

**Investigation on key molecular targets responsible for antidiabetic  
properties of *Symplocos cochinchinensis* (Lour.) S. Moore**

**Thesis submitted under the Faculty of Science of the  
Cochin University of Science and Technology  
for the award of the degree of**

**Doctor of Philosophy  
in  
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**By**

**ANTU K. ANTONY  
(Reg. No. 3869)**

*Under the supervision of*

**Dr. K. G. RAGHU**



**Agroprocessing and Natural Products Division  
Council of Scientific and Industrial Research–National Institute for  
Interdisciplinary Science and Technology (CSIR-NIIST)  
Thiruvananthapuram – 695 019, Kerala, India.**

**DECEMBER 2014**



Telephone: 91-471-2515486

Fax: 91-471-2491712

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**Dr. K. G. Raghu**  
**Principal Scientist**  
**Agroprocessing and Natural Products Division**

**CERTIFICATE**

*This is to certify that the work embodied in the thesis entitled “Investigation on key molecular targets responsible for antidiabetic properties of **Symplocos cochinchinensis (Lour.) S. Moore**” has been carried out by **Ms. Antu K. Antony**, under my supervision and guidance at Agroprocessing and Natural Products Division of Council of Scientific and Industrial Research-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram in partial fulfillment of the requirements for the award of degree of Doctor of Philosophy in Biotechnology under Faculty of Science, Cochin University of Science and Technology, Kochi, Kerala, India and the same has not been submitted elsewhere for any other degree. All the relevant corrections, modifications and recommendations suggested by the audience and the doctoral committee members during the pre-synopsis seminar of **Ms. Antu K. Antony** has been incorporated in the thesis.*

**K. G. Raghu**  
**(Thesis Supervisor)**

Thiruvananthapuram,  
December, 2014.

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**e-mail:** raghukgopal2009@gmail.com; raghukgopal@rediffmail.com

## DECLARATION

I hereby declare that thesis entitled “**Investigation on key molecular targets responsible for antidiabetic properties of *Symplocos cochinchinensis* (Lour.) S. Moore**” embodies the results of investigations carried out by me at Agroprocessing and Natural Products Division of Council of Scientific and Industrial Research - National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram as a full time research scholar under the supervision of Dr. K. G. Raghu and the same has not been submitted elsewhere for any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described is based on the findings of other investigators.

**Antu K. Antony**

Thiruvananthapuram,  
December, 2014

*Dedicated to...*

*My Lord and My God Jesus Christ and His Holy Spirit...*

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## List of Abbreviations

% HbA1c	:Percentage of glycated hemoglobin
AAS	:Atomic absorption spectrophotometer
ABTS	:2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADA	:American Diabetes Association
AG	:Alpha-glucosidase
AGEs	:Advanced glycation end products
AGI	:Alpha glucosidase inhibitor
ALT	:Alanine aminotransferase
AR	:Aldose reductase
AST	:Aspartate aminotransferase
BCA	:Bicinchoninic acid
BGL	:Blood glucose level
BSA	:Bovine serum albumin
DMSO	:Dimethyl sulphoxide
DPPH	:2,2 diphenyl-1-1-picryl hydrazyl
DPP-IV	:Dipeptidyl peptidase-IV
DTT	:Dithiothreitol
EDTA	:Ethylene diamine tetraacetic acid
ELISA	:Enzyme linked immunosorbant assay
FAS	:Fatty acid synthase
G6Pase	:Glucose 6 phosphatase
GIP	:Glucose-dependent insulintropic polypeptide
GK	:Glucokinase
GLP-1	:Glucagon-like peptide-1
GPDH	:Glyceraldehyde 3-phosphate dehydrogenase
Gpx	:Glutathione peroxidase
GSH	:Reduced glutathione
GSIS	:Glucose-stimulated insulin secretion
HDL	:High density lipoproteins
HEPES	:4-(2-Hydroxyethyl)-1-piperazine ethane sulfonic acid

HFS diet	:High fructose saturated fat diet
HOMA-IR	:Homeostatic model assessment - insulin resistance
HPLC	:High performance liquid chromatography
IBMX	:2-deoxyglucose, 3-isobutyl-1-methylxanthine
IDDM	:Insulin dependent diabetes mellitus
IDF	:International Diabetes Federation
IL-6	:Interleukin-6
ITT	:Insulin Tolerance Test
LDL	:Low density lipoproteins
MCP-1	:Monocyte chemoattractant protein-1
MDA	:Malondialdehyde
mg/kg bwd	:mg per kg body weight per day
NADPH	:Nicotinamide adenine dinucleotide phosphate
OGTT	:Oral Glucose Tolerance Test
OSTT	:Oral Sucrose Tolerance Test
PEPCK	:Phosphoenol pyruvate carboxy kinase
PPAR	:Peroxisome proliferator - activated receptor
PPH	:Postprandial hyperglycemia
PTP-1B	:Protein tyrosine phosphatase-1B
qRT-PCR	:Quantitative reverse transcriptase-polymerase chain reaction
RFU	:Relative fluorescent unit
RIPA buffer	:Radio-immunoprecipitation assay buffer
ROS	:Reactive oxygen species
SC	: <i>Symplocos cochinchinensis</i>
SCD	:SC dichloromethane fraction
SCD-1	:Stearoyl CoA desaturase-1
SCE	: <i>Symplocos cochinchinensis</i> ethanolic extract
SCEC	:SC ethyl acetate fraction
SCEL	:SC ethyl alcohol fraction
SCH	:SC hexane fraction
SD	:Sprague Dawley
SEM	:Scanning electron microscope
SGLT-2	:Sodium / glucose cotransporter-2

SIRT-1	:sirtuin (silent mating type information regulation 2 homolog) - 1
SLM	:Sucrose loaded mild diabetic rat model
SOD	:Superoxide dismutase
SREBP-1c	:Sterol regulatory element binding protein-1c
STZ	:Streptozotocin
STZ-S	:Sucrose-challenged streptozotocin- diabetic rat model
T1DM	:Type 1 diabetes
T2DM	:Type 2 diabetes
TBS	:Tris borate buffer
TFC	:Total flavonoid content
TG	:Triglyceride
TNF alpha	:Tumor necrosis factor alpha
TPC	:Total phenolic content
TTC	:Total tannin content
WHO	: World Health Organization

## Synopsis

Diabetes mellitus is a heterogeneous metabolic disorder characterized by hyperglycemia with disturbances in carbohydrate, protein and lipid metabolism resulting from defects in insulin secretion, insulin action or both. Currently there are 387 million people with diabetes worldwide and is expected to affect 592 million people by 2035. Insulin resistance in peripheral tissues and pancreatic beta cell dysfunction are the major challenges in the pathophysiology of diabetes. Diabetic secondary complications (like liver cirrhosis, retinopathy, microvascular and macrovascular complications) arise from persistent hyperglycemia and dyslipidemia can be disabling or even life threatening. Current medications are effective for control and management of hyperglycemia but undesirable effects, inefficiency against secondary complications and high cost are still serious issues in the present prognosis of this disorder. Hence the search for more effective and safer therapeutic agents of natural origin has been found to be highly demanding and attract attention in the present drug discovery research. The data available from *Ayurveda* on various medicinal plants for treatment of diabetes can efficiently yield potential new lead as antidiabetic agents. For wider acceptability and popularity of herbal remedies available in *Ayurveda* scientific validation by the elucidation of mechanism of action is very much essential. Modern biological techniques are available now to elucidate the biochemical basis of the effectiveness of these medicinal plants. Keeping this idea the research programme under this thesis has been planned to evaluate the molecular mechanism responsible for the antidiabetic property of *Symplocos cochinchinensis*, the main ingredient of *Nishakathakadi Kashayam*, a well-known *Ayurvedic* antidiabetic preparation. A general introduction of diabetes, its pathophysiology, secondary complications and current treatment options, innovative solutions based on phytomedicine etc has been described in Chapter 1.

The effect of *Symplocos cochinchinensis* (SC), on various *in vitro* biochemical targets relevant to diabetes is depicted in Chapter 2 including the preparation of plant extract. Since diabetes is a multifactorial disease, ethanolic extract of the bark of SC (SCE) and its fractions (hexane, dichloromethane, ethyl acetate and 90 % ethanol) were evaluated by *in vitro* methods against multiple targets such as control of postprandial hyperglycemia, insulin resistance, oxidative stress, pancreatic beta cell proliferation, inhibition of protein glycation, protein tyrosine phosphatase-1B (PTP-1B) and dipeptidyl peptidase-IV (DPP-

IV). Among the extracts, SCE exhibited comparatively better activity like alpha glucosidase inhibition, insulin dependent glucose uptake (3 fold increase) in L6 myotubes, pancreatic beta cell regeneration in RIN-m5F and reduced triglyceride accumulation in 3T3-L1 cells, protection from hyperglycemia induced generation of reactive oxygen species in HepG2 cells with moderate antiglycation and PTP-1B inhibition. Chemical characterization by HPLC revealed the superiority of SCE over other extracts due to presence of bioactives (beta-sitosterol, phloretin 2'glucoside, oleanolic acid) in addition to minerals like magnesium, calcium, potassium, sodium, zinc and manganese. So SCE has been subjected to oral sucrose tolerance test (OGTT) to evaluate its antihyperglycemic property in mild diabetic and diabetic animal models. SCE showed significant antihyperglycemic activity in *in vivo* diabetic models.

Chapter 3 highlights the beneficial effects of hydroethanol extract of *Symplocos cochinchinensis* (SCE) against hyperglycemia associated secondary complications in streptozotocin (60 mg/kg body weight) induced diabetic rat model. Proper sanction had been obtained for all the animal experiments from CSIR-CDRI institutional animal ethics committee. The experimental groups consist of normal control (NC), N + SCE 500 mg/kg bwd, diabetic control (DC), D + metformin 100 mg/kg bwd, D + SCE 250 and D + SCE 500. SCEs and metformin were administered daily for 21 days and sacrificed on day 22. Oral glucose tolerance test, plasma insulin, % HbA1c, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total protein etc. were analysed. Aldose reductase (AR) activity in the eye lens was also checked. On day 21, DC rats showed significantly abnormal glucose response, HOMA-IR, % HbA1c, decreased activity of antioxidant enzymes and GSH, elevated AR activity, hepatic and renal oxidative stress markers compared to NC. DC rats also exhibited increased level of plasma urea and creatinine. Treatment with SCE protected from the deleterious alterations of biochemical parameters in a dose dependent manner including histopathological alterations in pancreas. SCE 500 exhibited significant glucose lowering effect and decreased HOMA-IR, % HbA1c, lens AR activity, and hepatic, renal oxidative stress and function markers compared to DC group. Considerable amount of liver and muscle glycogen was replenished by SCE treatment in diabetic animals. Although metformin showed better effect, the activity of SCE was very much comparable with this drug.

The possible molecular mechanism behind the protective property of *S. cochinchinensis* against the insulin resistance in peripheral tissue as well as dyslipidemia in *in vivo* high fructose saturated fat diet model is described in Chapter 4. Initially animal were fed a high fructose saturated fat (HFS) diet for a period of 8 weeks to develop insulin resistance and dyslipidemia. The normal diet control (ND), ND + SCE 500 mg/kg bwd, high fructose saturated fat diet control (HFS), HFS + metformin 100 mg/kg bwd, HFS + SCE 250 and HFS + SCE 500 were the experimental groups. SCEs and metformin were administered daily for the next 3 weeks and sacrificed at the end of 11<sup>th</sup> week. At the end of week 11, HFS rats showed significantly abnormal glucose and insulin tolerance, HOMA-IR, % HbA1c, adiponectin, lipid profile, liver glycolytic and gluconeogenic enzyme activities, liver and muscle triglyceride accumulation compared to ND. HFS rats also exhibited increased level of plasma inflammatory cytokines, upregulated mRNA level of gluconeogenic and lipogenic genes in liver. HFS exhibited the increased expression of GLUT-2 in liver and decreased expression of GLUT-4 in muscle and adipose. SCE treatment also preserved the architecture of pancreas, liver, and kidney tissues. Treatment with SCE reversed the alterations of biochemical parameters, improved insulin sensitivity by modifying gene expression in liver, muscle and adipose tissues. Overall results suggest that SC mediates the antidiabetic activity mainly via alpha glucosidase inhibition, improved insulin sensitivity, with antiglycation and antioxidant activities.

The conclusions of all the results described above and suggestions for future work are presented in last section of the thesis.

### Introduction

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#### Prelude

A dramatic change in the lifestyle and dietary patterns has occurred worldwide during the last century across the populations. The marked variations in human environments and the accompanied globalization accelerated these changes. The switching of work culture from heavy labour to sedentary, increased mechanization, improved transportation facilities are some of the changes that have had an impact on human health<sup>1</sup>. Physical inactivity and excessive intake of energy-dense and nutrient-poor food options are the two major behavioural contributors to the incidence and prevalence of non-communicable diseases (NCDs)<sup>2</sup>. NCDs such as diabetes and cardiovascular diseases have now become the main public health challenges for the 21<sup>st</sup> century and affect the productivity of people<sup>1</sup>. In both developed and developing nations, diabetes epidemic, particularly type 2 diabetes (T2DM) remains as a major threat<sup>3</sup>. Paradoxically, this also relates to the achievements in public health care during the 20<sup>th</sup> century that resulted in the increase of average life expectancy owing to the elimination of many of the communicable diseases<sup>4, 5</sup>. In the absence of effective and affordable interventions for both types of diabetes, the frequency of diabetes will be further escalating worldwide<sup>6, 7</sup>. Even though, researchers and clinicians are involved in the study and development of various pharmacological interventions, diabetes and its secondary complications continue to be a global epidemic and there is high demand for novel treatment options without much adverse effects. The increasing prevalence of diabetes and obesity has already imposed a huge burden on health-care systems and this will continue to increase in the future<sup>1, 8</sup>. Although T2DM is numerically more prevalent in the general population and type 1 diabetes is restricted to a small population of children; the condition may be reversed within one to two decades because of the increasing prevalence of T2DM in children and adolescents<sup>9, 10</sup>.



## 1. 1 Diabetes

“Diabetes is a mysterious illness”, the statement by Greek physician Araetaus around 130 AD is still relevant<sup>11</sup>. Diabetes mellitus is a major epidemic of this century<sup>12</sup> which has increased in incidence by 50% over the past 15 years<sup>13</sup>. Despite, this mysterious and multifactorial illness has become the modern epidemic; the surprising fact is that, diabetes is one of the world’s oldest diseases, described in historical records of civilizations such as those found in ancient Egypt, Persia, and India<sup>14-16</sup>. Diabetes mellitus is a heterogeneous disorder characterized by chronic hyperglycaemia and the etiological heterogeneity is suggested by genetic inheritance and its interplay with environmental factors<sup>17</sup>. One theory concerning the etiology of diabetes tells that it is the result of the evolution of a thrifty genotype that had survival benefits in the past but is detrimental in the current environment; the opposing theory is that diabetes represents an adult metabolic response to fetal malnutrition<sup>18</sup>.

## 1. 2 History of diabetes

The first reference to diabetes mellitus in the history was described about 3500 years ago by the ancient Egyptians in ‘Ebers Papyrus’ (1550 BC) that mentions remedies for the treatment of excessive urination or polyurea and has detected by German Egyptologist Georg Ebers in 1872 from Thebes<sup>19, 20</sup>. Initially, the term ‘diabetes’ was introduced by Araeteus of Cappodocia (81-133 AD) from the Greek word for siphon<sup>14</sup>. The Greek medical writer Galen, a contemporary of Aretaues mentioned the condition as “diarrhea of the urine” and “the thirsty disease.” Indian physicians Charaka and Sushruta noted the attraction of flies and ants to the urine of those affected by this ailment, coined the term ‘*madhumeha*’ or ‘honey urine’ and also detected that patients suffering from *madhumeha* exhibited extreme thirst and foul breath; a thousand years before the first Europeans recognized the sweet taste of urine in patients with diabetes<sup>19</sup>. The word ‘mellitus’ (honey sweet) was added by Thomas Willis from Britain in 1675 after rediscovering the sweetness of urine and blood of patients<sup>14</sup>. In 1776, British researcher Matthew Dobson gave first experimental evidence for the excretion of sugar in the urine of people suffering from diabetes in a paper presented to the medical society of London<sup>19</sup>. In 1857, Claude Bernard from France discovered the role of liver in diabetes and it was established

through the identification of the fact that glycogen is stored in liver as the precursor of glucose<sup>14</sup>.

