to their synergistic properties and minimal undesirable effects. In previous chapters, we have demonstrated the protective property of \textit{Symlocos cochinchinensis} (SC) in streptozotocin induced pathophysiological alterations as well as its antihyperglycemic activity via inhibition of alpha glucosidase and enhanced insulin sensitivity with antiglycation and antioxidant potential. In the present chapter, we report the insulin sensitizing effects of SC in high fructose and saturated fat fed insulin resistant rodent model and the contributing mechanisms involved.

\textbf{4.2 Experimental details}

\textbf{4. 2. 1 Chemicals and reagents}

All the chemicals and biochemicals were from Sigma (St. Louis, MO, USA). All the antibodies except for phospho-AKT were purchased from Santa Cruz Biotechnology, Inc. (USA) and phospho-AKT (Ser-473) antibody was obtained from Cell Signalling Technology (USA).

\textbf{4. 2. 2 Plant material}

The plant material was collected and processed as described in Chapter 2, section 2. 2. 2. Since SCE exhibited comparatively better activity with respect to various \textit{in vitro} targets and its high content of bioactives, SCE was taken for evaluation against high fructose and saturated fat fed insulin resistant animal model.
4. 2. 3 Animals, diet and experimental design

Male albino rats of Sprague Dawley strain (5 weeks old, 140±20 g), bred at animal facility of CSIR-CDRI, Lucknow were selected for this study. Rats were housed in polypropylene cages (5 rats per cage) under an ambient temperature of 23±2°C; 50-60% relative humidity; light 300 lux at floor level with regular 12 h light/dark cycle. Animals were maintained in a 4 h/day feeding protocol for 1 week with a standard laboratory diet. This kind of feeding regimen synchronizes metabolic conditions of animals, and can be used to study regulation of metabolism by nutrients\textsuperscript{14, 15}. The rats were fed high fructose saturated fat diet (HFS) for 11 weeks. The diet composition is given as Table 4.1. After 8 weeks (56 days) of feeding animals were divided into 4 groups of 6 animals in each group. The animals under these groups received test materials, metformin (positive control) and vehicle (1.0% gum acacia) from 57\textsuperscript{th} day to 78\textsuperscript{th} day. The 57\textsuperscript{th}, 64\textsuperscript{th}, 71\textsuperscript{st}, 77\textsuperscript{th} and 78\textsuperscript{th} days are designated as ‘day 0’, ‘day 7’, ‘day 14’, ‘day 20’ and ‘day 21’ in the preceding sections. In addition to these groups there were two more groups i.e., animal treated with normal diet with vehicle and normal diet with high dose of test material (SCE 500 mg/kg body weight to check toxicity). The schematic representation of details of experimental design is given in Figure 4.1.

Details of various groups with treatment are given below:

Group 1: Normal diet (ND) animals treated with vehicle (1.0% gum acacia) alone (control).

Group 2: Normal diet animals treated with SCE 500 mg/kg body weight per day (mg/kg bwd) (ND+SCE500; to rule out toxicity).

Group 3: High fructose saturated fat diet control animals (HFS) treated with vehicle (1.0% gum acacia) alone.

Group 4: HFS animals treated with SCE 250 mg/kg bwd (HFS+SCE250).

Group 5: HFS animals treated with SCE 500 mg/kg bwd (HFS+SCE500).

Group 6: HFS animals treated with metformin 100 mg/kg bwd (HFS+Met100).

Animals of control group were given an equal volume of 1.0% gum acacia. For all animal
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Figure 4.1. The schematic representation of details of experimental design.

groups, food and water intake was determined daily; body weight was checked weekly. After completion of experiments, animals were sacrificed on 79th day by cervical dislocation under light ether anaesthesia as per ethics committee guidelines.

4. 2.4 Oral glucose tolerance test (OGTT)

A glucose load (3 g/kg) was given to each animal orally 30 min after test sample or vehicle administration. BGL was determined at 30, 60, 90 and 120 min post-administration of sucrose. OGTT was performed on day 0, 7, 14 and 21 in all the groups.

4. 2.5 Insulin tolerance test (ITT)

On day 0 and day 20, an insulin tolerance test was performed. Briefly, the animals were treated with 1 U/kg bw of insulin (Humulin-R, Eli Lilly, Indianapolis, IN, USA) in physiological saline, intraperitoneally. BGL was determined at 15, 30, 45 and 60 min after insulin administration.

4. 2.6 HOMA-IR

HOMA-IR was calculated using the formula; HOMA-IR = (glucose × insulin)/405, where the concentration of glucose was expressed in mg/dL and that of insulin in mU/L.
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<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Diet components (g/kg)</th>
<th>Normal diet (ND)</th>
<th>High fructose saturated fat (HFS) diet</th>
</tr>
</thead>
<tbody>
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<td>36.5</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Whole wheat</td>
<td>23.5</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Fructose</td>
<td>-</td>
<td>60.0</td>
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<td>4</td>
<td>Ground nut oil</td>
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<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Saturated fat</td>
<td>-</td>
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<tr>
<td>6</td>
<td>Casein (fat free)</td>
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<td>20.0</td>
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<td>Methionine</td>
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<tr>
<td>8</td>
<td>Cholesterol</td>
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</tr>
<tr>
<td>9</td>
<td>Vitamin mixture</td>
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<td>0.2</td>
</tr>
<tr>
<td>10</td>
<td>Mineral mixture</td>
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<tr>
<td>11</td>
<td>Wheat bran</td>
<td>10.6</td>
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</tr>
</tbody>
</table>

Table 4.1 Composition of diet used in High Fructose Saturated fat (HFS) diet model.

4.2.7 Biochemical parameters

Blood samples were collected from retro-orbital plexus of each animal in heparin coated tubes for the separation of plasma and further analytical procedures on day 0 and day 21, during the experiment period. Plasma was separated by centrifugation at 3000×g for 20 min at 4°C, then aliquots were made. One lot was used for the immediate analysis of triglyceride, total cholesterol, HDL-C, LDL-C, % HbA1c and the remaining portion was immediately frozen and stored at -80°C for further analysis. Plasma level of insulin, adiponectin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), uric acid, creatinine, albumin and total protein were measured. Insulin level was assayed using an ELISA kit from Mercodia (Uppsala, Sweden). ELISA kit from Cayman chemicals (Ann Arbor, MI, USA) was used for adiponectin measurement. Kits from BD Biosciences (San Jose, CA, USA) were used for the ELISA analysis of MCP-1, IL-6 and TNF alpha.
from plasma and tissues. All other plasma parameters were quantified using kits from Agappe diagnostics (Knonauerstrasse, Switzerland).

4.2.8 Tissue collection and storage for biochemical and genetic analysis

Immediately after sacrifice of animal, liver, adipose, kidney and muscle tissues were excised, frozen in liquid nitrogen prior to storage at -80 °C until further analysis. The adipose tissue for various analysis were isolated from the epididymal fat pads of rats. Soleus muscle, the oxidative slow-twitch muscle type was collected for the biochemical and genetic analysis. Specifically, insulin-stimulated glucose transport is greater in skeletal muscle enriched with slow-twitch muscle fibers\textsuperscript{16-18}. Hence, soleus muscle was collected for the study.

4.2.9 Tissue collection for histopathology

Immediately after dissection, pancreas, liver and kidney were taken out, cleaned and fixed in 10% neutral buffered formalin solution for preparation of histopathological slides. The tissues were processed for preparation of paraffin section as described in Chapter 3 (section 3.2.9).