### **1. 2. 1 Discovery of insulin**

The key milestone in the history of diabetes is the discovery of role of the pancreas in pathogenesis of diabetes by Austrian researchers Mering and Minkowski in 1889<sup>21</sup>. Minkowski hypothesised that pancreas produces a substance which regulate blood glucose level. In 1893, French scientist Gustave-Edouard Laguesse suggested that tiny islands of pancreatic tissue described by Paul Langerhans in 1869 might be the source of the substance involved in blood glucose control. Distinguished German pathologist, Paul Langerhans, a student of Rudolf Virchow described small groupings of pancreatic cells that were not drained by pancreatic ducts in his doctoral thesis. Belgian physician Jean de Mayer named the presumed substance produced by the islets of Langerhans “insulin” in 1909<sup>22</sup>. Moses Barron reported a rare case of a pancreatic stone in 1920 that blocked the main pancreatic duct and the blockage caused the degeneration of the acinar glandular cells but not the islet cells. This report by Barron as well as the hypothesis put forward by Minkowski stimulated research ideas of Frederick Grant Banting, an orthopedic surgeon who joined in John J. R Macleod’s laboratory at University of Toronto, Canada. The idea was to surgically ligate the pancreas to stop the flow of nutrients towards acinar glandular cells, which may cause the disintegration of these exocrine cells that produces digestive pancreatic juice and preservation of the islet cells for the preparation of pancreatic islet extracts<sup>23</sup>. In 1921, Banting along with the physiology student Charles Best and biochemist James Collip succeeded in purifying an active extract from atrophied pancreatic glands of the laboratory dogs and was effectively reversed the diabetic symptoms in pancreatectomized diabetic dogs<sup>24</sup>. Banting named the isolated antidiabetic substance as ‘isletin’ but Macleod suggested the name ‘insulin’ without knowing that the name ‘insuline’ had already been coined by de Mayer in 1909<sup>23</sup>. Till the early 20<sup>th</sup> century the life expectancy of children with type 1 diabetes was only about 2 years. The news of the discovery of insulin was accepted internationally with tremendous enthusiasm and thus many children suffering from diabetes were reinstated to health. In 1923, Banting and Macleod were awarded Nobel Prize for the discovery of insulin.

### 1. 3 Definition and classification of diabetes

According to World Health Organization (WHO) diabetes is defined as group of metabolic disorders characterized by hyperglycemia with disturbances in carbohydrate, protein and lipid metabolism resulting from defects in insulin secretion, insulin action or both<sup>25</sup>. Diabetes can be raised from a variety of abnormalities. These ranges from mechanisms that cause pancreatic beta-cell degeneration or cessation of insulin production and secretion to conditions that cause insulin insensitivity to peripheral tissues<sup>26</sup>. The insensitivity or lack of insulin results in deficient insulin action on its target organs and results in deranged carbohydrate, protein and lipid metabolism<sup>27</sup>. Based on the pathogenesis of hyperglycemia diabetes is classified into 3 etiological types along with some minority cases due to various specific metabolic or genetic causes<sup>28</sup>. The various forms of diabetes is listed in Table 1. 1.

<b>Classification of Diabetes Mellitus</b>	
Type 1 diabetes mellitus	
Type 2 diabetes mellitus	
Gestational diabetes	
<b>Other forms:</b>	
Pancreatic disease:	chronic pancreatitis, pancreatectomy, cystic fibrosis, haemochromatosis, pancreatic carcinoma
Endocrine:	acromegaly, Cushing's syndrome, thyrotoxicosis, phaeochromocytoma
Drug induced:	corticosteroids, thiazide diuretics
Conditions with specific genetic causes:	e.g., maturity-onset diabetes of the young (MODY)

**Table 1. 1.** Classification of diabetes mellitus (reproduced from the reference 28 with permission).

#### 1. 3. 1 Type 1 Diabetes

Type 1 diabetes (T1DM) was previously termed as insulin dependent diabetes mellitus (IDDM) or juvenile onset diabetes. T1DM accounts for about 5-10% of diabetes prevalence results from a cellular mediated autoimmune destruction of insulin producing pancreatic beta cells, leading to absolute insulin deficiency<sup>26</sup>. Patients with T1DM has been found to carry a number of autoantibodies in their blood targeted to pancreas, the

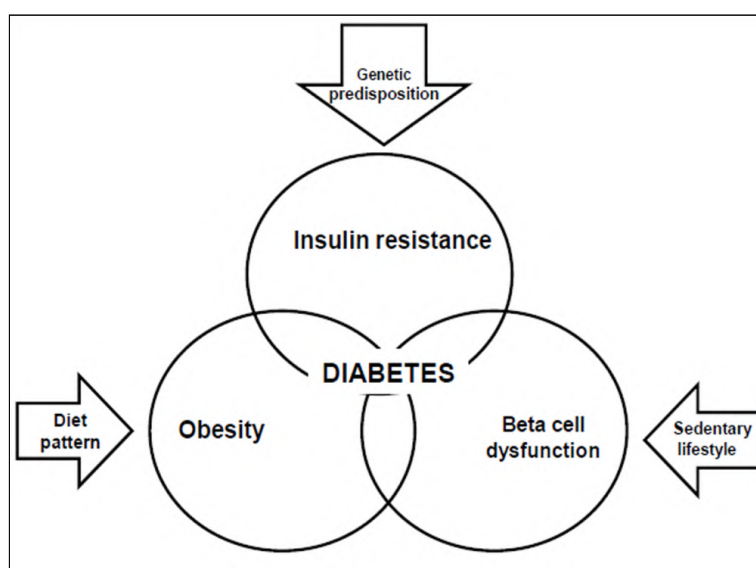
site of production and secretion of insulin such as islet cell antibodies (ICA), insulin autoantibodies (IAA), and antibodies against glutamic acid decarboxylase (GAD) and insulinoma-associated autoantigen-2 (IA-2), a proteins found in pancreatic beta-cells<sup>29</sup>. Development of chronic hyperglycemia and keto-acidosis are the major manifestations of IDDM. The rate of pancreatic beta cell degeneration varies from individual to individual under this condition which may be rapid in infants and children; this can be reason behind the early classifications like juvenile onset diabetes<sup>26</sup>.

### **1. 3. 2 Type 2 Diabetes**

Type 2 diabetes is the global epidemic that constitutes about 90-95% of those with diabetes and referred to as non-insulin dependent diabetes (NIDDM) or adult onset diabetes. The advancement of the etiology of T2DM results from the combination of impaired biological response to insulin i. e insulin resistance and relative insulin deficiency and the subsequent derangement in carbohydrate, protein and lipid metabolism<sup>25, 26</sup>. The progression from normal glucose tolerance to abnormal glycemia results from these two fundamental defects in the pathogenesis of T2DM and is caused by the combination of genetic and environmental factors (Figure 1. 1). The genetic factors may be primary or secondary; primary genetic factors include the genes responsible for diabetes or 'diabetogenes' and the diabetes induced modulations in gene expression constitute secondary factors<sup>30</sup>. Environmental factors such as diet, obesity and physical activity may act as initiating factors or progression factors for T2DM<sup>30</sup>.

### **1. 3. 3 Gestational Diabetes**

Any degree of glucose intolerance developed during the pregnancy period is termed as gestational diabetes mellitus (GDM). In the early phase of pregnancy i. e first trimester and first half of second trimester, the fasting and postprandial glucose concentrations are normally lower than in normal, non-pregnant women. So elevated fasting or postprandial plasma glucose levels at this time in pregnancy may well reflect the presence of diabetes<sup>27</sup>. One of the undesirable effects of GDM is that there are chances to continue to be in hyperglycaemic state by the person with GDM even after the delivery and further progression to T2DM<sup>26</sup>.

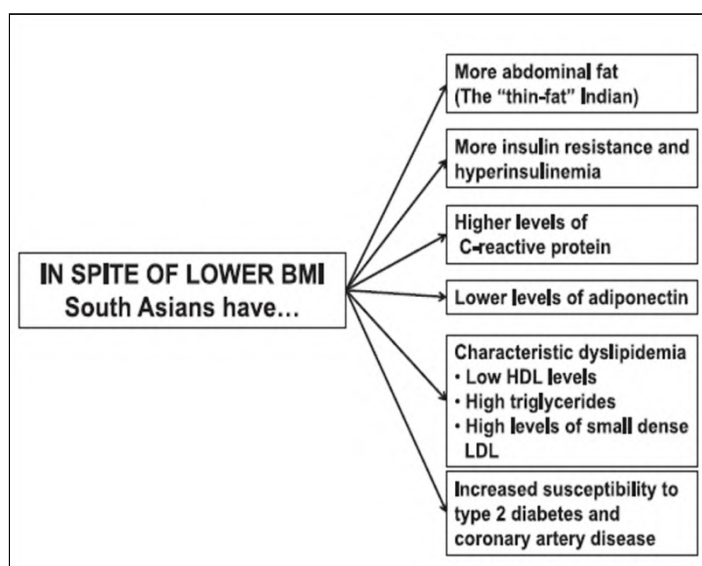


**Figure 1. 1.** Role of genetics and environment in the pathogenesis of diabetes.

#### 1. 4 Diabetes prevalence - Global burden and ethnicity

The number of people living with diabetes and mortality due to diabetes across the world is shocking<sup>31</sup>. Currently there are 387 million people with diabetes worldwide whereas 175 million people with diabetes are undiagnosed and is expected to affect 592 million people by 2035<sup>13</sup>. Diabetes caused 4.9 million deaths in 2013 and in every 7 seconds a person dies from diabetes<sup>13</sup>. The incidence (number of new cases per year per unit of population) and prevalence (number of known cases per unit of population) of diabetes vary significantly with geographical locations<sup>28</sup>. Europe has the highest prevalence of T1DM in children and lowest prevalence in Japan<sup>13</sup>. T2DM has the highest prevalence in Pima Indians, Pacific Islanders, South Asians, Hispanics, and Africans<sup>28</sup>. South Asian region which constitute India and China forms one of the epicenters of the global diabetes epidemic<sup>31, 32</sup>. Investigations in the South Asian population residing in the UK during the early 1980s suggested the possibility of an Asian Indian or South Asian phenotype with a characteristic metabolic profile as shown in Figure 1. 2. South Asian phenotype refers to a combination of characteristics that predisposes South Asian to the development of insulin resistance, T2DM, and cardiovascular disease<sup>32</sup>. T2DM occurs at younger ages and at

lower levels of BMI in South Asian compared with Caucasians<sup>33</sup>. In spite of a relatively lower rate of obesity as defined by BMI cut points, South Asian tend to have larger waist measurements and waist-to-hip ratios, indicating a greater degree of central body obesity<sup>34</sup>. In India, 65 million people are diabetic, the nationwide prevalence is around 9%, but in the relatively prosperous southern cities it is as high as 20%<sup>31</sup>. Surprisingly, Kerala the most literate and socially developed state of India is reported to have 27% of diabetes population even with the availability of high class medical facilities<sup>35</sup>. This is a very alarming situation and need detailed investigation and intervention to curtail the uncontrolled growth of this metabolic disorder. The study by Bakker et al. suggests that only a short term high fat, high calorie diet impairs insulin sensitivity in healthy, young South Asian men but not in Caucasian men<sup>36</sup>. Thus, Indians carrying the South Asian phenotype and having a tendency to follow a sedentary lifestyle along with a high fat, high carbohydrate, and high calorie dietary pattern suggest the possible means of epidemic of T2DM in this subcontinent.



**Figure 1. 2.** Metabolic profile of South Asian (Asian Indian) phenotype (reproduced from the reference 32 with permission).

### 1. 5 Diagnosis

The established glucose criteria for the diagnosis of diabetes include the fasting plasma glucose (FPG) and 2 hour postprandial glucose (2 h PG) during an oral glucose tolerance test (OGTT). The prescribed diagnostic cut point for FPG is  $\geq 126$  mg/dl (7.0 mM) and

that of diagnostic 2-h PG value is  $\geq 200$  mg/dl (11.1 mM)<sup>26</sup>. According to WHO, OGTT should be performed using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water to find out the 2-h PG value<sup>2</sup>. A random (or casual) plasma glucose analysis is also required for patients with severe classic hyperglycemic symptoms or hyperglycaemic crisis<sup>26</sup>. The American Diabetes Association (ADA) diagnostic criteria for diabetes are given in Table 1. 2. For the initial assessment of the severity of the diabetes an HbA1c test is also recommended by ADA as HbA1c is a widely used marker of chronic glycemia, reflecting average blood glucose levels over a 2 to 3 months period of time. Diagnostic cut point of HbA1c is 6.5% and this test correlates well with microvascular and, to a lesser extent, macrovascular complications and is widely used as the standard biomarker for the adequacy of glycemc management<sup>37</sup>. The HbA1c test has several advantages over the FPG such as greater convenience, fasting is not required, greater pre-analytical stability and less day-to-day perturbations during periods of stress and illness<sup>26</sup>.

Blood glucose parameter	% Glycated Hemoglobin (% HbA1c)	Fasting plasma glucose (FPG)	Postprandial plasma glucose (PPG)*
Normal	<6.5	<100 mg/dL	<140 mg/dL
Prediabetes	6.5 - 7	100 -125 mg/dL	140 -199 mg/dL
Diabetes	>7	>126 mg/dL	>199 mg/dL

**Table 1. 2.** Criteria for the diagnosis of diabetes (reproduced from ADA guidelines, 2011 with permission)  
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\*2-h plasma glucose  $>199$  mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

OR

In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose  $>199$  mg/dl (11.1 mmol/l).

### 1. 5. 1 Prediabetes

Prediabetes is a condition where blood sugar level is higher than normal, but not enough to be classified as diabetes. The term impaired glucose tolerance (IGT) or pre-diabetes was first coined in 1979 by the World Health Organization and the National Diabetes Data Group to replace the terms *borderline* and *asymptomatic diabetes mellitus*.

In 1997, an expert committee of the American Diabetes Association recommended the following criteria for IGT: a normal fasting plasma glucose (<126 mg/dL) with a postprandial plasma glucose of >140 mg/dL but <200 mg/dL 2 h after a 75 g oral glucose challenge<sup>38</sup>. The pre-diabetic stage is an extremely useful marker of patients at risk for the eventual development of type 2 diabetes. Patients with IGT may benefit from timely patient education and perhaps even more aggressive forms of intervention or medication<sup>39</sup>. Prediabetic stage is the warning sign for diabetes; subjects in the prediabetic stage may be return to normal stage with careful diet and exercise.

### **1. 6 Insulin**

Insulin is a peptide hormone secreted by the beta-cells of the pancreatic islets of Langerhans and maintains normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism and promoting cell division and growth through its mitogenic effects<sup>40</sup>. Insulin is the dipeptide containing A chain and B chain linked by disulphide bridges, and composed of 51 amino acids with a molecular weight of 5802. The A chain comprises 21 amino acids and the B chain 30 amino acids (Figure 1.3A). Its iso-electric point is pH 5.5<sup>41</sup>. In 1958, Frederick Sanger was awarded his first Nobel Prize for determining the sequence of the amino acids that make up insulin.

Insulin is coded on the short arm of chromosome 11 and synthesised as its precursor, proinsulin<sup>42</sup>. Initially, Pre-proinsulin is formed by sequential synthesis of a signal peptide, the B chain, the connecting (C) peptide and then the A chain comprising a single chain of 100 amino acids in the ribosomes of the rough endoplasmic reticulum (RER) from the mRNA. Removal of the signal peptide gives rise to proinsulin, which acquires its characteristic 3 dimensional structure in the endoplasmic reticulum. Proinsulin is transferred from the RER to the Golgi apparatus through secretory vesicles; aqueous zinc and calcium rich environment in the Golgi favours formation of soluble zinc-containing proinsulin hexamers<sup>43</sup>. The immature storage vesicles are formed from the Golgi and enzymes acting outside the Golgi convert proinsulin to insulin and C peptide<sup>44</sup>. Insulin forms zinc-containing hexamers which are insoluble, precipitating as chemically stable crystals at pH 5.5. When mature granules are secreted into the circulation by exocytosis,



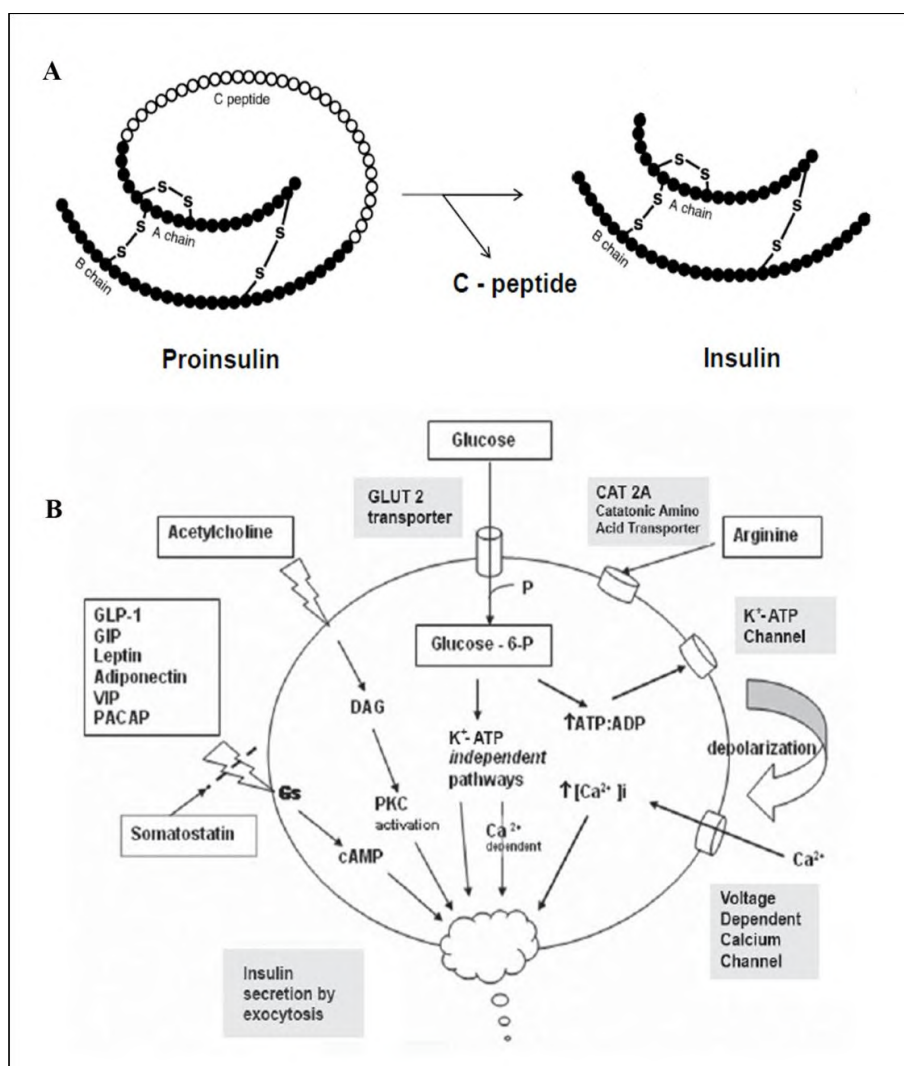
insulin, and an equimolar ratio of C-peptide are also released. Proinsulin and zinc typically comprise no more than 6% of the islet cell secretion<sup>43</sup>.

### **1. 6. 1 Insulin secretion and factors involved**

Elevated blood glucose level induces the 'first phase' of glucose stimulated insulin secretion (GSIS) by release of insulin from secretory granules of the beta-cell. Glucose entry into the beta cell is sensed by glucokinase, which phosphorylates glucose to glucose-6-phosphate (G6P) which increases rate of glycolysis and ATP production<sup>45</sup>. High intracellular ATP level cause the closure of K<sup>+</sup>-ATP-dependent channels which results in membrane depolarization and activation of voltage dependent calcium channels leading to an increase in intracellular calcium concentration; this triggers pulsatile insulin secretion<sup>46</sup>. Other mediators of insulin release include activation of phospholipases and protein kinase C (for example by acetylcholine) and by stimulation of adenylyl cyclase activity and activation of beta cell protein kinase A, which potentiates insulin secretion. This adenylyl cyclase mediated mechanism may be activated by hormones, such as vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide, glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (Figure 1. 3B). These factors appear to play a significant role in the second phase of glucose stimulated insulin secretion, after refilling of secretory granules translocated from reserve pools<sup>47</sup>.

It was found that insulin secretion from the islet cells into the portal veins is characteristically pulsatile, by the summation of coordinate secretory bursts from millions of islet cells<sup>40</sup>. Reports also suggest that insulin is more effective at reducing blood glucose levels if it is delivered in pulses rather than continuously<sup>48</sup>. In healthy individuals glucose stimulated insulin secretion from pancreas is biphasic: an initial component (first phase), which develops rapidly but lasts only a few minutes, followed by a sustained component (second phase)<sup>49, 50</sup>. The first phase starts with a rapid rise in insulin 1-3 minutes after the plasma glucose elevation and it returns towards baseline 6-10 minutes after glucose stimulation; in the second phase the insulin level raises gradually once again<sup>51</sup>. Loss or diminished first-phase secretion and reduced second-phase secretion are characteristic features of T2DM and it is well known that a decrease in the first phase of

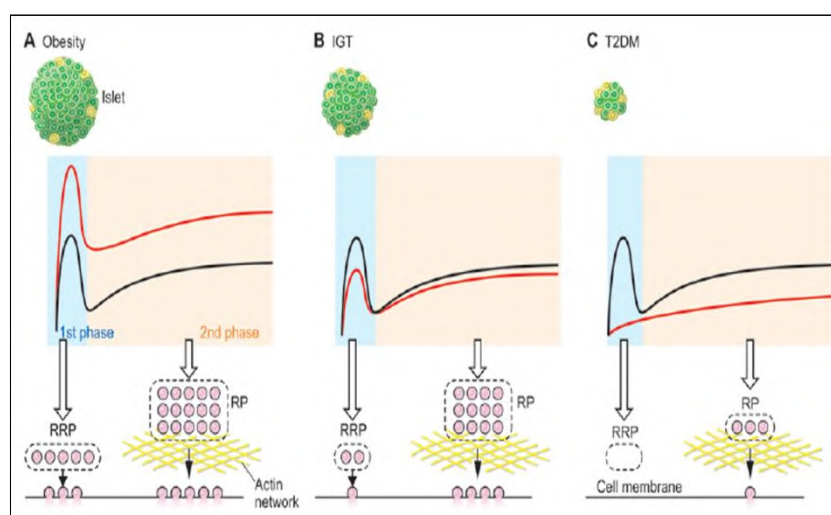
GSIS is found in the early stage of T2DM (Figure 1. 4) and also in impaired glucose tolerance (IGT) <sup>49, 52</sup>. The first phase of insulin secretion represents release of insulin already synthesised and stored in secretory granules; the second phase represents secretion of both stored and newly synthesised insulin.



**Figure 1. 3.** (A) Formation of insulin from proinsulin. (B) Schematic representation of factors involved in the regulation of insulin secretion (reproduced from the reference 40 with permission).

Synthesis and secretion of insulin is regulated by nutrient and non-nutrient secretagogues, in addition to environmental stimuli and the interactions of other hormones<sup>44</sup>. Nutrient secretagogues such as glucose appear to trigger insulin secretion from the beta-cell by increasing intracellular ATP and closing of K<sup>+</sup>-ATP channels. Other factors involved are amino acids, fatty acids, pituitary adenylate cyclase-activating polypeptide, GIP, GLP-1,

acetylcholine, adipokines like leptin and adiponectin<sup>47, 53</sup>. Non-nutrient secretagogues may act via cholinergic and adrenergic pathways, or through peptide hormones and cationic amino acids. When food is seen, smelled or acutely ingested, islet cell cholinergic muscarinic receptors activate phospholipase C, with subsequent intracellular events activating protein kinase C, phospholipase A2 and mobilizing intracellular calcium and promote insulin secretion. Catecholamines, through alpha 2-adrenoceptors, typically inhibit insulin release during stress and exercise. Nutrients in the GI tract stimulate the secretion of hormones known as incretins which amplify glucose-induced insulin release. These account for the greater insulin response to oral, as opposed to intravenous, glucose.



**Figure 1. 4.** Dynamics of insulin secretion in obesity, IGT, and T2DM (reproduced from the reference 226 with permission) (A) In obesity, the function of individual beta-cells appears to be normal and both phases of GSIS are enhanced primarily due to an increase in beta-cells. (B) In IGT, the first phase is slightly impaired because of a decrease in the size of the readily releasable pool (RRP) and/or partial defect in the exocytotic process of the granules in this pool, and the second phase is only moderately reduced. (C) In T2DM, the first phase is absent because of a complete loss of the RRP and/or a complete defect in the exocytotic process. The second phase is also reduced, probably due to the decreased releasable pool (RP) and/or disturbance of cortical actin network in T2DM. Black lines indicate dynamics of insulin secretion in normal state, red lines indicate insulin secretion under T2DM.

GIP and GLP-1 are the two most important incretin hormones. GLP-1 enhances insulin action by cAMP generation and activation of a cAMP-responsive protein kinase<sup>44, 53</sup>. Adiponectin appear to act via glucose phosphorylation, calcium influx and protein kinase C<sup>54</sup>. Marked variation in insulin secretion may occur via modulation on beta cell mass and differentiation<sup>55</sup>.

### **1. 6. 2 Physiological actions of insulin**

Insulin is a key player in the regulation of intermediary metabolism and it organizes the use of fuels for either storage or oxidation. By controlling these processes, insulin has profound influences on both carbohydrate and lipid metabolism, and significant effects on protein and mineral metabolism. Hence, derangements in insulin signalling either due to insulin deficiency or insulin resistance have widespread and devastating effects on many organs and tissues.

### **1. 6. 3 Effect of insulin in glucose metabolism**

Insulin has multiple actions on different tissues of the body and is the major regulator of blood glucose concentrations. As a result of overnight fasting, insulin levels remains low and blood glucose concentrations are maintained by the hepatic glucose supply. Under the fed state, the intestine digests the food and the glucose produced is absorbed from the gut, causing an increase in blood glucose concentration. The rise in blood glucose concentration stimulates the release of insulin by pancreatic beta-cells. Insulin reduces blood glucose mainly in two ways; by reducing the supply of glucose from the liver as well as by increasing the uptake of glucose by organs such as muscle and fat tissue. The concentration of insulin required to reduce glucose supply by the liver is probably lower than that needed to increase the uptake of glucose by muscle and fat<sup>28</sup>.

### **1. 6. 4 Effect of insulin in protein metabolism**

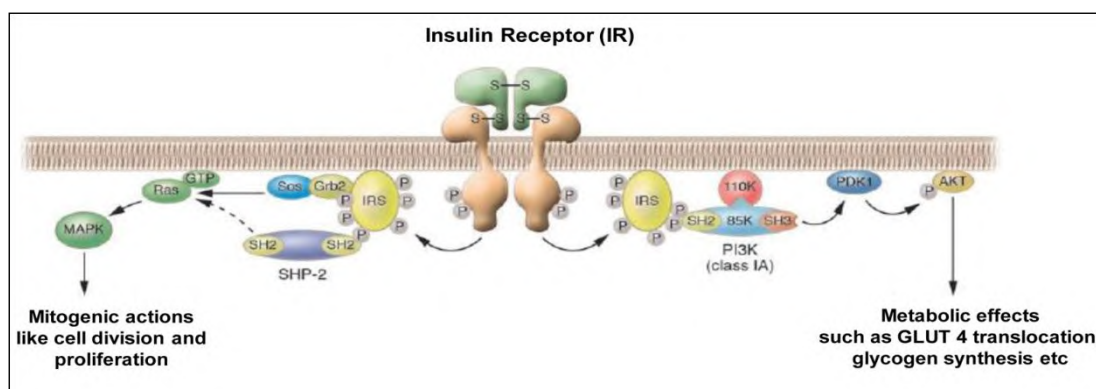
Insulin has an anabolic effect in protein metabolism in which it promotes amino acid uptake by cells and conversion of these amino acids into protein<sup>56, 57</sup>. *In vivo* studies report that the main effect of insulin is the reduction of proteolysis and maintenance of protein turnover in muscle and liver rather than a stimulation of protein synthesis<sup>58, 59</sup>. Negative nitrogen balance related to weight loss is one of the symptoms of diabetes which is promoted by the lack of insulin action on protein degradation under insulin deficient states.

### **1. 6. 5 Effect of insulin in lipid metabolism**

Major effect of insulin in fat metabolism is the reduction in the plasma free fatty acids (FFA) level. It inhibits hormone-sensitive lipase (HSL) which catalyses breakdown of triglycerides (lipolysis) in adipose tissue; thus decreasing the release of FFA from adipocytes<sup>60</sup>. FFA released from adipose tissue may be used by skeletal muscle and liver as a fuel source during fasting or under exercise, thus reserving the supply of glucose as a fuel for other tissues such as brain. Increased circulating insulin concentration inhibits hepatic VLDL secretion, largely via suppression of FFA availability<sup>61</sup>. Insulin promotes *de novo* lipogenesis by the induction of several key enzymes of lipogenic pathway, including fatty acid synthase and acetyl-CoA carboxylase and it also reduces the level of circulating ketone bodies<sup>62</sup>.

### **1. 6. 6 Molecular mechanism of insulin signalling**

Insulin exerts its metabolic and mitogenic effects on target organs such as liver, muscle, and fat through its cell surface receptors and by initiating a number of intracellular signalling cascades<sup>63, 64</sup>. Insulin receptors (IR) are located on the plasma membrane of the insulin-sensitive tissues and the hormone binds reversibly to these cell surface transmembrane receptors. When insulin binds to its receptor, it induces a conformational change in the insulin receptor molecule; this increases its tyrosine kinase activity, which then autophosphorylates multiple tyrosine residues within its intracellular region and insulin receptor substrates (IRS)<sup>64</sup>. The phosphorylated tyrosine residues on the insulin receptor create binding sites for a number of soluble intracellular effector proteins that attach to the insulin receptor and are phosphorylated by the insulin receptor tyrosine kinase<sup>65</sup>. The effector proteins/enzymes activated by phosphorylation causes the activation of a number of divergent intracellular signalling cascades involving other tyrosine kinases, serine kinases, and lipid kinases. The key enzymes participating in this intracellular signalling are lipid kinase PI 3-kinase, protein kinase B or AKT, glycogen synthase kinase-3, and certain isoforms of protein kinase C<sup>66</sup>. Schematic representation of insulin signalling pathways is given in Figure 1. 5.



**Figure 1. 5.** Schematic presentation of insulin signalling pathways (reproduced from the reference 227 with permission).

The major metabolic effects of insulin are acceleration of glucose uptake into skeletal muscle and adipose tissue, and stimulation of glycogen storage in the liver. Insulin promotes the translocation of the glucose transporter GLUT-4 from the intracellular storage vesicles to the cell membrane surface in insulin-sensitive tissues, allowing glucose to enter cells down its concentration gradient<sup>67, 68</sup>. This insulin action on cellular glucose uptake depends on the upstream signalling molecules PI 3- kinase and protein kinase C<sup>66</sup>. Glucose transporters are the members of major facilitator superfamily and they mediate the thermodynamically downhill movement of glucose across the plasma membrane of animal cells<sup>69</sup>. A brief description of major mammalian glucose transporters are given in Table 1. 3. The effect of insulin on glycogen synthesis is partly due to PI 3-kinase-dependent inhibition of glycogen synthase kinase-3<sup>66</sup>.

### 1. 7 Physiology and biochemistry of glucose homeostasis

Glucose is the essential nutrient and acts as the starting material for most of the biosynthetic pathways in the human body. Glucose is the major fuel for the tissues. Glucose is the exclusive energy source for brain and red blood cells under non starvation conditions, which depend on the bloodstream for a steady glucose supply. The liver plays a central role in the glucose homeostasis by balancing the uptake and storage of glucose via glycogenesis and the release of glucose via glycogenolysis and gluconeogenesis<sup>70</sup>. Once the liver glycogen store is full, the adipose tissue converts glucose into triacylglycerol for longer term storage as fat. Muscles utilises the major share of plasma glucose and it also efficiently accumulate glycogen for their own use. The brain is a

particular target organ that can use glucose and/or ketone bodies as an energy source. However, the fact that glucose represents the sole source of energy for some of its cells imposes a tight control over glycemia<sup>71</sup>.

ISOFORM	Tissue Distribution	Affinity for Glucose	Km	Characteristics	Gene location
<b>GLUT 1</b>	Brain micro vessels, Red blood cells Placenta Kidney All tissues	High	1 mmol/L	Ubiquitous Basal transporter	Chr 1
<b>GLUT 2</b>	Liver Kidney beta cell Small intestine	Low	15-20 mmol/L High Km	Insulin-independent transporter	Chr 3
<b>GLUT 3</b>	Brain neurons Placenta Foetal muscle All tissues	High	<1 mmol/L Low Km	Found in glucose dependent tissues	Chr 12
<b>GLUT 4</b>	Muscle cells Adipocytes Heart	Medium	2.5-5 mmol/L	Sequestered intracellularly and translocates to cell surface in response to insulin	Chr 17
<b>GLUT 5</b>	Small intestine Testes	Medium	6 mmol/L	High affinity for fructose	Chr 1

**Table 1. 3.** A brief description of major mammalian glucose transporters (reproduced from the reference 69 with permission).

### 1. 7. 1 Liver - the major metabolic regulatory organ

Liver is the key regulator of glucose metabolism and the absorbed glucose reach the liver before being delivered to muscle and adipose tissue. These are the notable factors that make liver as unique organ in carbohydrate homeostasis<sup>72</sup>. The glucose-rich blood from the digestive tract directly reaches to liver through the portal vein. The liver cells have a large number of GLUT-2 transporters for the intracellular glucose transport whose function is independent of insulin action. GLUT-2 has a high Km value or low affinity for



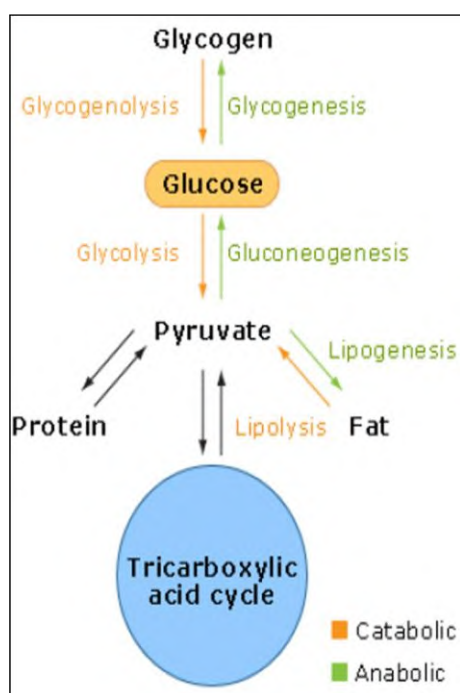
glucose which enables a rapid influx of glucose when plasma glucose levels are high<sup>69</sup>. Hence, levels of glucose inside and outside the cell can become equal in liver. The liver possesses a unique enzyme glucokinase (GK) for the conversion of glucose into glucose 6 phosphate (G6P) to trap inside the cell, rather than hexokinase, the enzyme present in other tissues. The peculiarity of GK is that it can produce G6P at a faster rate and is not inhibited by its product<sup>69</sup>. Thus, liver can work as major consumer of glucose and it buffers plasma glucose level through glycolysis, glycogenesis and lipogenesis under the influence of insulin<sup>70</sup>. Once trapped inside the liver cell, under the fed state, the high levels of insulin and low levels of glucagon stimulate glycolysis, which can be non-oxidative type, producing pyruvate or oxidative type, by releasing acetyl CoA which is further oxidized through the tricarboxylic acid (TCA) cycle to form ATP, carbon dioxide and water<sup>69</sup>. The generated ATP is used for anabolic and other energy-requiring processes in the cell. Non-oxidative glycolysis carbons undergo gluconeogenesis and the newly formed glucose is either stored as glycogen or released back into plasma. Elevated glucose and insulin both stimulate metabolic enzymes involved in glycogen synthesis and this accelerates further hepatic glucose uptake and storage as glycogen. Glycogenesis is promoted by insulin through the activation of glycogen synthase and inhibition of glycogen phosphorylase<sup>69</sup>.