4.2.10 Triglyceride assay

For extraction of tissue lipids, 100 mg tissue was homogenized in 2 mL chloroform: methanol (2:1)\textsuperscript{19}. The sample was centrifuged at 3,000 rpm for 30 min. Lipid in the lower phase was collected, dissolved in chloroform containing 1% Triton-x 100, dried and then resuspended in water. The triglyceride concentrations were measured by an enzymatic colorimetric assay with commercially available kits from Cayman chemicals (Ann Arbor, MI, USA).

4.2.11 Biochemical assays for glycogen content and enzyme activities (glucokinase, glucose 6- phosphatase and phosphoenol pyruvate carboxy kinase)

Liver and skeletal muscle glycogen content was estimated using commercially available kits from Cayman chemicals (Ann Arbor, MI, USA). The cytosolic and microsomal preparations of liver homogenates were prepared following the method of Hulcher and Oleson\textsuperscript{20} and the protein concentration was determined using BCA kit (Pierce BCA
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protein assay kit, Pierce Biotechnology, IL, USA). The activities of following enzymes; glucokinase (GK, EC 2.7.1.2), glucose-6-phosphatase (G6Pase, EC 3.1.3.9) and phosphoenol pyruvate carboxy kinase (PEPCK, EC 4.1.1.32) were assayed according to the method of Iynedjian et al., Alegre et al. and Bentle and Lardy respectively. For glucokinase activity assay, liver tissue was homogenized using LabGEN 7 homogenizer (Cole-Parmer, Vernon Hills, IL, USA) at 4°C, in three volumes of a buffer containing 20 mM Tris HCl (pH 7.5), 0.25 M sucrose, 80 mM KCl, 5 mM EDTA, 4 mM MgCl2, 2.5 mM dithiothreitol (DTT) and 10 μg/mL of a protease inhibitor cocktail (Sigma, St. Louis, USA). The homogenates were centrifuged at 17,000×g for 15 min. The resulting post mitochondrial supernatants were centrifuged at 180,000×g for 60 min in order to obtain cytosols. Glucokinase activity was measured by a spectrophotometric assay. The total reaction volume was 750 μL. The assay was conducted at 30°C for 15 min, in presence of 45 mM Tris HCl (pH 7.5), 110 mM KCl, 8 mM MgCl2, 0.5 mM NADP, 0.9 U/mL glucose 6-phosphate dehydrogenase from yeast, 0.5 or 100 mM glucose and 0 or 5 mM ATP. Enzyme activity was calculated as the ATP-dependent rate of NADPH formation at 100 mM glucose minus the rate at 0.5 mM. The reaction mixture for the assay of G6Pase was composed of 40 mM sodium HEPES (pH 6.5), 14 mM glucose-6-phosphate, 18 mM EDTA (pH 6.5), 2 mM NADP, 0.6 IU/mL mutarotase, and 0.6 IU/mL glucose dehydrogenase. The PEPCK activity was determined in the direction of oxaloacetate formation using the spectrophotometric assay. 1 mL of the reaction mixture (pH 7.0) contained 77 mM sodium HEPES, 1 mM inosine 5’-diphosphate, 1 mM MnCl2, 1 mM DTT, 0.25 mM NADH, 2 mM phosphoenol pyruvate, 50 mM NaHCO3, 7.2 units of malic dehydrogenase. To this, 1 mL of tissue lysate was added and the enzyme activity was then monitored for 2 min at 25°C based on a decrease in the absorbance at 340 nm.

4.2.12 Immunoblotting

Tissue lysates of liver, adipose and muscle were prepared for immunoblotting as reported previously. Briefly, tissues (100-200 mg) were homogenized in RIPA buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM EDTA at pH 7.4) containing 10 μg/mL of a protease inhibitor cocktail (Sigma, St. Louis, USA). Tissue homogenates were centrifuged at 12,000 rpm for 20 min at 4°C. Then the supernatants were collected and analysed for the determination of protein concentration.
using a commercial kit (Pierce BCA protein assay kit, Pierce Biotechnology, IL, USA). The tissue lysates (2 mg/mL) prepared were treated with Laemmli sample buffer\textsuperscript{25} containing 100 mM dithiothreitol, and heated at 90-100\degree C for 10 min followed by 1% SDS-PAGE in a mini-Protean apparatus (Bio-Rad Laboratories, Hercules, CA). The electrophoretic transfer of proteins to a PVDF membrane was done as described by Towbin et al.\textsuperscript{26} except for the addition of 0.02% sodium dodecyl sulfate to the transfer buffer to enhance the elution of high-molecular-mass proteins. The PVDF membrane was blocked for 1 h in a blocking agent (3% bovine serum albumin, BSA) at 25 \degree C. The blot was washed 3 times in Tris borate buffer (TBS) with 0.1% Tween. The membrane was then incubated overnight with the desired primary antibody in 1% BSA. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody in 1% BSA for 1 h at room temperature. The blot was then washed with TBS thrice, 10 min each. Immunoreactive bands were visualized by Pierce Enhanced Chemiluminescence according to manufacturer’s instructions (Thermo Fischer Scientific, Rockford, IL, USA) and quantified using Biorad Image Lab\textsuperscript{TM} software. To validate equal loading in each lane and to normalize the blots for protein levels, beta-actin was used as internal loading control.

4.2.13 Analysis of gene expression

The quantitative reverse transcriptase-PCR (qRT-PCR) was performed according to the method described previously\textsuperscript{27}. Total RNA was extracted from tissue samples, by guanidine isothiocynate-phenol-chloroform, TRIZOL (Invitrogen, Carlsbad, CA). Using agarose gel electrophoresis and spectrophotometry, RNA quality and concentration were determined. Primers were designed using the Primer Quest\textsuperscript{®} Tool (IDT) and listed in Table 4.2. All primers were purchased from IDT-Sigma (St. Louis, MO, USA). Reverse transcription reactions were carried out with 2 \mu g of RNA using the Super Script\textsuperscript{®} III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real time PCR reactions were carried out using the IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). The number of PCR cycles was titrated for each gene specific primer pair target to ensure linearity. Amplification efficiency of each primer set was verified before sample analysis. Real-time PCR was performed using CFX 96 Real Time system (Bio-Rad Laboratories, Hercules, CA). The mRNA abundance relative to beta-actin (Actb gene) was determined
using comparative critical threshold (Ct) method according to manufacturer's instruction.

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<th>mRNA</th>
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<th>Reverse primer (5' to 3')</th>
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**Table 4.2.** Nucleotide sequence of qRT-PCR primers.

### 4.2.14 Statistical analysis

Quantitative glucose tolerance of each group was calculated by the area under the curve (AUC) method using Graph Pad Prism software version 3 (Graph Pad Software Inc., La Jolla, CA, USA). All other results were analyzed using a statistical program SPSS/PC+, version 11.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± SD, n = 6. p≤0.05 was considered to be significant.

### 4.3 Results

#### 4.3.1 Induction of insulin resistance and dyslipidemia in animals by feeding HFS diet

Insulin resistance and dyslipidemia was induced in animals by the administration of HFS diet continuously for 8 weeks. Prolonged feeding of HFS diet caused a 1.5 fold increase
in the body weight of animals of HFS group compared to animals of ND group (p≤0.05, Fig. 4. 2A) on day 21. The summary of food intake, water intake and body weight is given in Fig. 4. 2A-C. During ITT, HFS animals exhibited a lack of glucose response towards insulin action, the characteristic feature of insulin resistance while the ND animals displayed a normal glucose response (Fig. 4. 3A-F). The ITT results show the development of insulin resistance in HFS animals (Fig. 4. 4A and B). The insulin resistance (HOMA-IR) of the HFS animals was found to be increased 2 fold when compared to SD group after 8 weeks of feeding (Fig. 4. 5C). Glucose intolerance was noticed in HFS group during OGTT. The HFS animals also showed hypertriglyceridemia (2.8 fold), Hypercholesterolemia (2.5 fold), hyperinsulinemia (1.67 fold), low level of adiponectin (0.32 fold decrease), and hepatic TG accumulation (2 fold) after 8 weeks of feeding.