When the liver glycogen stores get replenished, the excess glucose in the liver is converted to triacylglycerols (TG). TG possesses two components; the glycerol and the fatty acid moieties and both can be synthesized from glucose. Under normal physiological conditions, liver does not store triacylglycerols, instead the TG is packaged along with proteins, phospholipids, and cholesterol into the lipoprotein complexes known as very low-density lipoproteins (VLDL), which are secreted into the bloodstream. Some of the fatty acids from the VLDL are taken up by tissues for their immediate energy needs, but most are stored in adipose tissue as triacylglycerols<sup>73</sup>.

When the plasma glucose level falls, the liver has a critical role in regulating endogenous glucose production from de novo synthesis (gluconeogenesis) or the catabolism of glycogen (glycogenolysis)<sup>74</sup>. The previous studies suggest that approximately 75% of hepatic glucose release is derived from glycogenolysis, and the remainder (25%) from gluconeogenesis, primarily by the conversion of lactate and alanine to glucose<sup>75, 76</sup>. The



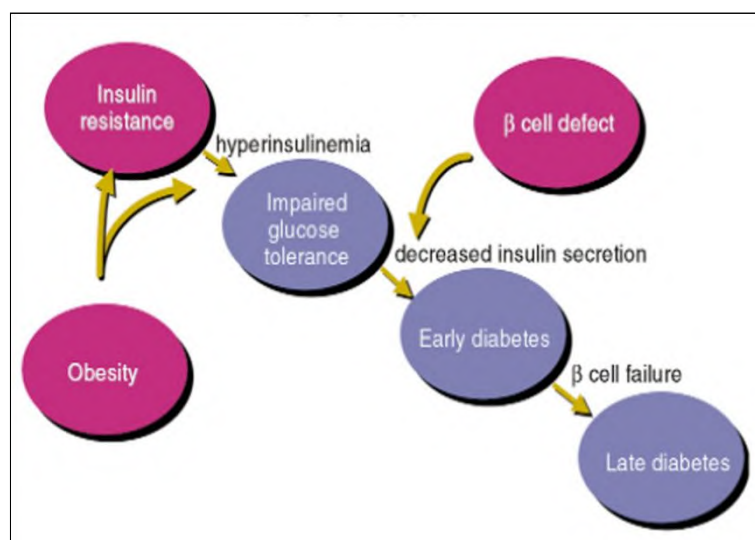
Figure 1. 6 shows the anabolic and catabolic pathways occurring in the liver.



**Figure 1. 6.** Anabolic and catabolic reactions occurring in liver. Glucose metabolism involves both energy producing (catabolic) and energy consuming (anabolic) reactions. (reproduced from the reference 228 with permission).

## 1. 8 Pathophysiology of diabetes

The core patho-physiologic defects in T2DM are insulin resistance in peripheral tissues like liver, adipose and muscle as well as beta cell failure. It is now recognized that the beta-cell failure occurs much earlier and is more severe than previously thought. Individuals with an elevated impaired glucose tolerance (IGT) are insulin resistant and have lost over 80% of their beta-cell function. In addition to the insulin resistance in muscle, liver, and beta-cell, accelerated lipolysis in adipose, incretin deficiency/resistance in the gastrointestinal tract, hyperglucagonemia in beta-cell, increased glucose reabsorption by the kidney, and insulin resistance in brain all play important roles in the development of glucose intolerance in type 2 diabetic individuals<sup>77</sup>. The epidemic of diabetes that has enveloped westernized countries is related to the epidemic of obesity and physical inactivity<sup>77,78</sup>. The metabolic staging of diabetes is illustrated in Figure 1. 7.



**Figure 1. 7.** Metabolic staging of diabetes (reproduced from the reference 63 with permission).

### 1. 8. 1 Insulin resistance

Insulin resistance is defined as reduced responsiveness towards normal circulating concentrations of insulin exhibited by the insulin dependent peripheral tissues such as liver, muscle and adipose towards insulin action<sup>63, 79, 80</sup>. Genetic predisposition is a major factor in the origin and development of the insulin resistance<sup>81, 82</sup>.

In liver, the insulin resistance is manifested by the overproduction of glucose during the basal state despite the presence of fasting hyperinsulinemia and an impaired suppression of hepatic glucose production in response to insulin after food intake<sup>82-84</sup>. The insulin resistance in the muscle is developed by impaired glucose uptake following ingestion of a carbohydrate meal and results in postprandial hyperglycemia<sup>85-88</sup>. Inflammation has an important role in the pathogenesis of insulin resistance, especially in adipocytes<sup>89</sup>. Nowadays, it is clear that obesity is associated with a state of chronic, low-grade inflammation, particularly in white adipose tissue<sup>90</sup>. The proinflammatory cytokines or adipokines like TNF alpha, MCP-1, IL-6, leptin, resistin, visfatin and PAI-1 are able to induce insulin resistance<sup>89, 91</sup>. The primary mechanism through which inflammatory signalling leads to insulin resistance is the inhibition of insulin signalling downstream of the insulin receptor. Exposure of cells to TNF alpha or elevated levels of free fatty acids stimulates inhibitory phosphorylation of serine residues of IRS-1<sup>92-94</sup>. This phosphorylation reduces both tyrosine phosphorylation of IRS-1 in response to insulin

and the ability of IRS-1 to associate with the insulin receptor and thereby inhibits downstream signalling and insulin action<sup>94-96</sup>.

Excessive intracellular lipid accumulation in “ectopic sites,” such as liver and muscle is another proposed mechanism for insulin resistance; the increased skeletal muscle triglyceride concentrations and hepatic steatosis are typical concomitant abnormalities of insulin resistance states<sup>97</sup>. Metabolism of excessive fatty acid loads can result in the generation of bioactive lipid products such as diacyl glycerols (DAGs). This stimulates protein kinase C (PKC) activity, which leads to inhibitory serine phosphorylation of insulin signalling components causing insulin resistance<sup>97, 98</sup>. Similarly, saturated fatty acids can be converted to ceramides that inhibit AKT activity and decrease insulin sensitivity<sup>99</sup>.

### **1. 8. 2 Pancreatic beta-cell dysfunction**

Insulin resistance causes an increased metabolic demand for insulin in several tissues that can result in the development of hyperglycemia. By increasing pancreatic beta-cell mass and insulin gene expression, beta-cells synthesize and secrete more insulin to compensate the increased demand. Thus, period of near-normal glycemia or IGT can exist in which pancreatic beta-cells compensate for insulin resistance by hypersecretion of insulin. But at a particular point, the beta-cell compensation is followed by beta-cell failure, in which the pancreas fails to secrete sufficient insulin. This is the time point where prediabetic state or IGT is converted to diabetic state. The link between insulin resistance, beta-cell dysfunction and T2DM and the time line of development of diabetic complications is demonstrated in Figure 1. 8A and B. Chronic hyperglycemia or glucotoxicity<sup>100</sup>, chronic dyslipidemia or lipotoxicity<sup>101</sup>, or the combination of both, glucolipotoxicity<sup>102</sup> have been postulated to contribute to the worsening of beta-cell function over time, creating a vicious cycle by which metabolic abnormalities impair insulin secretion, which further aggravates metabolic perturbations<sup>103</sup>. Moreover, the antioxidant defence system of pancreatic beta cell is not strong enough to withstand the deleterious free radical generated by the glucolipotoxicity<sup>104</sup>.

### **1. 8. 3 Physiological starvation and ketoacidosis**

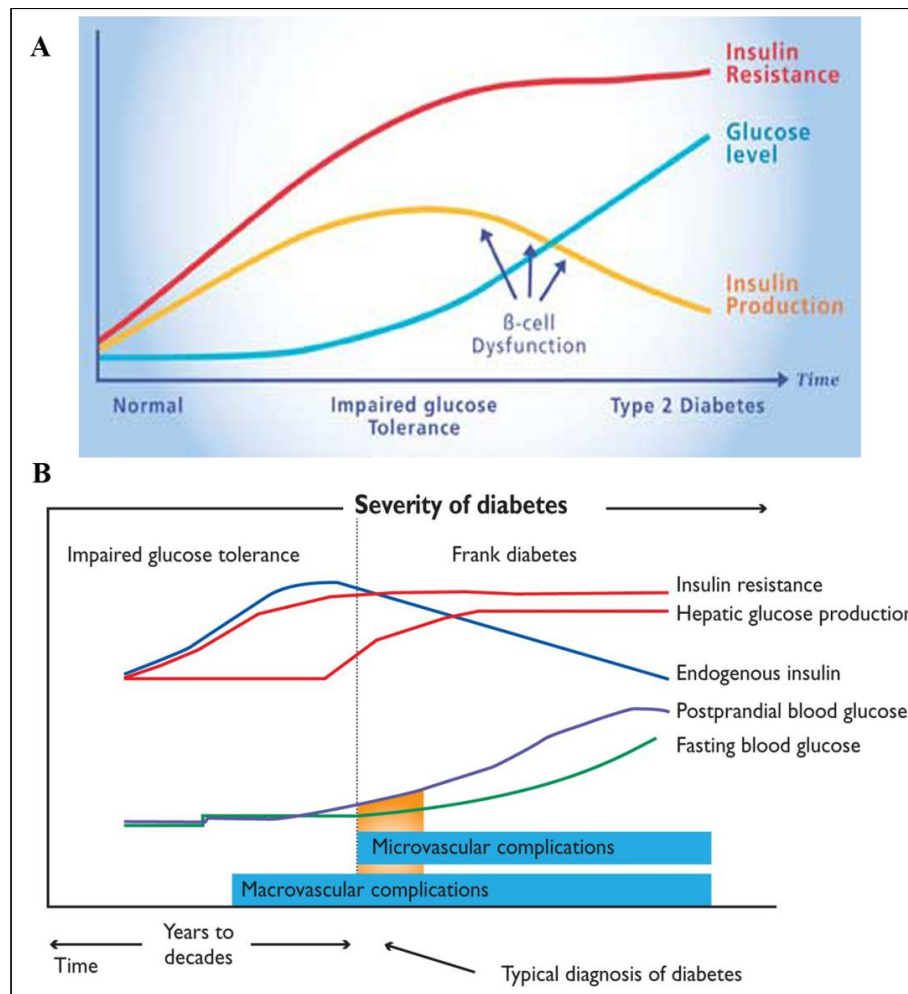
T1DM and also the end stage T2DM can be often referred to as “starvation in the midst of plenty”<sup>105</sup>. Under diabetes, the extracellular glucose levels may be extremely high, but the cells are unable to uptake and utilise the glucose. As in starvation, these individuals use non-glucose sources of energy, such as fatty acids and ketone bodies, in their peripheral tissues. An uncontrolled production of ketone bodies may occur; these ketone bodies are weak acids and it acidifies the blood<sup>106</sup>. The result is the metabolic state of diabetic ketoacidosis (DKA). Hyperglycemia and ketoacidosis are the hallmarks of insulin deficient diabetes, either type 1 or type 2<sup>107</sup>. Hypertriglyceridemia can also be noticed in DKA. In diabetic subjects, the enzyme lipoprotein lipase that normally degrades lipoproteins is inhibited by the low level of insulin and the high level of glucagon. So, the levels of VLDL and chylomicrons derived from the lipid components of the diet are high under DKA<sup>105</sup>.

### **1. 9 Diabetes secondary complications**

Secondary complications of diabetes occur in the majority of individuals with both type 1 and type 2 diabetes. Prolonged hyperglycemia increases the likelihood of developing secondary damage to numerous systems, and these complications represent a substantial cause of morbidity and mortality<sup>108</sup>. The duration and degree of hyperglycaemia, the protracted increase in blood glucose levels beyond the usual fasting or postprandial ranges positively correlates the occurrence of secondary complications<sup>109</sup>. The most common complications of diabetes are ‘microvascular disease’ (due to damage to small blood vessels) and ‘macrovascular disease’ (due to damage to the arteries) which share numerous mechanisms by which hyperglycaemia can disrupt cell and organ functions.

Microvascular complications include eye damage or ‘retinopathy’, brain damage, kidney dysfunction termed ‘nephropathy’ and neural damage or ‘neuropathy’ and the major macrovascular complications include accelerated cardiovascular disease resulting in myocardial infarction and cerebro-vascular disease manifesting as strokes<sup>110</sup>. The organs that are susceptible to diabetic complications carries the cells that exhibit insulin independent glucose uptake and possess the glucose metabolizing enzyme aldose reductase; for example capillary endothelial cells in the retina, mesangial cells in the renal

glomerulus, and neurons and Schwann cells in peripheral nerves<sup>108, 111</sup>. Hence, diabetes selectively damages cells, like endothelial cells and mesangial cells, whose glucose transport rate does not depend on insulin levels, leading to the accumulation of glucose inside the cell<sup>111</sup>.



**Figure 1. 8.** (A) The link between insulin resistance, beta cell function and diabetes (B) time line of development of macro and microvascular complications. (reproduced from the references 38 and 39 with permission).

Hyperglycemia accelerates the development of vascular complications via several mechanisms: activation of the polyol and hexosamine pathways, activation of protein kinase C, increased oxidative stress, increased production of advanced glycation end-products (AGEs), increased synthesis of growth factors, cytokines and angiotensin II<sup>112</sup>. These factors in turn, induce endothelial dysfunction and lead to the progressive development of micro- and macrovascular complications and multiorgan damage<sup>112</sup>.

Reports suggest that increased oxidative stress, induced by several hyperglycemia activated pathways, is a key factor in the pathogenesis of endothelial dysfunction and vascular disease<sup>113</sup>. A number of intervention studies in type 2 diabetic patients have shown that intensive treatment is required to reduce the long-term complications of the disease<sup>18</sup>.

### **1. 10 Diabetes treatment options**

The first step in the progression from normal glucose tolerance to T2DM is IGT<sup>81, 114</sup>. Impaired fasting glucose (IFG) and IGT are recognized as prediabetic states by the ADA<sup>26</sup>. The characteristic features of IGT or IFG are impaired beta-cell function and insulin resistance. Hence, the first line diabetic interventions must be followed to prevent/slow IGT progression to diabetes that can preserve or augment beta-cell function and can ameliorate insulin resistance<sup>115</sup>. The treatment regimen for diabetes must be designed to achieve specific target goals which may depend on the age of the patient, the years of anticipated survival, other concomitant illnesses, and the patient's willingness to comply with specific treatment regimens<sup>18</sup>. Diet and exercise, insulin and oral therapies as well as traditional remedies are the available options for the control and management of diabetes. But the universally accepted fact is that effectiveness of the diabetes therapies are solely based on individualised care and management of glycemia.

#### **1. 10. 1 Diet and exercise**

Obesity and physical inactivity are major risk factors for type 2 diabetes<sup>116</sup>. Obesity is the single most important factor responsible for the marked increase in both the incidence and prevalence of T2DM over the last 20 years due to the pronounced changes in the human environment, behaviour and lifestyle<sup>1</sup>. These have resulted in escalating rates of both obesity and diabetes and the recent adoption of the term 'diabesity', first suggested by Shafir a decade ago<sup>117, 118</sup>. Insulin resistance the key etiological factor of T2DM can be resulted from weight gain and physical inactivity. Obese individuals with a predominance of intra- abdominal or mesenteric fat have higher rates of FFA mobilization and greater resistance to the anti-lipolytic effects of insulin when compared with individuals with lower body obesity<sup>119, 120</sup>. In contrast to this, insulin sensitivity and glucose tolerance can be improved in non-diabetic and diabetic subjects by weight loss and exercise<sup>121</sup>.

Different prospective studies demonstrated that a treatment regimen using diet and exercise reduces IGT progression to T2DM<sup>122, 123</sup>. Even though, weight loss decreases the diabetes risk by 50-60%, about 40-50% of IGT subjects still progress to T2DM. This indicates that lifestyle intervention alone is not sufficient to prevent diabetes in a large percentage of individuals. In addition to behavioural intervention, pharmacological therapy (thiazolidinediones, metformin, GLP-1 receptor agonists etc) at the stage of IGT uniformly has been shown to effectively prevent IGT conversion to T2DM<sup>124</sup>.

### **1. 10. 2 Insulin and oral therapies**

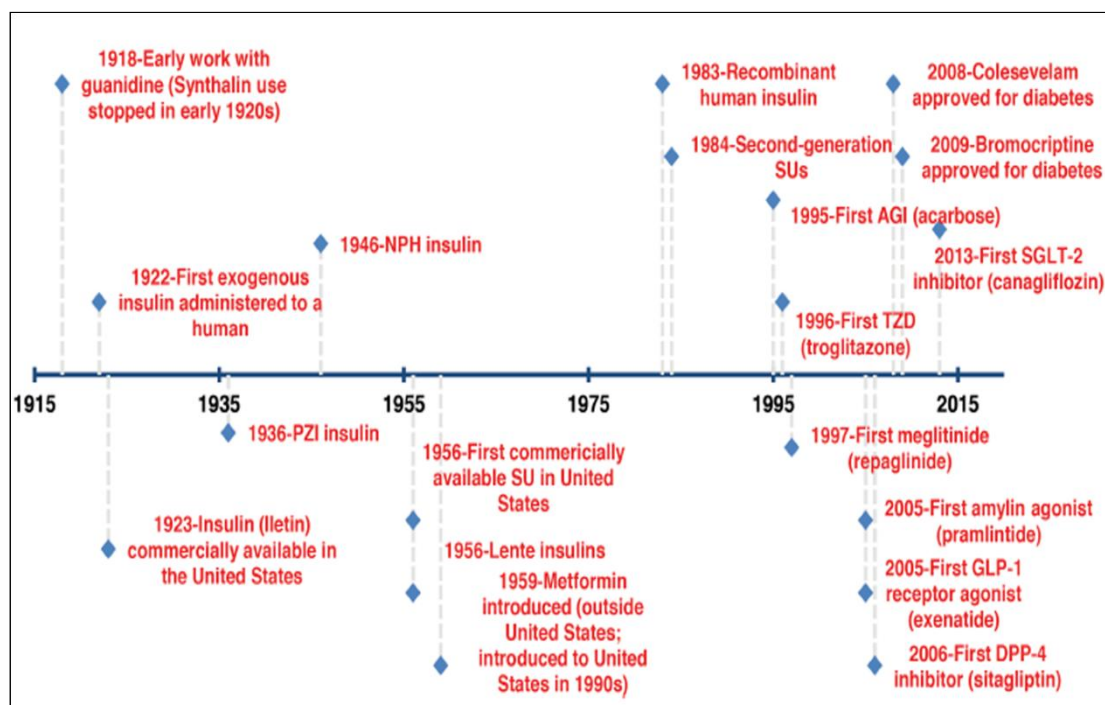
Current therapies for diabetes have mainly based on elevating plasma insulin levels via direct insulin administration or oral agents that promote insulin secretion which improve insulin sensitivity of tissues and eventually reducing the rate of carbohydrate absorption from the gastrointestinal tract<sup>125</sup>. The currently used drugs are sulfonylureas, glinides, GLP-1 receptor agonists, metformin, thiazolidinediones, and alpha-glucosidase inhibitors that target insulin resistance or beta-cell dysfunction by increasing insulin secretion or tissue insulin sensitivity<sup>125</sup>. The history of diabetes medication is illustrated in Figure 1. 9.

#### **1. 10. 2. 1 Insulin therapy**

There were no effective pharmacological agents for the management of diabetes, until Banting et al. discovered the insulin therapy in 1921. The first commercial insulin was 'Iletin' developed by Eli Lilly in 1922. The next major advancement was crystallization of insulin in 1926 which improved purity of soluble insulin and also led to the insulin formulation modifications with different time-action profiles<sup>126</sup>. In 1936, the first extended-action insulin, Protamine Zinc Insulin (PZI), composed of an amorphous combination of protamine, zinc, and insulin was released. In 1946, release of the second extended-action insulin, Neutral Protamine Hagedorn (NPH) by the Nordisk Insulin Laboratory which contained about 10% of the protamine found in PZI along with zinc insulin crystals<sup>126</sup>. This insulin was shorter acting than PZI and could be combined with regular insulin. In 1956, the lente series of insulin was introduced: ultralente, lente, and semilente. These formulations were synthesized by altering the content of the excess zinc. All insulin preparations available before 1983 were derived from animal sources. By the



development of recombinant DNA technology, the first recombinant human insulin, got approval in 1983<sup>127</sup>. The search for insulin that acts more closely with physiological insulin secretory patterns accelerated after the release of human insulin.



**Figure 1. 9** The history of diabetes medications (reproduced from the reference 20 with permission).

In the last years, increased advancement was noticed in the insulin preparations, i. e the development of insulin analogs, which were designed to overcome the disadvantages of traditional human insulins in the treatment of both T1DM and T2DM. Insulin analogs more closely mimic the physiological insulin profile and are therefore associated with an improved balance between glycemic control and tolerability<sup>128</sup>. Individuals with T1DM lack endogenous insulin and therefore rely entirely on injected insulin. The types of insulin available for current clinical use are rapid acting (bolus), long acting (basal) and intermediate types<sup>129</sup>. Rapid-acting analogs, such as insulin aspart, insulin lispro, and insulin glulisine, are used as mealtime insulin replacement because they mimic the physiological insulin response to food intake<sup>130, 131</sup>. It reduces the risk of hypoglycemia in the intervals between meals that is often seen with human insulin<sup>129, 130</sup>. Long acting insulin analogues are insulin glargine and insulin detemir, which more closely



approximate the natural, constant physiological release of insulin<sup>130, 131</sup>. Insulin glargine is injected as a solution, but precipitates upon injection into subcutaneous tissue, delaying absorption<sup>132, 133</sup>. But, insulin detemir, forms hexamers and reversibly binds to albumin in the circulation, prolonging its absorption and bioavailability. The simultaneous supplementation of both prandial and basal insulin with a limited number of injections has been achieved the development of premixed insulin analogs<sup>130, 131</sup> which are a mixed suspension of a rapid-acting analog along with its protamine-crystallized form. Several premixed insulin analogs are currently available, including biphasic insulin aspart (BIAsp 70/30; 30% rapid-acting insulin aspart and 70% protaminated insulin aspart) and biphasic insulin lispro (lispro mix 75/25; 25% rapid-acting lispro and 75% protaminated insulin lispro). The development of insulin pens and insulin pumps greatly improved the flexibility and convenience of insulin administration<sup>127, 134</sup>. Results from large scale prospective studies such as the Diabetes Control and Complications Trial show that an intensive treatment regimen consisting of a rapid acting plus a long-acting insulin can help patients with T1DM to achieve better glycemic control, with a lower risk of complications, than less intensive therapy<sup>135</sup>.

During the onset of type 2 diabetes, blood glucose levels can often be controlled with changes in diet and physical activity along with insulin secretagogues. But, the beta-cell dysfunction that leads to impaired insulin secretion is progressive, and eventually patients will require treatment strategies that include insulin, either alone or with oral agents<sup>136</sup>. T2DM patients also develop both bolus (meal time) and basal (between meal) defects in insulin function, thus bolus and basal glucose levels are increased, resulting in hyperglycemia<sup>137</sup>. So insulin therapy is recommended to these individuals by ADA. Intensive insulin therapy study in patients with T2DM showed that the insulin therapy partially reversed the post binding defect in peripheral insulin action, produced near-normal basal hepatic glucose output, and enhanced insulin secretion, thereby maintaining lower glucose values. In addition, the mean daily insulin requirement was reduced by 23% after about 2 weeks of therapy<sup>138</sup>.

### **1. 10. 2. 2 Metformin**

Metformin was developed from the antidiabetic folk remedy used in Southern and Eastern Europe called French lilac or goat's rue, *Galega officinalis*<sup>139</sup>. The antihyperglycemic

moiety in this plant, 'guanidine' was isolated, in the early 20<sup>th</sup> century and later Frank et al. synthesized a guanidine compound called synthalin in Germany and used it to treat diabetes during the 1920s<sup>126</sup>. Many synthetic analogues such as synthalin, phenformin and buformin were analysed by clinical trials, but due to hepatotoxicity and lactic acidosis led to their withdrawal from the market.

Metformin (1, 1-dimethyl biguanide), a biguanide derivative was introduced as an antihyperglycemic agent in 1959 but was approved in the United States only in 1990s. Nowadays, metformin is the most widely used antihyperglycemic agent in the world and is the only clinically significant biguanide<sup>139, 140</sup>. Metformin is recommended as the first-line oral therapy in the recent guidelines of the ADA (American Diabetes Association) and EASD (European Association of the Study of Diabetes)<sup>141, 142</sup>. The main effect of this drug is to decrease hepatic glucose production through a mild inhibition of the mitochondrial respiratory chain complex I<sup>143</sup>. This results in transient decrease in cellular energy status promotes the activation of AMPK, a well-known cellular energetic sensor. Thus, metformin-induced AMPK activation is believed to promote the transcriptional inhibition of the hepatic gluconeogenic programme<sup>143</sup>. It also reduces plasma glucose via an increase in insulin-dependent glucose uptake<sup>140</sup>. This medication has got a good tolerance in both humans and animal models and is typically associated with a significant reduction in % HbA1c levels, about 1.5%<sup>140</sup>.

### **1. 10. 2. 3 Sulfonylureas**

Sulfonylurea has been used in the treatment for type 2 diabetes for >50 years<sup>144</sup>. Sulfonylureas increase the insulin secretion from pancreatic beta-cells by binding to receptors that block the K<sup>+</sup>-ATP-dependent channels, leading to cell depolarization and subsequently insulin exocytosis<sup>145</sup>. Chlorpropamide, acetohexamide, and tolazamide were the first-generation sulfonylureas<sup>145, 146</sup>. In 1984, glyburide and glipizide, which are more potent second-generation sulfonylureas, became available in the market<sup>147</sup>. Glimepiride, a third-generation sulfonylurea, was introduced in 1995<sup>148</sup>. Sulfonylureas are cheap, and predictable, but the incidence of hypoglycemia, a major side effect, limits their use. The % HbA1C is decreased by 1-2%, by the administration of sulfonylureas<sup>148</sup>.

### **1. 10. 2. 4 Thiazolidinediones**

Thiazolidinediones (TZDs), or ‘glitazones’ were introduced to the U.S. market in 1996. TZD are peroxisome proliferator activated receptor-gamma (PPAR gamma) activators whose mechanisms of action are enhancement of skeletal muscle insulin sensitivity and reduction in hepatic glucose production<sup>149</sup>. TZDs do not increase the risk of hypoglycemia and have a more durable effect than metformin or SUs and associated with an A1C decrease of 0.5-1% in most patients<sup>140</sup>. Troglitazone was the first FDA approved TZD<sup>150</sup>. But, the use of troglitazone was associated with hepatic failure, so FDA removed the drug from the market in 2000. The TZD which is currently available in the market is pioglitazone. Pioglitazone has been beneficial for cardiovascular disease moderately but it increases the incidence of bladder cancer<sup>149</sup>. Even though, the TZD drug rosiglitazone acted as an effective insulin sensitizer, due to the increased risk of myocardial infarction (MI) and fluid retention there is a restriction for its use by FDA. The RECORD (Rosiglitazone Evaluated for Cardiovascular Outcomes and Regulation of Glycemia in Diabetes) study findings unveiled that people treated with rosiglitazone did not have an elevated risk of MI compared to patients taking other antihyperglycemic medications<sup>151</sup>.

### **1. 10. 2. 5 Alpha glucosidase inhibitors**

Alpha glucosidase inhibitors (AGIs) bring about a site specific effect in the small intestine, inhibiting alpha glucosidase enzymes resides in the brush border cells. Alpha-glucosidase is a collective term referring to membrane-bound enzymes of the small intestinal villi involved in the breakdown of alpha-linkages of oligosaccharides and disaccharides into glucose<sup>28</sup>. The enzymes included are maltase, isomaltase, glucoamylase, and sucrase. AGIs reduce the rate of absorption of carbohydrates by prolonging the absorption time but do not alter the absolute absorption. Thus AGIs slow down the elevation of postprandial glucose levels<sup>152</sup>. The reduction of HbA1c observed with AGIs is typically 0.5-1.0%. AGI drugs available in market are acarbose, miglitol and voglibose. Besides the slowing down of carbohydrate absorption, AGIs also augment incretin hormone secretion and enhances beta-cell function; by altering gut microbiota flora. AGIs also cause inhibition of platelet aggregation, attachment of macrophage to

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vascular endothelium, ameliorate the development of atherosclerosis and help to maintain elasticity of blood vessels<sup>153</sup>.

### **1. 10. 2. 6 GLP-1 mimetics or analogues and DPP-IV inhibitors**

GLP-1, the hormone secreted by the L cells of the small intestines within minutes of carbohydrate or lipid rich meal stimulates insulin synthesis and glucose dependent insulin secretion<sup>154, 155</sup>. GLP-1 also suppresses glucagon release and delays gastric emptying<sup>154, 155</sup>. It has a short half-life of 1-2 min because of rapid degradation by the enzyme dipeptidyl peptidase-IV (DPP-IV)<sup>155</sup>. Due to this physiological importance, the GLP-1 receptor agonists and DPP-IV inhibitors have been emerged as the treatment options for T2DM<sup>156</sup>.

The first GLP-1 agonist introduced in the market is exenatide. It is a mimetic of exendin-4, a peptide isolated from the saliva of the *Heloderma suspectum* (Gila monster), and has a 53% similarity to the human GLP-1<sup>157, 158</sup>. It is more resistant to DPP-IV degradation, thus has a longer half-life<sup>158</sup>. Exenatide injection, twice daily, given 40-60 min before breakfast and dinner was approved in 2005<sup>159</sup>. In 2012, a once-weekly formulation of exenatide (long acting release) was approved<sup>160</sup>. A modified form of human GLP-1, with 97% homology named liraglutide was approved in 2010 as daily injection<sup>161</sup>. About 1% decrease of HbA1c level was attained by GLP-1 receptor agonists. The gastrointestinal disorders and pancreatitis are reported in some patients<sup>162, 163</sup>. There are three approved DPP-IV inhibitors: sitagliptin, saxagliptin, and linagliptin. The HbA1C reduction exhibited by DPP-IV inhibitors is up to 0.8%. These are administered alone or in combination with metformin. The most attractive feature of GLP-1 agonist or DPP-IV is the absence of hypoglycaemia<sup>164-166</sup>.

### **1. 10. 2. 7 Aldose reductase inhibitor**

Aldose reductase inhibitors (ARIs) can block the metabolism of the polyol pathway and significantly reduces intracellular sorbitol accumulation in tissues where glucose uptake is independent of insulin<sup>167</sup>. Hence it is used to slow or reverse the progression of diabetic secondary complications like retinopathy, neuropathy or nephropathy<sup>168</sup>. Epalrestat, ranirestat, fidarestat are the currently available ARIs for the clinical use.

### **1. 10. 2. 8 SGLT-2 inhibitors**

The sodium / glucose co-transporter 2 (SGLT-2) are high-capacity, low-affinity glucose transporter found primarily in the kidney<sup>169</sup>. This transporter is responsible for about 90% of glucose reabsorption in the kidney<sup>169</sup>. If SGLT-2 inhibitors are used, excess glucose in the renal tubules is not reabsorbed, and glucose is excreted in the urine. The result is the net loss of glucose and a reduction in hyperglycemia. Canagliflozin and Dapagliflozin A are the FDA approved SGLT-2 inhibitors<sup>170</sup>. Recent meta-analysis studies evaluating SGLT-2 inhibitors reported HbA1C reductions of 0.5-0.6% in patients treated with these agents<sup>171</sup>.

### **1. 10. 2. 9 Emerging targets - AGE inhibitors, SIRT-1 activators, PTP-1B inhibitors**

AGEs and AGE precursors damages the cells in several ways and cause secondary complication; by modifying the structure and function of transcription factors, components of extracellular matrix, circulating proteins. The circulating proteins with modified structure can then bind to AGE receptors (RAGEs) and activate them, thereby causing the production of inflammatory cytokines and growth factors, which in turn cause vascular pathology<sup>172, 173</sup>. *In vivo* studies have been shown that, pharmacologic inhibition of AGEs prevents late structural changes of experimental diabetic secondary complication such as retinopathy<sup>174</sup>. The example for the inhibitors of renal AGE accumulation under clinical trial is alagebrium which is a specific AGE cross-link breaker<sup>175</sup>.

SIRT-1 is a conserved NAD-dependent protein deacetylase. SIRT-1 influences several metabolic processes such as lipid and glucose metabolism, insulin signalling, suppression of the PTP-1B gene and others involved in energy maintenance and also regulate transcription coactivator PPAR-gamma coactivator-1 alpha or PGC-1alpha<sup>176</sup>.

Protein tyrosine phosphorylation is a fundamental mechanism for the intracellular control of cell growth and differentiation. It is governed by the opposing activities of protein tyrosine kinases, which catalyze phosphorylation, and protein tyrosine phosphatases (PTPs), which are responsible for dephosphorylation<sup>125</sup>. PTP-1B also dephosphorylates thereby inactivates (down-regulation) insulin receptors and insulin receptor substrates<sup>177, 178</sup>. PTP-1B inhibition represents a therapeutic approach for insulin resistance and obesity

in T2DM<sup>179, 180</sup>.