**Figure 4. 2A-C.** (A)Body weight, (B)food intake, (C)water intake at 3 time points, on day 0, 10 and 21 in HFS fed SD rats. Data are expressed as the mean ± SD, n = 6. ‘a’ represents groups differ significantly from ND group (p≤0.05). ‘b’ represents groups differ significantly from HFS group (p≤0.05). ‘ab’ represents groups differ significantly from both HFS and ND group (p≤0.05). SCE, *S. cochinensis* (SC) ethanolic extract; ND, normal diet control group treated with vehicle (1% gum acacia); ND+ SCE 500 normal diet group treated with SCE 500 mg/kg bwd; HFS, high fructose saturated fat diet vehicle group; HFS+SCE 250, HFS group treated with SCE 250 mg/kg bwd; HFS+SCE 500, HFS group treated with SCE 500 mg/kg bwd and HFS+Met 100, HFS group treated with metformin 100 mg/kg bwd.
4.3.2 SCE administration reversed glucose intolerance and lack of glucose response during OGTT and ITT

From OGTT result it was clear that, the administration of SCE significantly improved the glucose tolerance of HFS rats and there was a marked reduction of blood glucose level in HFS+SCE 250 and 500 groups compared to the HFS group at every time point. Day 7 OGTT exhibited 7.65%, 11.37% and 15.02% reduction in glycemic response in SCE 250, 500 and Met 100 treated groups respectively (Fig. 4. 3A and B). Day 14 OGTT showed 15.74%, 20.21% and 25.40% significant reduction in glycemic response in SCE 250, 500 and Met 100 groups respectively (p≤0.05, Fig. 4. 3C and D) while on day 21, OGTT exhibited further improvement of 21.59%, 25.14% and 30.26% reduction in glycemic response in SCE 250, 500 and Met 100 groups respectively (p≤0.05, Fig. 4. 3E and F). Both extracts and metformin showed dose and duration dependent reduction in glycemic responses. In ITT, treatment with SCE 250, 500 and Met 100 significantly showed the insulin-induced reduction in plasma glucose levels significantly on day 20 (p≤0.05, Fig. 4. 4A and B).

4.3.3 SCE treatment improved insulin sensitivity and normalised plasma insulin and adiponectin levels

Based on fasting plasma glucose and insulin levels, insulin sensitivity was determined using HOMR-IR. The HFS group showed around 4 fold increase in the insulin resistance on day 21 compared to ND group. The SCEs and metformin treatment for 21 days in HFS groups significantly improved the insulin sensitivity comparable to that of ND animals (p≤0.05, Fig. 4. 5C). The HFS animals also exhibited hyperinsulinemia (2.2 fold) and decreased level of plasma adiponectin (0.32 fold decrease) compared to that of ND animals (Fig. 4. 5A and B). The histopathology of pancreas of HFS animals displayed abnormal pancreatic islets morphology (Fig. 4. 5D c). However, administration of SCE 250/500 for 21 days prevented the histopathological alterations in HFS group; endocrine cells were found to be in normal morphology (Fig. 4. 5D e and f) and in metformin group the pancreas exhibited almost similar morphology to that of normal control without any change in endocrine cell population (Fig. 4. 5D d).
Figure 4.3A-F. The improved glucose tolerance exhibited by HFS rats after 7, 14 and 21 days of SCE treatment (A) The glycemic response curve and (B) incremental AUC$_{0-120}$ min on day 7, (C) The glycemic response curve and (D) incremental AUC$_{0-120}$ min on day 14, (E) The glycemic response curve and (F) incremental AUC$_{0-120}$ min on day 21. Data are expressed as the mean ± SD, n = 6. ‘a’ represents groups differ significantly from ND group (p≤0.05). ‘b’ represents groups differ significantly from HFS group (p≤0.05). ‘ab’ represents groups differ significantly from both HFS and ND group (p≤0.05). SCE, S. cochinchnensis (SC) ethanolic extract; ND, normal diet control group; HFS, high fructose saturated fat diet vehicle group; HFS+SCE 250, HFS group treated with SCE 250 mg/kg bwd; HFS+SCE 500, HFS group treated with SCE 500 mg/kg bwd and HFS+Met 100, HFS group treated with metformin 100 mg/kg bwd.

4.3.4 SCE corrected hepatic steatosis and plasma hypertriglyceridemia

HFS animals displayed significantly higher levels of plasma (2.9 fold) and liver triglyceride level (3.1 fold) compared to ND animals on day 21 (p≤0.05, Fig. 4. 6A and B). Total cholesterol in plasma (4 fold) and LDL-C (5.5 fold) were also significantly (p≤0.05) higher in HFS group but level of HDL-C (0.50 fold decrease) was found to be significantly lower compared to the ND group (Fig. 4. 6C - E). Three weeks of SCE treatment substantially reduced the triglyceride content in liver of HFS animals and the plasma lipid profile as well (p≤0.05, Fig. 4. 6A-E).
4.3.5 Renal and hepatic renal function and morphology was improved by SCE administration

The decrease in plasma albumin level of HFS animals (0.30 fold) indicated the diminished liver and kidney function (Fig. 4.7A). The significantly (p≤0.05) elevated plasma uric acid level (2.10 fold) reveal the deranged metabolic pathways of liver and renal dysfunction due to intake of high fructose through the diet (Fig. 4. 7B). The % HbA1c level was about 8.05 in HFS animals, but SCE 250, 500 and Met 100 groups showed reduction in % HbA1c i.e., 7.01, 6.75 and 6.35% respectively (Fig. 4. 7C).