### 1. 10. 3 Traditional remedies for diabetes – role of *Ayurveda*

The human use of plants as medicines started in ancient times at least to the Middle Paleolithic age some 60,000 years ago<sup>181</sup>. Traditional medicine has been defined by WHO as “diverse health practices, approaches, knowledge and beliefs incorporating plant animal or mineral based medicines, spiritual therapies, manual techniques or exercise applied singularly or in combination to maintain well being as well as to treat, diagnose or prevent illness”<sup>182</sup>. According to WHO, almost 65% of the world’s population depends on complementary or alternative system for primary health care<sup>183</sup>. *Ayurveda* is one of the oldest, health traditions in the world, originated in India. It is based on *Sankhya* philosophy, which means ‘rational enquiry into the nature of the truth’. Sanskrit meaning of *Ayu* is life and *Veda* is knowledge or science<sup>184</sup>. *Charak Samhita* (1000 BC) and *Sushrut Samhita* (100 AD) are the main classics. *Ayurveda* materia medica gives detailed descriptions of over 1500 herbs and 10,000 formulations<sup>184</sup>. Diabetes was known to ancient *Ayurvedic* physicians as *Madumeha* (*madhu* means ‘honey’ and *meha* means ‘urine’) and has given an elaborate description about this disease<sup>19</sup>. Many herbs and herbal preparations in the form of *kashayam* or *kwadha* (decoction), *rasayanam*, *bhasmam* etc have been used to treat diabetes. WHO expert committee on diabetes has been recommended for the promotion of traditional methods for the treatment of diabetes<sup>185, 186</sup>. A reverse pharmacology approach, inspired by traditional medicine and *Ayurveda*, can offer a smart strategy for new drug candidates to facilitate discovery process and also for the development of rational synergistic botanical formulations<sup>187</sup>.

### 1. 11 Diabetes research tools for traditional plant based medications

The test material can be analysed for its antidiabetic effects using *in vivo* and *in vitro* test systems. Preliminary testing of the study material under *in vivo* conditions can give valuable information on the type of extract to be made, a suitable dose, toxic effects, as well as it also helps to confirm efficacy. *In vitro* tests can play an important role in the elucidating the molecular mechanism of the test material<sup>28</sup>.

### 1. 11. 1 *In vitro* models for diabetes

*In vitro* assays rely on a specific biological process relevant to the disease and its treatment. Perfused whole organs, isolated tissues, cells in primary or immortal culture, subcellular membranes or purified receptors, and enzymes are the *in vitro* systems generally used. The advantages of *in vitro* assays in ethnobotanical research are reduced variability, low cost, minimal use of animals, it needed only less amount of material and above all of these, the mechanism of action of the test material can be elucidated as it uses a simple system compared to whole organism<sup>28</sup>. The major *in vitro* test methods to evaluate antidiabetic potential are the following:

*Antioxidant activity:* Antioxidant activities of test material with respect to various reactive radicals (ABTS, DPPH, hydroxyl etc.) can be screened in cell free systems to see the possible role of these materials to minimise reactive oxygen species induced complications in diabetes.

*Inhibition of carbohydrate-digesting enzymes:* Inhibition of carbohydrate digestive enzymes (alpha glucosidase) can be measured to check the potential of the test material to control postprandial hyperglycemia.

*Antiglycation assay:* *In vitro* antiglycation screening is recommended in recent years to have active antiglycation agents against protein glycation during hyperglycemia.

*Inhibition of aldose reductase:* Aldose reductase inhibitors are potential candidates in diabetic therapy due to serious issue of retinopathy in diabetes. So there is high demand for development of aldose reductase inhibitors and *in vitro* system can be utilised for the same.

*Models based on the liver as an insulin target tissue:* Hyperglycemia induced oxidative stress can be developed in *in vitro* system utilizing HepG 2 cell lines. This system could be used to evaluate potential of test material in reducing hyperglycemia induced oxidative stress which is one of the main causes for diabetes secondary complications.

*Models based on adipocytes as an insulin target tissue:* Adipogenesis can be evaluated to test the TZD like activity of test material. Various factors associated with adipogenesis

like TG content, activity of GPDH and DGAT1 can also be utilised for the evaluation of adipogenic potential. The mouse-derived 3T3-L1 fibroblast cell line provides an alternative to the use of freshly isolated primary adipocytes. The cells are commercially available in preadipocyte form and can be induced to differentiate into adipocytes by the inclusion of a glucocorticoid (eg., dexamethasone), insulin and an agent that elevates intracellular cAMP (eg., isobutyl methyl xanthine, IBMX) in the culture medium<sup>188</sup>. The differentiation process, as well as glucose uptake, lipogenesis, and inhibition of lipolysis in differentiated state can be assessed in this cell line as these are mediated by insulin.

*Models based on muscle as an insulin target tissue:* The uptake and utilization of glucose in the muscle is under control of insulin. A rat skeletal muscle cell line, L6 may be used for the study of antidiabetic agents. Cells are obtained as myoblasts that are induced, by adjusting the medium, to differentiate into an alignment stage and then into fused myotubes. Glucose uptake assay using <sup>3</sup>H-2-deoxyglucose is most sensitive to insulin in the myotubes, corresponding to an increase in muscle specific GLUT-4 transporters<sup>189</sup>.

### **1. 11. 2 *In vivo* models for diabetes**

The classical animal model employed by Banting and Best was pancreatectomy in dogs<sup>24</sup>. Today, most of the experiments in diabetes are carried out in rodents, although some studies are still performed in larger animals. Due to the heterogeneity of diabetic conditions, single animal model that exhibit all the features of diabetes is not available. Hence, different *in vivo* models, each displaying a different characteristic of diabetic states can be selected for the study<sup>190</sup>. Normal nondiabetic animals and animals with impaired glucose tolerance and insulin resistance (but not overt diabetes) have also been used to demonstrate antihyperglycemic and insulin sensitizing activities and to investigate the mode of action of antidiabetic test materials<sup>191</sup>. There is a marked overlap between type 1 and 2 diabetes mellitus either in human beings or in animal models, so the *in vivo* models for both the types of this disease are described here<sup>192, 193</sup>.

*Pharmacological models:* Streptozotocin (STZ) and alloxan are the most frequently used drugs for the generation of drug induced model and is useful for the study of multiple aspects of the disease. The cytotoxic action of these diabetogenic agents is mediated by reactive oxygen species, but the mechanism of action of both drugs are different<sup>194, 195</sup>.



Alloxan and the product of its reduction, dialuric acid, establish a redox cycle with the formation of superoxide radicals. The superoxide radicals undergo dismutation to hydrogen peroxide with a simultaneous elevation in cytosolic calcium concentration, which causes rapid destruction of pancreatic beta-cells<sup>196</sup>. The most frequently used intravenous dose of alloxan in rats is 65 mg/kg<sup>194</sup>. Streptozotocin enters the pancreatic beta-cell via a glucose transporter, GLUT-2 and causes alkylation of deoxyribonucleic acid (DNA). Then, STZ induces activation of poly adenosine diphosphate ribosylation and nitric oxide release. As a result of STZ action, pancreatic beta-cells are destroyed by necrosis<sup>197</sup>. In adult rats, 60 mg/kg is the most common dose of STZ to induce insulin dependent diabetes<sup>198</sup>.

*Diet induced models:* The basic diet-induced models are the following: high-fat diet, high-carbohydrate diet, combined high-fat and high-carbohydrate diet, diet with a high content of NaCl and fructose etc. High fat intake of saturated fatty acids and cholesterol causes obesity, insulin resistance, hepatic steatosis and increased content of triglycerides in muscles in rats<sup>199, 200</sup>. Administration of high-fructose and high-sucrose diets allows the studies on muscle and liver abnormalities in a state of insulin resistance<sup>201, 202</sup>. In order to stimulate hypertension and metabolic syndrome in rats, diet manipulations with high concentration of NaCl and fructose are used<sup>203</sup>.

*Surgical models:* The induction of diabetes can also be done with the complete surgical removal of the pancreas. Some reports are available with surgical model to explore effects of natural products with animal species such as rats, pigs, dogs and primates<sup>178, 204</sup>.

*Genetic models:* Animal strains that spontaneously develop diabetes and displaying complex and heterogeneous characteristics of diabetes such as insulin resistance, obesity, dyslipidemia and hypertension are considered in this category. This provides valuable insights to study some events that are observed in human T2DM. Some strains like ob/ob mouse may maintain euglycemia due to a robust and persistent compensatory pancreatic beta-cell response, matching the insulin resistance with hyperinsulinemia. On the other hand, the diabetic C57BL/KsJ db/db mouse rapidly develops hyperglycemia since their pancreatic beta-cells are unable to maintain the high levels of insulin secretion required throughout life<sup>205</sup>. Thus, food intake is important in determining the severity of the

diabetic phenotype and restriction of energy intake reduces both the obesity and hyperglycemia seen in this strain of mice. Another example is the spontaneously diabetic Goto-Kakizaki rat which is a genetic lean model of T2DM originating from selective breeding over many generations of glucose-intolerant nondiabetic Wistar rats<sup>206</sup>. The NOD mouse shows resemblance to type 1 hyperglycemia between 12 and 30 weeks of age, whereas in BB rats it occurs around 12 weeks of age<sup>205</sup>.

*Transgenic models:* The transgenic or knockout rodent models targeting proteins thought to play a key role in glucose metabolism are helpful in giving insights into gene regulation and development, pathogenesis and finding new targets and the treatment of disease<sup>207</sup>. The tissue specific knockout mouse models allows further insight into the insulin action with respect to particular target tissues like muscle, adipose or liver<sup>208, 209</sup>.

### **1. 12 Aims and Objectives of the study**

Most of current therapies for diabetes and its complications were developed by serendipity. Emerging knowledge of key pathogenic mechanisms, such as the impairment of glucose-stimulated insulin secretion and the role of ‘lipotoxicity’ as a probable cause of hepatic and muscle resistance to insulin’s effects on glucose metabolism, has led to a host of new molecular drug targets<sup>74</sup>. Moreover, in spite of the introduction of various antidiabetic agents, diabetes and its secondary complications continue to be a global epidemic and there is high demand for novel treatment options without much side effects. Today, drug discovery strategies based on natural products and traditional medicines are re-emerging as attractive options<sup>210</sup>. Rationally designed, carefully standardized, synergistic traditional herbal formulations and botanical drug products with robust scientific evidence can also be alternatives<sup>184</sup>. Traditional knowledge is excellent source of novel drugs because of the increased tolerance, low cost, minimum side effects, local availability as well as synergistic effects. *Ayurveda* is the ‘great Indian tradition’ of ancient therapeutic approach with sound philosophical and experimental basis<sup>187</sup>. ‘*Nishakathakadi Kashayam*’ is an effective *Ayurvedic* preparation for diabetes as per *Ayurvedic* script ‘*Sahasrayogam*’<sup>211</sup>. The ingredients of *Nishakathakadi Kashayam* are *Curcuma longa*, *Strychnos potatorum*, *Ixora coccinia*, *Embllica officinalis*, *Symplocos cochinchinensis*, *Aerva lanata*, *Salacia reticulate* and *Vetiveria zizanioids*.



**Figure 1. 10.** The plant selected for the study - *Symplocos cochinchinensis* (Lour.) S. Moore.

In the present study, we selected *Symplocos cochinchinensis* (Lour.) S. Moore. from the family *Symplocaceae*, also known as *pachotti* in Malayalam or *lodhra* in Hindi, is widely distributed in tropical and subtropical areas in Asia, Oceania, and America (Figure 1. 10). The leaves and bark of *S. cochinchinensis* are reported to have antioxidant, antihyperglycemic, hypolipidemic and antidiabetic properties<sup>212-215</sup>. *S. cochinchinensis* is also used in *Ayurveda* for the treatment of various disorders, such as leprosy, tumors, diarrhea, dysentery, inflammation, and uterine disorders<sup>216</sup>. The leaves have been used as a vegetable salad along with cucumber juice for the treatment of diabetes<sup>217</sup>. Anti-HIV<sup>218</sup>, inhibitory activities against phosphodiesterase<sup>219</sup>, antimicrobial<sup>220</sup>, antiinflammatory<sup>221</sup>, and antitumor<sup>222</sup> applications have also been reported. The phytochemical investigation of the genus *Symplocos* had resulted in the isolation of confusoside, trilobatin, beta-sitosterol, symplocoside, salireposide, benzoylsalireposide, oleanolic acid, beta-amyrin, and stigmasterol<sup>223</sup>.

The increasing use of traditional therapies demands more scientifically sound evidence for the principles behind therapies and for effectiveness of medicines<sup>187</sup>. Recent advancements in the analytical and biological sciences, along with innovations in genomics and proteomics can play an important role in validation of these therapies<sup>210, 224</sup>. Even though, *S. cochinchinensis* is known traditionally for its antidiabetic property as a component of *Nishakathakadi Kashayam*, the mechanism of action of its antidiabetic activity was not clear. So, we decided to carry out a thorough mechanistic analysis of its antidiabetic potential utilizing various *in vitro* and *in vivo* methods for the scientific validation of its traditional use.

The main aims and objectives of the present study are

1. To evaluate the effect of *Symplocos cochinchinensis* on *in vitro* druggable targets relevant to diabetes.
2. To study the action of *Symplocos cochinchinensis* on hyperglycemia induced secondary complications in rat model of streptozotocin diabetes (type 1).
3. To analyze the possible molecular mechanism behind the protective property of *Symplocos cochinchinensis* against peripheral tissue (liver, muscle and adipose) insulin resistance and dyslipidemia in diet induced *in vivo rodent* model (type 2).

### **1. 13 Societal impact of the study**

Kerala, the land of rich biodiversity and traditional and indigenous knowledge is located in the windward side of Western Ghats. The region of Western Ghats situated in Kerala is the richest in all three levels of biodiversity such as ecosystem diversity, species diversity and genetic diversity<sup>225</sup>. Thousands of medicinal plants with excellent therapeutic potential are available in this region. Nowadays, the possibilities of sustainable utilization of biodiversity and indigenous knowledge remain unexplored. Recently, National Rural Health Mission reported that the diabetes prevalence is about 27% in Kerala<sup>35</sup>, even though Kerala is state of high literacy and improved healthcare facilities. So, it is high time to develop low cost and effective medication as well as lifestyle intervention strategies to reduce the incidence and prevalence of this epidemic. The prevalence of

diabetes can be minimised to a certain extent by utilizing the biodiversity and indigenous knowledge. The plant selected for present study is widely available in Western Ghats and it is already reported to have antidiabetic properties. The study is expected to uncover more scientific information for its use in diabetes which may scientifically validate the traditional knowledge for universal acceptance and wider use. Altogether, the outcome of the present study can be incorporated for lead generation in future for control and management of diabetes.

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### Effect of *Symplocos cochinchinensis* on biochemical targets relevant to diabetes

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#### 2.1 Introduction

Diabetes mellitus is a global health threat associated with increased morbidity, mortality and poor quality of life which is characterized by chronic hyperglycemia<sup>1</sup>. Hyperglycemia leads to vascular complications via glucose toxicity and oxidative stress<sup>2</sup> and proper glycaemic control is an important therapeutic strategy to prevent diabetic complications<sup>3</sup>. Major determinants of postprandial hyperglycemic variations include gut digestion and absorption rate, available insulin response and tissue insulin sensitivity<sup>4</sup>. A medication that can address these abnormalities along with oxidative stress may be quite beneficial to diabetes. Current therapies include insulin and various oral agents such as sulfonylureas, biguanides, alpha-glucosidase inhibitors and gliptins, which are used as monotherapy or in combination to achieve better glycaemic regulation<sup>3</sup>. These medications have some undesirable effects<sup>5</sup> and managing diabetes without side effects is still being a challenge. Hence the search for more effective and safer therapeutic agents of natural origin has been found to be valuable.

Traditional medicines are frequently used in urban settings as an alternative in daily healthcare and it recommends complex herbal mixtures and multi-compound extracts<sup>6</sup>. Synergistic properties of herbal medicines due to the presence of variety of components within a single herbal extract are beneficial to multifactorial diseases like diabetes<sup>7</sup>. Herbal medicines have played an important role in treating diabetes in various parts of the world for centuries. *Ayurveda*, a system of traditional medicine native to Indian subcontinent always plays major role in primary health care of both rural and urban populations of India<sup>8</sup>. *Symplocos cochinchinensis* (Lour.) S. Moore. (SC) from the family Symplocaceae, is a

medicinal plant with anti-inflammatory, antitumor, antimicrobial and antidiabetic properties<sup>9, 10</sup>. The bark of SC is one of the key ingredients of *Nisakathakadi Kashayam* (decoction); a very effective *Ayurvedic* preparation for diabetes mentioned in the ancient script '*Sahasrayogam*'<sup>11</sup>. For wider acceptability of the health benefits of SC, a detailed scientific investigation on its mode of action on various biochemical targets relevant to diabetes is mandatory. But any thorough study illustrating the mechanism of action of SC or its biochemical targets relevant to diabetes is not available in literature. Here, attempts were made to see the main bioactives responsible for its antidiabetic property and to elucidate the mode of action of SC using selected biochemical targets relevant to diabetes. The outline of the experimental design is given in Figure 2. 1.

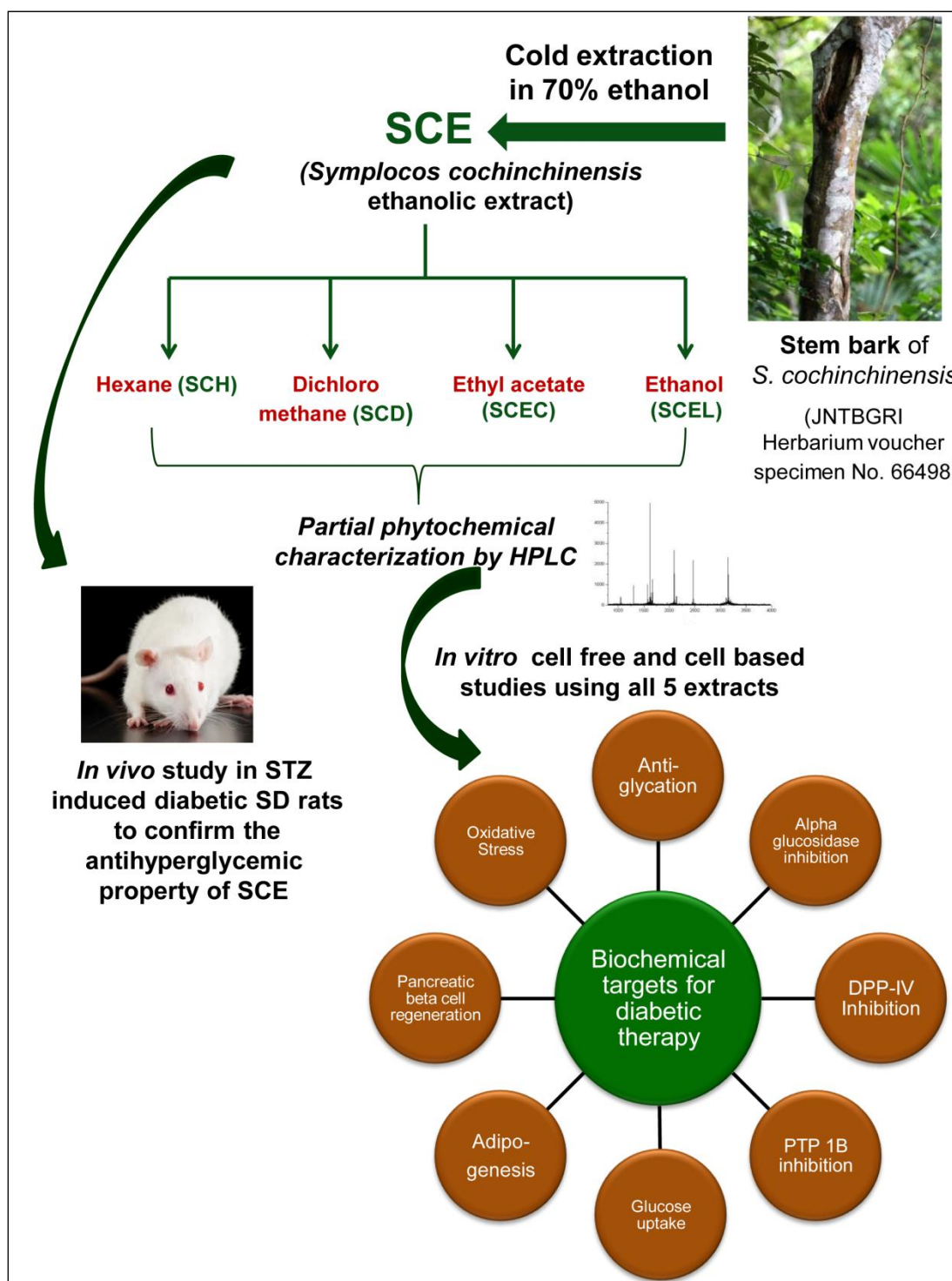
## 2. 2 Experimental details

### 2. 2.1 Chemicals and reagents

Streptozotocin ( $\geq 98\%$ ), 2,2 diphenyl-1-picryl hydrazyl (DPPH), 4-nitro phenyl alpha-D- glucopyranoside, yeast alpha-glucosidase, acarbose, gallic acid, tannic acid, quercetin, trolox, diprotin A, suramin, beta-sitosterol, phloretin 2'-glucoside, oleanolic acid, rosiglitazone, metformin, cytochalasin B, 2-deoxyglucose, 3-isobutyl -1-methylxanthine (IBMX), dexamethasone, insulin, dimethyl sulphoxide (DMSO) and all other chemicals and biochemicals unless otherwise noted were from Sigma (St. Louis, MO, USA). 2-deoxy-d-[<sup>3</sup>H]-glucose (2-DG) was from GE Healthcare, UK. All the positive controls used were of HPLC grade.

### 2. 2. 2 Plant material

The bark of SC was collected from Palode, Thiruvananthapuram (8° 29' N, 76° 59' E) during July 2011 and authenticated by Taxonomist from Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Thiruvananthapuram, Kerala. A voucher specimen (No. 66498) was stored at the herbarium of JNTBGRI. The bark of SC is used for the preparation of *Nisakathakadi Kashayam*. Keeping this in mind we also used bark of SC in this study. This plant is plenty available in this specific area (Palode, Thiruvananthapuram) and there is no restriction for the collection of the plant. It is



**Figure 2.1 Schematic representation of experimental design.** The bark of *Symplocos cochinchinensis* was dried, powdered, extracted in 70% ethanol and vacuum dried to obtain SCE. The sequential extraction of SCE using solvents like hexane, dichloromethane, ethyl acetate and ethanol resulted in 4 fractions; SCH, SCD, SCEC and SCEL. All these 5 extracts (SCE, SCH, SCD, SCEC and SCEL) were undergone partial phytochemical characterisation, various *in vitro* cell free and cell based assays. The antihyperglycemic activity of SCE was confirmed by *in vivo* studies involving streptozotocin induced diabetic Sprague Dawley (SD) rats.

not an endangered or protected species. The location is not privately-owned or protected in any way. The bark of SC was dried in an oven at 40<sup>0</sup>C and powdered. 2 kg dry powder was extracted by maceration at 35-37<sup>0</sup>C; five times for 18 to 20 hrs with 70% ethanol<sup>12</sup>. Then it was filtered under vacuum and dried using rotary evaporator (Heidolph, Schwabach, Germany) at 35-40<sup>0</sup>C. This *Symplocos cochinchinensis* hydroethanol extract was designated as SCE. SCE was fractionated using 4 different solvents based on polarity; n-hexane (SCH), dichloromethane (SCD), ethyl acetate (SCEC) and 90% ethyl alcohol (SCEL). The SCE and its fractions were stored at 4<sup>0</sup>C, protected from light and humidity.

### **2. 2. 3 HPLC analysis**

The HPLC analysis was carried out as described previously<sup>13</sup> on LC-20AD HPLC system (Shimadzu, Tokyo, Japan) equipped with the PDA detector, SPD-M20A and LC solutions software. The chromatographic separations were performed using Phenomenex Luna C-18 Column (150 mm×4.6 mm, internal diameter-5µm), with a flow rate of 0.5 mL/min and a sample injection volume of 20 µL. The mobile phase used was acetonitrile (A) and water (B) with an isocratic elution ratio of 85:15 (A:B (v/v)) in 20 min. The sample was monitored with UV detection at 210 nm at 40<sup>0</sup>C.

### **2. 2. 4 Atomic Absorption Spectrophotometer (AAS) analysis**

SCE (25 mg/mL) was digested in dilute HCl (7:3). The concentration of minerals was quantified (mg/g of sample) by atomic absorption spectrophotometer (Perkin Elmer Inc. USA).

### **2. 2. 5 Quantification of Total Phenolic Content (TPC), Total Tannin Content (TTC) and Total Flavonoid Content (TFC)**

TPC was determined as described previously<sup>14</sup>, and were expressed as milligram gallic acid equivalents per gram of extract (mg GAE/g). Tannin estimation was done by the indirect method<sup>15</sup>. TTC was expressed as milligram tannic acid equivalents per gram of extract (mg TAE/g). For both TPC and TFC, the colour developed was measured at 760 nm using a UV-visible spectrophotometer (UV-

2450PC; Shimadzu, Kyoto, Japan). TFC estimation was done as described previously<sup>16</sup> and expressed as milligram quercetin equivalents per gram of extract (mg QE/g). The absorbance was measured at 510 nm using a UV–visible spectrophotometer (UV-2450PC; Shimadzu, Kyoto, Japan). The entire work of the thesis was performed using the single lot of extracts stored at 4<sup>0</sup>C; the stability of these extracts were checked at every 6 months by TPC and TFC analysis. There was no significant difference in the TPC and TFC quantities throughout the period of the work.

### **2. 2. 6 *In vitro* alpha glucosidase (AG), dipeptidyl peptidase-IV (DPP-IV) and protein tyrosine phosphatase-1B (PTP-1B) inhibition assay**

Yeast and rat intestinal AG (EC 3.2.1.20) inhibitory properties of the extracts were determined as described previously<sup>17</sup> using acarbose as standard. All the extracts were checked for DPP-IV (EC 3.4.14.5) inhibition using the kit from Cayman chemicals (Ann Arbor, MI, USA). Diprotin A was used as the standard. PTP-1B (EC 3.3.3.48) inhibitory property of extracts was evaluated using the kit from Calbiochem (Darmstadt, Germany). The absorbance was measured using microplate reader (BioTek, Winooski, VT, USA). Percentage inhibition values were plotted against the corresponding concentrations of the sample to obtain IC<sub>50</sub> value.

### **2. 2. 7 Determination of antioxidant potential and metal chelation activity**

The antioxidant activity of extracts was assessed by DPPH method<sup>18</sup> with gallic acid as standard. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity was determined using assay kit (Zen-Bio Inc., NC, USA) and trolox was the standard. The hydroxyl radical scavenging activity was measured by the deoxyribose method<sup>19</sup> with catechin as standard. The chelation of ferrous ions by the extracts was estimated using ferrozine method<sup>20</sup> and EDTA was used as the standard. IC<sub>50</sub> values were calculated and compared with the respective standards.

### **2. 2. 8 Determination of antiglycation activity**

Advanced glycation end products (AGEs) derived from bovine serum albumin

(BSA) were quantified using the previous method<sup>21</sup>. BSA (10 mg/mL) in PBS (pH 7.4) containing 0.02% (w/v) sodium azide was preincubated with various concentrations (100, 500 and 1000 µg/mL) of the extracts (SCE and SCEL) in PBS for 30 min at 25<sup>0</sup>C. Ribose (500 mM) was added to the reaction mixture and incubated at 37<sup>0</sup>C and investigations (fluorescence/microstructure analysis) were done at two time intervals (i.e., one after 24 h and another after 7 days). BSA in the presence of ribose in phosphate buffered saline was served as control. AGE fluorescence ( $\lambda_{\text{ex}}$ 370 nm;  $\lambda_{\text{em}}$  440 nm) was measured in terms of relative fluorescence unit (RFU) after 24 h and 7 days of incubation using microplate reader (BioTek, Winooski, VT, USA). Investigations after 24 h and 7 days incubation are designated as day1 and day7 experiments respectively for future references. The data was compared with the reference compound quercetin (100 µM). AGEs formed were also processed for complexity analysis to check whether test material has capacity to block the formation of glycated products<sup>21</sup> using scanning electron microscope (SEM; Carl Zeiss, Munich, Germany). For this sample was sputter coated with gold on a polaron SC 7620 sputtering machine (Quorum Technologies Ltd, Lewes, UK) fitted with gold palladium target for duration of 270 s at 10 mA volt current. The sample was then analyzed on EVO 18 special edition model of SEM with accelerating voltage ranging from 12 KV to 20 KV. For comparing the microstructure, all samples were visualized at 16,000 × magnification.

## **2. 2. 9 Cell culture**

HepG2 and L6 cell lines were obtained from National Centre for Cell Science, Pune, India. 3T3-L1, RIN-m5F, MIN-6, PANC-1 and P8 cell lines were procured from ATCC (Manassas, VA, USA). The HepG2 cells were maintained in low glucose (5.5 mM) DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution (10,000 U/mL penicillin G, 10 mg/mL streptomycin, 25 µg/mL amphotericin B), with 5% CO<sub>2</sub> at 37<sup>0</sup>C. L6 skeletal muscle cells were maintained in alpha-MEM supplemented with 10 % FBS and 1% antibiotic/antimycotic solution at 5% CO<sub>2</sub> at 37<sup>0</sup>C. Differentiation was induced by switching confluent cells to medium supplemented with 2% FBS. Experiments were performed in differentiated myotubes. RIN-m5F cells derived

from rat insulinoma cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% antibiotic/antimycotic solution at 5% CO<sub>2</sub> at 37<sup>0</sup>C. MIN-6 cells derived from mouse insulinoma cells were maintained in DMEM supplemented with 10% FBS, 1% antibiotic/antimycotic solution, 100 µg/mL L-glutamine, 10 µL/L beta - mercaptoethanol at 5% CO<sub>2</sub> at 37<sup>0</sup>C. PANC-1 cells are used as non-endocrine pancreatic cancer derived from ductal cell. P8 cell line, obtained by fusing human PANC-1 cells with human beta cells and is a gluco-responsive insulin secreting cell line. PANC-1 cells, P8 cells and 3T3-L1 murine preadipocytes were cultured in DMEM supplemented with 10% FBS and antibiotics. Differentiation was induced by switching to DMEM with 500 µM 3-isobutyl-1-methylxanthine (IBMX), 10 µM dexamethasone and 500 nM insulin (MDI) for 48 h. Differentiation was then maintained in DMEM containing 10% FBS and 500 nM insulin for 8 days.

### **2. 2. 10 Determination of cell viability**

The extracts were dissolved in DMSO for application to cell cultures and final concentration of DMSO was fixed at 0.1% for all cell based assays. The cytotoxicity was checked by the mitochondrial function assay (MTT assay) using kit from Cayman chemicals (Ann Arbor, MI, USA). It is based on the ability of viable cells to reduce the MTT to insoluble formazan crystals by mitochondrial dehydrogenase. HepG2, L6 and 3T3-L1 cells were seeded at a density of  $4 \times 10^4$  cells/well in 24 well plate and incubated for 24 h. Cells were treated with various concentrations of extract and incubated for 24 h. Media was replaced with fresh media and 10 µL MTT dye was added. Plates were incubated at 37<sup>0</sup>C for 4 h. The resulting formazan crystals were solubilised in 100 µL SDS and incubated for 4 hrs. The absorbance was read at 570 nm using microplate reader (BioTek, Winooski, VT, USA). Then, cell viability was evaluated in HepG2, L6 and 3T3-L1 with all extracts.

### **2. 2. 11 Evaluation of cell proliferation**

MIN-6, RIN-m5F, P8 and PANC-1 cells were seeded in a 96-well plate at a density of 6000 cells/well. After 24 h incubation, cells were treated with four



different concentrations of SCE (1.25, 2.5, 5, 10 µg/ml) and incubated for 24 h. Then, cell proliferation in MIN-6, RIN-m5F, P8 and PANC-1 was evaluated by MTT assay as described in section 2. 2. 10.

### **2. 2. 12 Hyperglycemia-induced oxidative stress**

The cells were maintained in low glucose medium (5.5 mM) for the initial 24 h, then switched over to high glucose (25 mM) medium with or without the extracts (SCE, SCEC and SCEL) or quercetin (positive control) to check whether the extracts prevent the generation of oxidative stress. The intracellular reactive oxygen species (ROS) production was monitored with the fluorescent probe CM-H<sub>2</sub>DCFDA<sup>22</sup>. Briefly, cells were incubated with CM-H<sub>2</sub>DCFDA (10 µM) in serum-free medium for 30 min at 37<sup>0</sup>C, washed with PBS. Images were taken using pathway 855 bio-imaging system (BD Biosciences, San Jose, CA, USA).

### **2. 2. 13 Glucose uptake**

The 2-deoxy glucose uptake in L6 myotubes was performed for all extracts as described previously<sup>23</sup>. Briefly myotubes were incubated with extracts or standards for 16 h with final 3 h in serum-deprived medium (alpha-MEM) and a sub-set of cells were stimulated with 100 nM insulin for 20 min. Glucose uptake was assessed for 5 min in HEPES-buffered saline [140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub> (pH 7.4)] containing 10 µM 2-DG (0.5 µCi/mL 2-[<sup>3</sup>H] DG) at room temperature. Subsequently cells were rinsed with an ice-cold solution containing 0.9% NaCl and 20 mM D-glucose. To quantify the radioactivity incorporated, cells were lysed with 0.05 N NaOH and were counted in a beta-counter (Perkin Elmer, Waltham, MA, USA). Nonspecific uptake was determined in the presence of cytochalasin B (50 µM) during the assay, and these values were subtracted from all other values. Glucose uptake measured in triplicate and normalized to total protein, was expressed as fold induction with respect to unstimulated cells. Rosiglitazone and metformin were the standards.

### **2. 2. 14 Adipocyte differentiation**

The adipogenic potential of all the extracts (30 µg/mL) was assessed in 3T3-L1



preadipocyte over untreated cells by quantifying the accumulation of triglycerides using oil red O staining on day 8<sup>24, 25</sup>. Rosiglitazone was used as standard. The cell lysates from all experimental groups were prepared according to the previous method<sup>21</sup> and assayed for GPDH (EC 1.1.1.8) activity using a Takara GPDH Assay Kit (Takara Bio Inc, Otsu, Japan). The membrane fraction for DGAT-1 assay was collected as described previously<sup>26</sup>. DGAT-1 (EC 2.3.1.20) activity was measured using the kit from MyBioSource (San Diego, CA, USA). Total cellular TG was extracted as reported previously<sup>27</sup>. TG content was assayed using a TG assay kit (Cayman Chemicals). The protein content was measured and normalized for GPDH, DGAT-1 and TG assays using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL USA). The adiponectin level in the residual media was measured using adiponectin assay kit (Cayman Chemicals).