Figure 4.4A-B. Effect of SCE on insulin tolerance of HFS fed SD rats on day 0 and day 20. (A) The insulin tolerance test (ITT) conducted in various experimental groups on day 0. (B) The ITT conducted in various experimental groups on day 20. Data are expressed as the mean ± SD, n = 6. ‘a’ represents groups differ significantly from ND group (p≤0.05). ‘ab’ represents groups differ significantly from both HFS and ND group (p≤0.05). SCE, S. cochinchinensis (SC) ethanolic extract; ND, normal diet control group treated with vehicle (1% gum acacia); ND+ SCE 500 normal diet group treated with SCE 500 mg/kg bwd; HFS, high fructose saturated fat diet vehicle group; HFS+SCE 250, HFS group treated with SCE 250 mg/kg bwd; HFS+SCE 500, HFS group treated with SCE 500 mg/kg bwd and HFS+Met 100, HFS group treated with metformin 100 mg/kg bwd.
Figure 4. 5A-D. Plasma insulin, adiponectin levels, insulin sensitivity on day 0, 21 and histopathology of pancreas after SCE treatment. (A) Plasma insulin level, (B) Plasma adiponectin level and (C) Homeostatic model assessment-insulin resistance (HOMA-IR). Data are expressed as the mean ± SD, n = 6. ‘a’ represents groups differ significantly from ND group (p≤0.05). ‘b’ represents groups differ significantly from HFS group (p≤0.05). ‘ab’ represents groups differ significantly from both HFS and ND group (p≤0.05). (D) Representative microscopic scans of pancreas stained by Hematoxylin and Eosin (H and E 400×) (a) ND, (b) ND+SCE 500, (c) HFS, (d) HFS+ Met 100, (e) HFS+SCE 250 and (f) HFS+ SCE 500. SCE, S. cochinchinensis (SC) ethanolic extract; ND, normal diet control group treated with vehicle (1% gum acacia); ND+ SCE 500 normal diet group treated with SCE 500 mg/kg bwd; HFS, high fructose saturated fat diet group treated with vehicle; HFS+SCE 250, HFS group treated with SCE 250 mg/kg bwd; HFS+SCE 500, HFS group treated with SCE 500 mg/kg bwd and HFS+Met 100, HFS group treated with metformin 100 mg/kg bwd.
The HFS diet also modified renal tissue architecture and caused mesangial matrix expansion and inflammatory infiltration in the renal tissue of HFS animals. The renal tissue integrity was found to be maintained by the treatment with SCE 250/500 or Met 100 compared to that of HFS vehicle control (Fig. 4. 7D a-f). ALT, the specific biomarker for hepatic damage was significantly (p≤0.05) increased (1.62 fold) in HFS group compared to ND animals (Fig. 4. 8A). The second important indicator of hepatic injury, AST was also significantly (p≤0.05) elevated (1.43 fold) in HFS rats compared to animals of ND group (Fig. 4. 8B). The HFS diet intake resulted in an altered hepatic histology in the animals of HFS group. But, the administration of SCE 250/500 or Met 100 preserved the hepatic tissue morphology (Fig. 4. 8C a-f).

**Figure 4. 6A-E. Effect of SCE treatment in plasma lipid profile and liver triglyceride (TG) content of HFS rats** (A) plasma concentration of TG, (B) liver TG content, (C) plasma concentration TC, (C) plasma concentration HDL-C, and (D) plasma concentration LDL-C in various groups. Data are expressed as the mean ± SD, n = 6. 'a' represents groups differ significantly from ND group (p≤0.05). 'b' represents groups differ significantly from HFS group (p≤0.05). 'ab' represents groups differ significantly from both HFS and ND group (p≤0.05). SCE, *S. cochinchinensis* (SC) ethanolic extract; ND, normal diet control group treated with vehicle (1% gum acacia); ND+ SCE 500 normal diet group treated with SCE 500 mg/kg bwd; HFS, high fructose saturated fat diet vehicle group; HFS+SCE 250, HFS group treated with SCE 250 mg/kg bwd; HFS+SCE 500, HFS group treated with SCE 500 mg/kg bwd and HFS+Met 100, HFS group treated with metformin 100 mg/kg bwd.
The potential of SC against HFS diet induced insulin resistance model

**Figure 4. 7A-D. Plasma albumin, uric acid, % HbA1c concentration and histopathology of kidney after SCE administration** (A) Plasma albumin level, (B) Plasma uric acid level and (C) %HbA1c. Data are expressed as the mean ± SD, n = 6. ‘a’ represents groups differ significantly from ND group (p≤0.05). ‘b’ represents groups differ significantly from HFS group (p≤0.05). ‘ab’ represents groups differ significantly from both HFS and ND group (p≤0.05). (D) Representative microscopic scans of kidney stained by Hematoxylin and Eosin (H and E, 400×) (a) ND, (b) ND+SCE 500, (c) HFS, (d) HFS+SCE 250 and (e) HFS+ SCE 500, (f) HFS+ Met 100. SCE, *S. cochinchinensis* (SC) ethanolic extract; ND, normal diet control group treated with vehicle (1% gum acacia); ND+ SCE 500 normal diet group treated with SCE 500 mg/kg bwd; HFS, high fructose saturated fat diet group treated with vehicle; HFS+SCE 250, HFS group treated with SCE 250 mg/kg bwd; HFS+SCE 500, HFS group treated with SCE 500 mg/kg bwd and HFS+Met 100, HFS group treated with metformin 100 mg/kg bwd.
Figure 4. 8A-C. Plasma Alanine amino transferase (ALT), Aspartate amino transferase (AST) and histopathology of liver after 21 days of SCE administration (A) Plasma ALT level. (B) Plasma AST level. Data are expressed as the mean ± SD, n = 6. ‘a’ represents groups differ significantly from ND group (p≤0.05). ‘b’ represents groups differ significantly from HFS group (p≤0.05). ‘ab’ represents groups differ significantly from both HFS and ND group (p≤0.05). (C) Representative microscopic scans of liver stained by Hematoxylin and Eosin (H and E, 400×) (a) ND, (b) ND+SCE 500, (c) HFS, (d) HFS+SCE 250 and (e) HFS+ SCE 500, (f) HFS+ Met 100. SCE, *S. cochinchinensis* (SC) ethanolic extract; ND, normal diet control group treated with vehicle (1% gum acacia); ND+ SCE 500 normal diet group treated with SCE 500 mg/kg bwd; HFS, high fructose saturated fat diet group treated with vehicle; HFS+SCE 250, HFS group treated with SCE 250 mg/kg bwd; HFS+SCE 500, HFS group treated with SCE 500 mg/kg bwd and HFS+Met 100, HFS group treated with metformin 100 mg/kg bwd.
Figure 4. 9A-F. Effect SCE administration on proinflammatory cytokines in plasma, kidney and liver after 21 days of treatment. (A) Plasma concentration of MCP-1 (B) Plasma concentration of IL-6 and TNF-alpha (C) MCP-1, IL-6 and TNF-alpha in kidney tissue (D) mRNA level of MCP-1 in renal tissue (E) IL-6 in liver tissue (F) TNF-alpha in liver tissue. Data are expressed as the mean ± SD, n = 6. ‘a’ represents groups differ significantly from ND group (p≤0.05). ‘b’ represents groups differ significantly from HFS group (p≤0.05). ‘ab’ represents groups differ significantly from both HFS and ND group (p≤0.05). MCP-1, Monocyte chemo-attractant protein-1; IL-6, Interleukin-6; TNF alpha, Tumor necrosis factor-alpha.
4.3.6 Elevated level of proinflammatory cytokines were reduced by the administration of SCE

Plasma levels of proinflammatory cytokines like MCP-1, IL-6 and TNF alpha were found to be significantly (p≤0.05) increased by 1.76, 1.24 and 1.42 fold respectively in HFS animals compared to the ND animals (Fig. 4. 9A and B). MCP-1, IL-6 and TNF alpha content in renal and IL-6 and TNF alpha content hepatic tissue was also found to be elevated significantly (p≤0.05) in HFS vehicle group (Fig. 4. 9 C, E and F). The relative mRNA content of MCP-1 in kidney was also found to be increased significantly (p≤0.05) in HFS vehicle group (Fig. 4. 9D). The administration of SCE 250/500 or Met 100 to HFS rats significantly reduced the proinflammatory cytokines in comparison with ND rats (Fig. 4. 9A - E).

Figure 4. 10A-C. Liver glycolytic and gluconeogenic enzyme activities, liver and muscle glycogen content after 21 days of SCE treatment (A) Activities of Glucokinase (GK), Glucose 6 phosphate dehydrogenase (G6Pase) and Phosphoenol pyruvate carboxy kinase (PEPCK) in liver tissue (B) Liver glycogen content (C) Muscle glycogen content. Data are expressed as the mean ± SD, n = 6. ‘a’ represents groups differ significantly from ND group (p≤0.05). ‘b’ represents groups differ significantly from HFS group (p≤0.05). ‘ab’ represents groups differ significantly from both HFS and ND group (p≤0.05).
4. 3. 7 Hepatic glycolytic enzyme activity was increased, PTP-1B and gluconeogenic enzyme activities were decreased by SCE treatment

Under HFS diet, animals exhibited decreased (0.36 fold) activity of hepatic glycolytic enzyme glucokinase while elevated activity of gluconeogenic enzymes like glucose 6 phosphatase (1.73 fold) and phosphoenol pyruvate carboxy kinase (1.42 fold) compared to the ND animals. But, with the administration of SCEs hepatic glucokinase enzyme activity was increased; glucose 6 phosphatase and phosphoenol pyruvate carboxy kinase enzyme activities were found to be decreased (p≤0.05, Fig. 4. 10A). The PTP-1B was found to be elevated (1.58 fold) in liver tissue of HFS animals (Fig. 4. 11D). The treatment with SCE 250/500 or Met 100 significantly (p≤0.05) reduced PTP-1B activity in these animals compared to that of HFS vehicle control (Fig. 4. 11D). Also, SCE treatment for 21 days in HFS animals replenished hepatic and skeletal muscle glycogen content (Fig. 4. 10B and C).

4. 3. 8 Regulation of gene expression by SCE

HFS feeding significantly (p≤0.05) up regulated the expression of genes involved in the lipid metabolism like SCD-1 (4.29 fold), SREBP-1c (2.03 fold) and FAS (2.76 fold) while the mRNA level of PPAR alpha (0.35 fold) was found to be down regulated compared to that of ND animals (Fig. 4. 11A). The expression levels of genes like G6Pase (5.34 fold), PEPCK (2.85 fold), GLUT-2 (6.54 fold) which are involved in the carbohydrate metabolism were up regulated, but glucokinase (0.29 fold) was down regulated in HFS group (Fig. 4. 11B). HFS feeding also significantly (p≤0.05) up regulated the expression PTP-1B (1.91 fold) mRNA and down regulated the gene expression of SIRT-1 (0.5 fold) in liver (Fig. 4. 11C). In adipose tissue of HFS rats, mRNA level of PPAR gamma 2, Akt-2 and GLUT-4 were found to be down regulated (Fig. 4. 13A). In skeletal muscle also expression levels of Akt-2 and GLUT-4 were down regulated with HFS diet, but SCD-1 was upregulated compared to ND animals (Fig. 4. 14A). SCE administration moderately suppressed the hepatic over expression of genes like SCD-1, SREBP-1c, FAS, G6Pase, PEPCK, GLUT-2 and PTP-1B significantly restored the levels of PPAR alpha, glucokinase and SIRT-1 compared to the vehicle treated HFS group (Fig. 4. 11A-C). A significant restoration was observed in the expression profile of the markers of insulin signalling like Akt-2 and GLUT-4 in adipose
4.3.9 Effect of SCE on the expression of insulin signalling markers and transcription factors

The expression of pattern of various proteins in the insulin signalling pathway like IRS-1, total AKT (tAKT), phosphorylated AKT (pAKT, Ser-473) and GLUT-2 of the liver tissue were analysed using western blotting (Fig. 4. 12A-C). We have also assessed the protein expression of IRS-2, pAKT, and GLUT-4 for skeletal muscle (Fig. 4. 14B-D) and IRS-1,
pAKT, and GLUT-4 for adipose tissue (Fig. 4. 13C-E). The protein levels of transcription factor, PPAR gamma2 was also checked in adipose tissue (Fig. 4. 13B). From these results, it was found that animals of HFS group shares an impaired protein expression pattern in the peripheral tissues like liver, muscle and adipose under high fructose feeding. HFS group displayed a decrease in the expression of IRS-1 (0.35 fold) and tAKT (0.39 fold) of the liver tissue; IRS-2 (0.33 fold), tAKT (0.41 fold) and GLUT-4 (0.47 fold) of skeletal muscle; IRS-1 (0.41 fold), tAKT (0.44 fold), GLUT-4 (0.52 fold) and PPAR gamma 2 (0.54 fold) of adipose tissue compared to the animals of ND group. However, we have noticed an elevated protein expression of GLUT-2 (2 fold) in liver tissue of HFS animals compared to the ND animals (Fig. 4. 12C). SCE supplementation for 3 weeks significantly (p≤0.05) improved the protein expression pattern in the peripheral tissues of HFS+SCE 250/500 or HFS+Met 100 animals. HFS group also exhibited the decreased pAKT/tAKT ratio in liver (0.45 fold), muscle (0.51 fold) and adipose (0.57 fold) compared to the ND group. SCE treatment at 500 mg/kg bwd dose significantly improved pAKT/tAKT ratio in liver (1.79 fold), muscle (2.1 fold) and adipose (2.65 fold) compared to the HFS group (Fig. 4. 12A-C; Fig. 4. 13C-E; Fig. 4. 14B-D). The absolute pAKT level was also lower in HFS compared to the ND group, but it was restored in SCE 250/500 and Met groups to the normal levels.

4.4 Discussion

The excessive fructose intake (>50 g/day) is one of the underlying etiologies of metabolic syndrome and T2DM. Unlike other sugars, the ingestion of excessive fructose induces features of metabolic syndrome in both laboratory animals and humans\(^\text{10}\). High intakes of saturated fat along with elevated level of dietary fructose worsen the situation again which may accelerate the development of insulin resistance and dyslipidemia\(^\text{13, 28}\). We have seen SC is an effective antidiabetic medicinal plant based on our studies using in vitro and in vivo streptozotocin diabetes model. Herein we investigate the insulin sensitizing effects of \textit{S. cochinchinensis} using high fructose and saturated fat fed insulin resistant rodent model and the contributing mechanisms involved. It has been reported that chronic feeding of high fructose (65%) together with saturated fat (12%) can bring about an array of disturbances in the carbohydrate and lipid metabolism of animal during 8 weeks period\(^\text{9, 29}\). HFS induced metabolic derangement subsequently developed
Figure 4. 12A-C. Effect of SCE on the protein expression in liver tissue isolated from HFS fed SD rats after 21 days of extract treatment. (A) insulin receptor substrate- 1 (IRS-1), (B) pAKT-2 (C) GLUT-2. Data are expressed as the mean ± SD, n = 6. ‘a’ represents groups differ significantly from ND group (p≤0.05). ‘b’ represents groups differ significantly from HFS group (p≤0.05). ‘ab’ represents groups differ significantly from both HFS and ND group (p≤0.05).

Symptoms like insulin resistance, hepatic steatosis, glucose intolerance, dyslipidemia and abnormal level of inflammatory markers in HFS vehicle group. Our research show that SCE administration significantly (p≤0.05) improved the general metabolic derangements (insulin resistance, glucose tolerance, lipid profile, inflammatory status) and reversed the hepatic lipid accumulation in HFS fed insulin resistant rodent model.

Generally, diets rich in saturated fat and fructose are associated with hyperphagia and weight gain9, 30. In our study also, HFS animals displayed symptoms like increased food intake, water intake and weight gain during the initial 8 weeks period. Increased weight gain and fat deposition is also responsible for insulin resistance31. SCE administration for 3 weeks modulated the feeding pattern of HFS animals in a similar way as noticed in metformin treated group (Fig. 4. 2B). The weight gain of HFS animals was also limited to an extent by SCE and metformin treatment (Fig. 4. 2A). Glucose intolerance and hyperinsulinemia are the major abnormalities exhibited by rats under high fructose diet32. In the present study, OGTT patterns of HFS rats show the impaired glucose tolerance.
Figure 4. 13A-E. Effect of SCE on the gene and protein expression in adipose tissue isolated from HFS fed SD rats after 21 days of treatment (A) Relative mRNA level of genes: PPAR gamma, Akt-2 and GLUT-4 (B) Protein expression of PPAR gamma (C) insulin receptor substrate-1 (IRS-1). (D) pAKT-2 (E) GLUT-4. Data are expressed as the mean ± SD, n = 6. ‘a’ represents groups differ significantly from ND group (p≤0.05). ‘b’ represents groups differ significantly from HFS group (p≤0.05). ‘ab’ represents groups differ significantly from both HFS and ND group (p≤0.05).
compared to the ND rats. From the OGTT results, it was evident that SCE treatment in these animals improved the glucose tolerance in a dose dependent manner from day 7 to day 21 (Fig. 4. 3A - F). ITT is a measure of whole body insulin sensitivity and it helps to assess whole-body glucose disposal in response to an insulin bolus and reflects both increased skeletal muscle glucose uptake and decreased liver glucose production\textsuperscript{33,34}. ITT result revealed that SCE treatment for 3 weeks substantially enhanced the insulin sensitivity of HFS animals compared to respective controls (Fig. 4. 4A and B).

The fructose-fed rat model develops an insulin-resistance syndrome with a very similar metabolic profile to the human condition, including hyperinsulinemia and insulin resistance\textsuperscript{35-37}. The animals in the HFS group showed hyperinsulinemia (Fig. 4. 5A). However, SCE treatment significantly (p≤0.05) reduced the elevated plasma insulin level. HOMA-IR analysis of fasting glucose and insulin serve as reasonable and reliable method to measure the insulin sensitivity and beta-cell function of rodent models as well as human subjects\textsuperscript{38}. From the HOMA-IR results, it was clear that the degree of insulin resistance is high in HFS vehicle group towards the end of the experiment period from day 0 to day 21 (Fig. 4. 5C). Adiponectin is the unique adipokine secreted from adipocytes and its expression is downregulated under obese conditions\textsuperscript{39}. Also, plasma adiponectin levels negatively correlate with visceral fat accumulation and insulin resistance\textsuperscript{40,41}. Chronic fructose consumption reduces adiponectin responses, contributing to insulin resistance\textsuperscript{42}. Adiponectin plays a protective role against insulin resistance by regulating glucose and lipid metabolism\textsuperscript{43}. Concordant with previous reports, the plasma adiponectin level of HFS animals was significantly reduced compared to that of ND group but it was brought back to normal level to certain extent by SCE application (Fig. 4. 5B). As we have seen in Chapter 2, in our \textit{in vitro} studies involving differentiated 3T3-L1 adipocytes also, adiponectin level was found to be elevated under SCE treatment. These identifications confirmed the insulin sensitizing action of SCE. It is reported that the bioactive compound, phloretin 2’glucoside present in SCE improves both expression and secretion of adiponectin\textsuperscript{44}. So, the presence of phloretin 2’glucoside in SCE may be one of the reasons behind its effect in the plasma adiponectin level of HFS animals. In addition, both the doses of SCE and metformin also assisted in protecting the pancreas from its structural as well as functional alterations (Fig. 4. 5D a - f).
Postprandial hypertriglyceridemia resulting from the intake of high fructose is the earliest metabolic perturbation associated with fructose consumption\textsuperscript{45}. This is due to the accelerated production of pyruvate and glycerol-3-phosphate which promote the elevated hepatic \textit{de novo} lipogenesis and upregulated VLDL production and secretion\textsuperscript{45}. According to Thorburn et al. and others relatively mild, short term elevation of triglyceride is sufficient to impair insulin action in peripheral tissues\textsuperscript{29, 46}. Subsequently, diet rich in fructose and saturated fat causes the development of an atherogenic lipid profile with elevated concentration of triglyceride rich remnant lipoproteins, small dense LDL-C and decreased concentration of HDL-C\textsuperscript{47}. Here also, HFS vehicle group exhibited hypertriglyceridemia, hypercholesterolemia, elevated LDL and low HDL level compared to the normal control group (Fig. 4. 6A - E). But in comparison with the HFS vehicle group, SCE 250/500 or Met 100 treated HFS groups showed a good TG-HDL ratio (2.84, 2.63 and 2.36 respectively) and lower TC and LDL levels indicating protective property of SC against fructose and saturated fat induced dyslipidemia. We have already noticed the beneficial effect of SC against lipid abnormalities associated with hyperglycemia in Chapter 3.

High energy food consumption affects not only the general metabolism but also our excretory system. There are a number of reports to link diet related complications and kidney function. High fructose intake for several weeks stimulates uncontrolled uric acid production in SD rats and elevated plasma uric acid levels\textsuperscript{48}. Initially, fructose causes overproduction of ATP; as ATP is consumed AMP accumulates and stimulates AMP deaminase, resulting in uric acid production and elevated plasma uric acid level, even though the rats possess uricase enzyme\textsuperscript{49, 50}. Uric acid has potent effects on proximal tubular cells stimulating MCP-1 production and mediates renal inflammatory responses\textsuperscript{51}. The increasing level of uric acid in rats has also been shown to induce mild renal damage and promote established renal complications by the mechanisms like uric acid-dependent renal vasoconstriction and glomerular hypertension\textsuperscript{52, 53}. In the present study, HFS animals also exhibited elevated plasma uric acid levels compared to the ND animals (Fig. 4. 7B). We found that SCE administration significantly (p≤0.05) reduced plasma uric acid in HFS animals. The plasma albumin level of HFS animals was found to be decreased but SCE restored this abnormal albumin level (Fig. 4. 7A). Hyperuricemia induces renal inflammation by increased macrophage infiltration, upregulated expression of
Figure 4. 14A-D. The gene and protein expression in soleus muscle isolated from HFS fed SD rats after 21 days of SCE treatment (A) Relative mRNA level of genes: Akt-2, GLUT-4 and SCD-1 (B) Protein expression of insulin receptor substrate-2 (IRS-2), (B) pAKT-2 (C) GLUT-4. Data are expressed as the mean ± SD, n = 6. ‘a’ represents groups differ significantly from ND group (p≤0.05). ‘b’ represents groups differ significantly from HFS group (p≤0.05). ‘ab’ represents groups differ significantly from both HFS and ND group (p≤0.05).

proinflammatory cytokines like MCP-1 and TNF alpha. MCP-1 plays a crucial role in inflammation mediated renal damage via promoting IL-6 release. Expression level of IL-6 in the renal tissue correlates with the degree of mesangial hyperproliferation, tubular atrophy, and the intensity of interstitial infiltrates. Similarly, we have noticed elevated expression of MCP-1, TNF alpha and IL-6 in the renal tissue of HFS animals (Fig. 4. 9D and E). The abnormal levels of proinflammatory cytokines in renal tissue was substantially ameliorated by SCE 250/500 or Met treatment in HFS animals. In addition the improved renal histology observed in the SCEs and Met groups compared to the HFS vehicle group (Fig. 4. 7D a - f) again supports the beneficial effect of SCE against HFS induced renal damage.

The high caloric state generated by the consumption of a combination of fructose and saturated fat resulted in increased plasma ALT level in human subjects.
a diet rich in fructose and saturated fat showed significantly higher hepatic expressions of IL-6 protein and TNF alpha protein. We also observed, the liver abnormalities like elevated serum AST and ALT levels, inflammatory infiltrations and abnormal expression of inflammatory cytokines like IL-6 and TNF alpha in the liver tissue of HFS treated animals (Fig. 4. 8A and B; Fig. 4. 9E and F). Rats fed with fructose enriched (60%) diet are found to develop macrovesicular and microvesicular steatosis in the liver like T2DM patients. These inflammatory responses and hepatic lipid accumulation explains the reason behind the abnormal liver function. Administration of SCE 250/500 or metformin significantly improved various biomarkers and architecture of liver in HFS rats (Fig. 4. 8C a - f).

The glycolytic enzyme, GK and gluconeogenic enzymes like G6Pase and PEPCK are the key liver enzymes that regulate glucose homeostasis. GK is considered to be instrumental in regulating hepatic glucose uptake according to the glycaemia level. G6Pase catalyzes the dephosphorylation of glucose 6-phosphate, the terminal step of glycogenolysis and gluconeogenesis. The cytosolic PEPCK is a major flux generating enzyme for gluconeogenesis. The increased ratio of G6Pase to GK as well as the over expression of PEPCK in liver in response to chronic fructose intake contribute to the development of fasting hyperglycemia and progression into T2DM in rats under high fructose diet. Both gene expression studies and enzyme assays clearly showed that the mRNA levels as well as the enzyme activities of G6Pase and PEPCK were significantly elevated in HFS group compared to that of ND group (Fig. 4.10A and 4. 11B). Chronic fructose ingestion can lead to intra-hepatic accumulation of fructose-1-phosphate which indirectly affects hepatic glucose metabolism by decreasing the activity of GK, the hepatic sensor of glycemia. In our results also, the hepatic gene expression and enzyme activity of glucokinase was depleted (Fig. 4. 10A and 4. 11B). Thus, fructose induced insulin resistance in the liver results in the failure of insulin to inhibit hepatic glucose production via inhibiting the expression of key gluconeogenic regulatory enzymes - PEPCK and G6Pase. But, SCE administration resulted in diminished activity of gluconeogenic enzymes - PEPCK and G6Pase and improved activity of glycolytic enzyme GK in HFS animals (Fig. 4. 10A). Valverde et al. also reported that the Akt-FoxO1 signalling plays a key role in controlling the gene expression of G6Pase. FoxO1 is an important transcription factor involved in the regulation of hepatic gluconeogenesis. The pAKT
(protein kinase B) phosphorylates FoxO1 which leads to its expulsion from the nucleus for its inactivation and degradation\textsuperscript{65, 66}. Here, we have noticed the reduced expression and activity of G6Pase in HFS animals after SCE treatment which may be due to the improved insulin sensitivity via Akt-FoxO1 signalling. The oleanolic acid is reported to have ability to reduce hyperglycemia via suppression of hepatic gluconeogenesis mediated by enhanced Akt-FoxO1 signalling pathway in T2DM animal model\textsuperscript{67}. The reduced expression and activity of G6Pase and enhanced Akt-FoxO1 signalling may be partially due to the presence of oleanolic acid in SCE.

Hepatic steatosis can result from increased fatty acid influx, elevated de novo lipogenesis (DNL), and reduced fatty acid oxidation\textsuperscript{68, 69}. High fructose and saturated fat feeding caused marked increase in hepatic lipid accumulation or steatosis\textsuperscript{70}. Reports suggest that hepatic triglyceride accumulation is a major mediator of hepatic insulin resistance\textsuperscript{71}. The impaired insulin signalling in the liver leads to decreased glycogen synthesis, and increased glycogenolysis and gluconeogenesis\textsuperscript{45}. Insulin secretion increases as a compensatory response towards the elevated hepatic glucose output\textsuperscript{72}. Hyperinsulinemia leads to increased de novo lipogenesis due to insulin mediated activation of the master regulator of the hepatic lipogenesis, SREBP-1c\textsuperscript{73}. Under hyperinsulinemic conditions, the insulin-resistant liver is resistant to the effects of insulin in the carbohydrate metabolism like stimulation of glycogen synthesis and inhibition of gluconeogenesis and glycogenolysis. But, it no longer develops resistance to insulin’s effects in promoting lipogenesis\textsuperscript{74, 75}. According to Miyazaki et al.\textsuperscript{76} high fructose diet induces the expression of hepatic SREBP-1c, lipogenic gene expression including fatty acid synthase (FAS, EC 2.3.1.85) and stearoyl-CoA desaturase-1 (SCD-1, EC 1.14.19.1). SCD-1 is a microsomal rate limiting enzyme in the biosynthesis of mono-unsaturated fatty acids and high SCD-1 gene expression is highly correlated with SREBP-1c dependent and independent hepatic steatosis\textsuperscript{73, 76}. Fructose also increases the stability of FAS mRNA which is an important downstream component of lipid synthesis\textsuperscript{77}. In the present study, the expression of the lipogenic genes that promote hepatic steatosis like SREBP-1c, SCD-1 and FAS were found to be up regulated significantly in HFS group compared to ND group (Fig. 4. 11A). This upregulated lipogenic gene expression is the cause of elevated triglyceride content in the liver tissue of in HFS group in comparison with ND group (Fig. 4. 6B). Reversal of hepatic steatosis is the effective measure in improving insulin sensitivity\textsuperscript{78}. SCE 250/500
or Met treatment markedly corrected the expression levels of the lipogenic genes SREBP-1c, SCD-1 and FAS and thereby reduced the hepatic lipid content in HFS animals. From this it can be assumed that one of the mechanisms behind the improvement of insulin resistance and glucose tolerance by SCE is most probably via down regulation of SCD-1 gene expression which regulate SREBP-1c dependent and independent hepatic lipid accumulation\textsuperscript{79}. The gene expression of PPAR alpha, a transcription factor which plays central role in fatty acid oxidation was down regulated in the liver of high fructose fed rats\textsuperscript{80}. In our study, the expression level of PPAR alpha in HFS animals was found to be down regulated significantly (p ≤ 0.05). But, SCE partially restored the mRNA level of PPAR alpha in HFS rats which in turn ameliorated the abnormal lipid metabolism and the subsequent progression of hepatic insulin resistance and steatosis in these animals (Fig. 4.11A)\textsuperscript{70, 80}.

HFS group displayed a reduced protein expression level of insulin signalling markers like IRS-1 and AKT and also exhibited the decreased pAKT/tAKT ratio in the liver tissue. Diacyl glycerol, one of the key lipid intermediate that link hepatic steatosis to insulin resistance can activate protein kinases C, which in turn cause inhibitory phosphorylation of IRS-1 at serine 307 to interrupt tyrosine phosphorylation of IRS-1c and subsequent increases in the expression of FAS\textsuperscript{80, 83}. Our HFS model also exhibited dysregulated hepatic insulin signalling by exhibiting reduced expression of IRS-1 and AKT-2 and upregulated PTP-1B expression (Fig. 4.11C and 4.12A-C). So the elevated PTP-1B activity along with upregulated SCD-1 expression may be caused the increased expression of SREBP-1c and FAS in these animals. However, SCE treatment significantly alleviated these abnormalities of insulin signalling pathway.

Cellular expression and activity of SIRT-1 (EC 3.5.1) is essential in the maintenance of glucose and lipid homeostasis in the liver\textsuperscript{84}. SIRT-1 is a conserved NAD-dependent protein deacetylase and one of seven mammalian orthologs of the yeast protein silent information regulator 2 (Sir2), which performs a variety of functions ranging from energy homeostasis to extension of life span by chromatin remodeling and subsequent gene
silencing\textsuperscript{85-88}. Moderate upregulation of SIRT-1 expression might protect against metabolic disorders and hepatic steatosis induced by a high fat diet\textsuperscript{89}. Sun et al.\textsuperscript{90} reported that SIRT-1 improves insulin sensitivity by repressing PTP-1B transcription at the chromatin level. PTP-1B is the negative regulator of the insulin signal transduction cascade, which dephosphorylates insulin receptors and insulin receptor substrates\textsuperscript{91}. In the present study, we evaluated the gene expression of SIRT-1 and PTP-1B and enzyme activity of PTP-1B in the liver tissue of HFS animals treated with vehicle, SCE 250/500 or metformin (Fig. 4.11C). Concordant with the previous reports, we found that the expression level of SIRT-1 was down regulated in HFS vehicle animals while PTP-1B mRNA and enzyme activity was significantly elevated. At the same time, in the HFS animals an upregulation in the expression of SIRT-1 was noticed. This caused the diminished PTP-1B mRNA level and enzyme activity in these HFS animals treated with SCE 250/500 or metformin. Hence, it may be concluded that SIRT-1 mediated down regulation or inhibition of PTP-1B may be the one of the possible mechanisms behind the improved insulin sensitivity and hepatic glucose homeostasis exhibited by administration of SCE.

Reports show that major share of triglycerides produced in the liver in response to fructose consumption is transported by lipoproteins, such as VLDL and LDL, to the visceral and not to the subcutaneous adipose tissue. This scenario causes substantial accumulation of fat in the visceral adipose tissue\textsuperscript{45}. High fructose consumption can cause not only visceral fat accumulation but also macrophage infiltration and production of pro-inflammatory cytokines in the visceral adipose tissue and decrease in the release of adiponectin\textsuperscript{92-94}. The above conditions together with hyperlipidemia and overproduction of inflammatory cytokines in the adipocytes are responsible for the impaired insulin signalling in diet induced experimental models\textsuperscript{94}. In adipose tissue, HFS group exhibited a reduced expression of insulin signalling markers like IRS-1, AKT and GLUT-4 and pAKT/tAKT ratio was also decreased in HFS animals. In this study, SCE administration caused a partial improvement in the insulin signalling pathway in the adipose tissue (Fig. 4.13A-E). The reduced levels of plasma pro-inflammatory markers noticed by the administration of SCE have also played a role in improved insulin sensitivity in adipose tissue (Fig. 4. 9A and B). Previous reports showed that PPAR gamma, the regulator of adipocyte differentiation, insulin sensitivity and glucose homeostasis in adipose tissue...
was found to exhibit reduced expression in obese subjects and in high fat fed animal models\textsuperscript{95, 96}. The transcription factor PPAR gamma directly regulates the expression of genes such as adiponectin and GLUT-4 which can plays important role in insulin sensitivity\textsuperscript{97, 98}. We have also noticed a reduced expression of PPAR gamma in our HFS animals compared to normal group. But SCE 250/500 or Met 100 groups showed a moderate improvement in PPAR gamma expression in adipose tissue compared to HFS vehicle animals (Fig. 4. 13A and B). The elevated adiponectin level in plasma and enhanced mRNA and protein content of GLUT-4 may be due to this improvement in PPAR gamma expression. We have also noticed a moderate adipogenic potential of SCE in our \textit{in vitro} cell line studies using differentiated 3T3-L1 cells which is described in Chapter 2.

Skeletal muscle is the major peripheral tissue in which insulin responsive glucose uptake and utilization take place\textsuperscript{99}. Insulin resistance caused by prolonged high fructose feeding is associated with increased accumulation of lipids in liver and muscle tissues of rats\textsuperscript{12}. Investigations involving high-fat fed rodent models also detected the intra-muscle triglyceride accumulation as a causative factor in the initiation of insulin resistance\textsuperscript{100, 101} and it is confirmed as one of the most consistent markers of whole-body insulin resistance\textsuperscript{102}. The insulin sensitivity can be improved by the reduction of elevated intracellular TG content in muscle\textsuperscript{103}. Significantly elevated TG accumulation was noticed in the skeletal muscle of animals of HFS group (Fig. 4. 6C). These HFS animals also exhibited an up regulated skeletal muscle expression of the SCD-1 mRNA. Reports suggest that SCD-1 gene is highly expressed in skeletal muscle tissue of extremely obese humans\textsuperscript{104} and obese insulin resistant Zucker diabetic fatty rats\textsuperscript{105} and its elevated expression in skeletal muscle contributes to abnormal lipid metabolism and progression of obesity and type 2 diabetes\textsuperscript{106}. However, SCE administration down regulated SCD-1 gene expression and diminished the lipid accumulation significantly in skeletal muscle of HFS animals in this study (Fig. 4. 14A).

Diacyl glycerol (DAG) is an important intermediate of both triglyceride and phospholipid metabolism and its level may get accumulated in the muscle tissues of animals by high fat/fructose consumption\textsuperscript{107}. DAG is one of the major second messengers involved in intracellular signalling and it may be the causative factor for the activation of protein
Figure 4.15. Possible mechanism of action of SCE in ameliorating hepatic steatosis and insulin resistance: The high fructose saturated fat (HFS) diet induced hepatic triglyceride (TG) accumulation and impaired hepatic insulin signalling via modulating gene expression (SIRT-1, SCD-1, SREBP-1c, FAS etc) and phosphorylation state of various signalling proteins like AKT, thereby causing accelerated hepatic insulin resistance. In muscle and adipose tissues, HFS diet impaired glucose utilization by dysregulated gene expression and phosphorylation of markers of insulin signalling (IRS, AKT and GLUT-4). The administration of SCE partially reversed hepatic steatosis and impaired insulin action in liver, muscle and adipose via acting at multiple molecular targets (1-9). Black arrows show the deleterious effects of HFS diet, white arrows unveil the protective effect of SCE.
kinase C and thereby results in impaired insulin signalling cascade and phosphorylation events\textsuperscript{108}. We have noticed fructose and fat rich diet resulted in a reduced gene and protein expression level of mediators of insulin signalling pathway like IRS-1, AKT and GLUT-4 in skeletal muscle (Fig. 4. 14A). The ratio of pAKT/tAKT was also found to be decreased in HFS animals (Fig. 4. 14C). The intramuscular accumulation of lipid metabolism intermediates by high fat and fructose consumption and the subsequent activation of protein kinase C may be the rationale behind this impaired insulin action in skeletal muscle. But, SCE administration caused a partial restoration of the insulin signalling pathway of skeletal muscle. A summary of mechanism of action of SCE in HFS fed rat model of insulin resistance and dyslipidemia is illustrated in Fig. 4. 15.

### 4. 5 Conclusion

The SCE was found to be an effective agent against high fructose saturated fat diet model of insulin resistance and dyslipidemia in SD rats. It is also worth to mention that SCE showed dose dependent effect in most of the parameters studied. The overall study revealed the beneficial property of SCE against insulin resistance, glucose intolerance, and dyslipidemia which are the major predictors of T2DM via improving hepatic glucose homeostasis, lipid metabolism and peripheral insulin signalling in SD rats. SIRT-1 mediated down regulation or inhibition of PTP-1B enzyme may be the mechanisms behind the insulin sensitizing and improved hepatic glucose homeostatic property of SCE. In addition, down regulation of SCD-1 gene expression which modulate SREBP-1c dependent and independent hepatic lipid accumulation also contribute partially to its antidiabetic activity.
References