## **2. 2. 15 Animals**

Male albino rats of Sprague Dawley (SD) strain (7-8 weeks old, 160±20 g), bred at animal facility of Council for Scientific and Industrial Research-Central Drug Research Institute (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<sup>0</sup>C; 50-60% relative humidity; light 300 lux at floor level with regular 12 h light/dark cycle. Animals were maintained on a standard pellet diet and water *ad libitum*. According to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) formed by the Government of India in 1964, proper sanction had been obtained for animal experiments from CSIR-CDRI institutional animal ethics committee (Ethics Committee Approval Reference No. IAEC/2008/63/Renewal 04 dated 16.05.2012). Approval was obtained specifically for the animal experiments of this study from CSIR-CDRI institutional animal ethics committee. Animals were sacrificed by cervical dislocation under light ether anaesthesia as per ethics committee guidelines. All the animal experiments were carried out by me at Department of Biochemistry, CSIR-CDRI, Lucknow.

### 2. 2. 16 Oral Sucrose Tolerance Test (OSTT) in normal rats

For this normal SD rats were fasted for 16 h. Animals showing fasting blood glucose level (BGL) between 70 to 90 mg/dL were divided into 6 groups containing 6 animals each. Animals of experimental groups were orally administered SCE (100, 250 and 500 mg/kg body weight (bw)), metformin (100 mg/kg bw) or acarbose (50 mg/kg bw) dissolved in 1.0% gum acacia. The dose of SCE was selected on the basis of dosage of 'Nisakathakadi Kashayam' for human use. 10-15 mL of this preparation containing approximately 3.5 g of SC bark including other 7 herbs in equal amount, thrice in a day is generally prescribed for patients<sup>11</sup>. Ethanol extract has been used for *in vivo* study due to the yield of more bioactive molecules and less toxicity of the solvent<sup>28</sup>. Since its selective nature, 70% ethanol is the most suitable solvent for *in vivo* pharmacological evaluation compared to other solvents; it will dissolve only the required bioactive constituents with minimum amount of the inert materials<sup>28</sup>. Animals of control group were given an equal volume of 1.0% gum acacia. Rats were loaded with sucrose (10 g/kg bw) orally 30 min after administration of test sample or vehicle. BGL was estimated at 30, 60, 90 and 120 min post administration of sucrose. Food but not water was withheld during the course of experimentation<sup>29</sup>.

### 2. 2. 17 OSTT in sucrose loaded mild diabetic rat model (SLM)

Animals were made diabetic by injecting streptozotocin (60 mg/kg in 100 mM citrate buffer-pH 4.5) intraperitoneally after overnight fasting. Animals showing fasting BGL <200 mg/dL after 72 h were selected, termed as mild diabetic<sup>30</sup> and divided into 4 groups of 6 animals each. Animals of experimental group were administered SCE (500 mg/kg bw), metformin (100 mg/kg bw) or acarbose (50 mg/kg bw). Mild diabetic control group were given an equal amount of 1.0% gum acacia. A sucrose load (10 g/kg) was given to each animal orally 30 min after test sample or vehicle. BGL was determined at 30, 60, 90 and 120 min post-administration of sucrose<sup>29</sup>.

## 2. 2. 18 OSTT in sucrose-challenged streptozotocin-diabetic rat model (STZ-S)

Like SLM, rats were made diabetic. Animals of BGL >350 mg/dL after 72 h were selected, termed as diabetic<sup>30</sup>, and divided into 4 groups of 6 animals each. Experimental groups were administered with SCE, metformin or acarbose like SLM. Diabetic control group received equal amount of 1.0% gum acacia. Rats were loaded with sucrose (3 g/kg bw) orally 30 min after test sample or vehicle. BGL was checked at 30, 60, 90, 120, 180, 240, 300 and 1440 min (24 h), respectively<sup>29</sup>. Acarbose has been selected as one of the positive control as it is the alpha-glucosidase inhibitor which can improve long term glycemic control in patients with diabetes<sup>31</sup>. Metformin is the widely used antidiabetic to treat the cardinal symptoms of diabetes like polyphagia, polydipsia, polyuria and insulin resistance due to its pleiotropic effect via various targets and it shows wide tolerance and less toxicity compared to other antidiabetics<sup>32</sup>. Due to the wider acceptability of metformin as an antidiabetic drug, we used it as a positive control.

## 2. 2. 19 Statistical analysis

Quantitative glucose tolerance of each group was calculated by the area under the curve (AUC) method using GraphPad Prism software version 3 (GraphPad 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  $\pm$  SD, n = 6.  $p \leq 0.05$  was considered to be significant.

## 2. 3 Results

### 2. 3. 1 Phytochemical characterization

HPLC analysis showed the presence of beta-sitosterol (111.62 $\pm$ 4.12 mg/g), phloretin 2' glucoside (98.32 $\pm$ 4.87 mg/g) and oleanolic acid (63.89 $\pm$ 3.03 mg/g) in *Symplocos cochinchinensis* ethanolic extract (SCE) (Fig. 2. 2A-D); phloretin 2' glucoside (508.46 $\pm$ 11.63 mg/g) and oleanolic acid (39.09 $\pm$ 1.73 mg/g) in ethyl acetate fraction of SCE (SCEC) (Fig. 2. 2A, B and E); phloretin 2'glucoside (273.65 $\pm$ 7.63 mgg-1) in ethyl acetate fraction of SCE (SCEL) (Fig. 2. 3A and C); beta-sitosterol (152.29 $\pm$ 6.31 mg/g) and phloretin 2'glucoside (188.97 $\pm$ 6.41 mg/g)

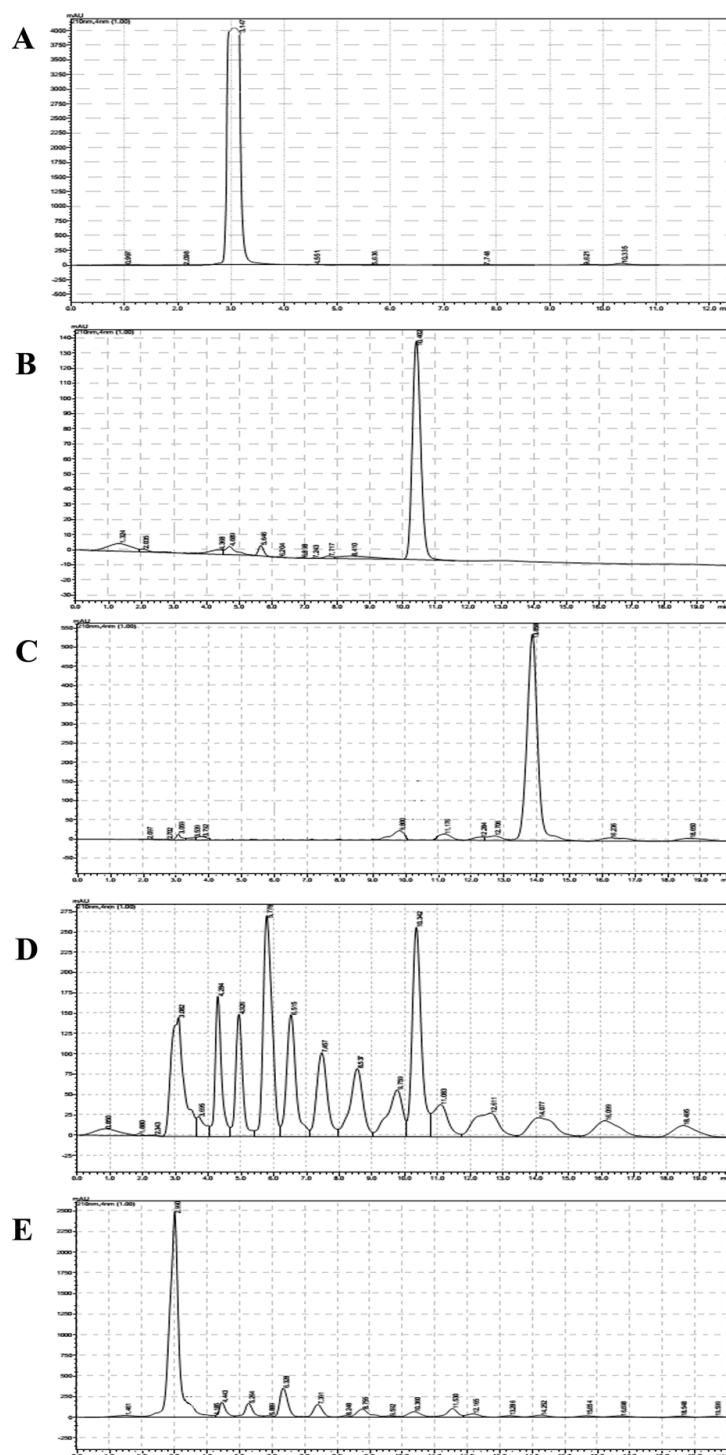
in dichloromethane fraction of SCE (SCD)(Fig. 2. 3A, B and D); beta-sitosterol (145.56±4.63 mg/g) in hexane fraction of SCE (SCH) (Fig. 2. 3B and E) <sup>33</sup>. Analysis of minerals by AAS for micro-nutrients revealed presence of various minerals like zinc (0.014±0.0005 mg/g) manganese (0.096±0.0041 mg/g), iron (0.147±0.005 mg/g), sodium (1.387±0.062 mg/g), potassium (2.496±0.11 mg/g), magnesium (4.368±0.203 mg/g) and calcium (46.799±2.15 mg/g). The dry yield, TPC, TTC and TFC of the extracts were shown in Table 2.1. Since SCE exhibited comparatively better activity with respect to various *in vitro* targets and its high content of bioactives, SCE was taken forward for *in vivo* study. Moreover, in Indian traditional system of medicine (*Ayurveda*) most of the decoctions are hydro-alcohol based (eg. *Arishta* and *Kashaya*).

Sample	Dry yield as % weight of dry plant material	Total phenolic content (TPC) in mg GAE/g	Total flavonoid content (TFC) in mg QE/g	Total tannin content (TTC) in mg TAE/g
SCE	12.35	53.72	19.35	10.47
SCH	0.50	13.40	8.56	-
SCD	0.32	36.27	19.85	-
SCEC	0.55	57.28	26.35	17.54
SCEL	2.91	54.68	22.85	13.26

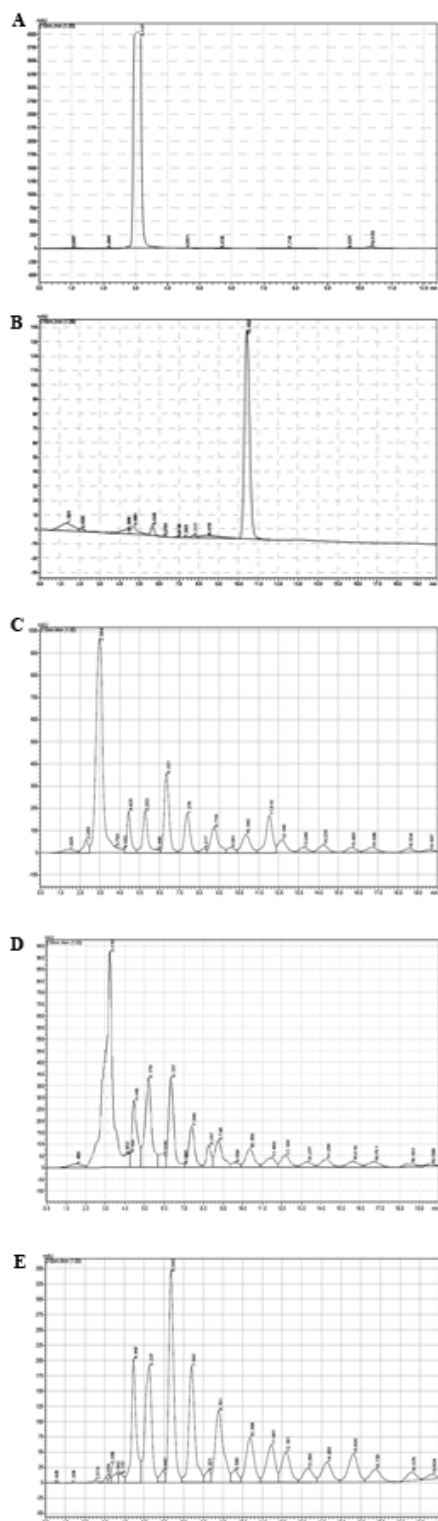
**Table 2. 1.** Dry yield, Total Phenolic Content (TPC), Total Tannin Content (TTC) and Total Flavonoid Content (TFC) of test materials.

### 2. 3. 2 *In vitro* AG, DPP-IV and PTP1-B inhibitory property

The extracts were evaluated for AG inhibition utilizing rat intestinal and yeast enzymes. SCEC, SCD and SCE showed significant yeast AG inhibition with IC<sub>50</sub> values of 62.30±1.53, 71.26±1.94 and 82.07±2.10 µg/mL respectively (Fig. 2. 4A). Rat intestinal AG inhibition (IC<sub>50</sub>) of the extracts was found to be 194.93±2.67 (SCEC), 143.02±2.91 (SCD) and 232.05± 3.34 µg/mL (SCE) (Fig. 2. 4B). Acarbose showed an IC<sub>50</sub> of 45±1.12 for yeast and 49.78±1.45 µg/mL for rat AG enzymes. SCEC fraction showed DPP-IV inhibition with an IC<sub>50</sub> of 87.63±1.88 µg/mL while IC<sub>50</sub> of SCE was 269.98±2.95 µg/mL (Fig. 2. 5A).



**Figure 2. 2A-E.** HPLC data of SCE and SCEC: HPLC chromatogram of standard compounds (A) phloretin 2'glucoside (retention time – 3.147min), (B) beta sitosterol (retention time – 10.402min) and (C) oleanolic acid (retention time – 13.858min). HPLC fingerprint analysis of (D) ethanol extract of *S. cochinchinensis* (SCE) and (E) its ethyl acetate fraction (SCEC). Peaks were detected at 210 nm.

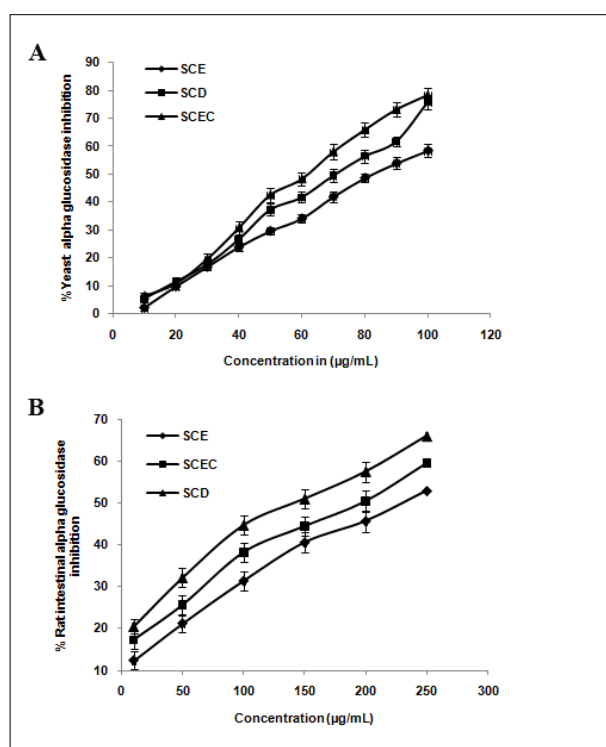


**Figure 2. 3A-E.** HPLC data of SCEL, SCD and SCH: HPLC chromatogram of standard compounds (A) phloretin 2'glucoside (retention time – 3.147min) and (B) beta sitosterol (retention time – 10.402min). HPLC fingerprint analysis of (C) ethanol fraction (SCEL), (D) dichloromethane fraction (SCD) and (E) hexane fraction (SCH). Peaks were detected at 210 nm.

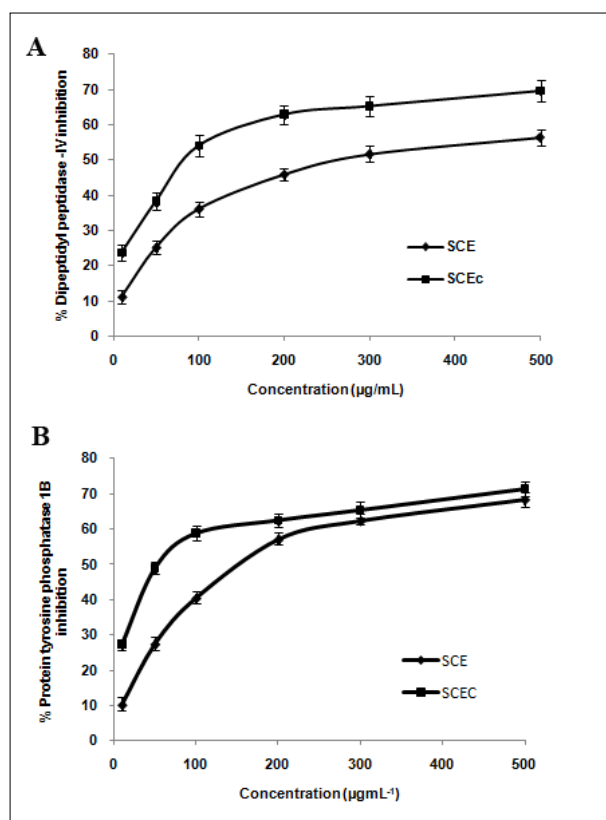
Standard compound diprotin A showed an  $IC_{50}$  of  $1540 \pm 11.2 \mu\text{g/mL}$ . PTP-1B inhibition was noticed in SCEC fraction with an  $IC_{50}$  of  $55.83 \mu\text{g/mL}$  and SCE exhibited an  $IC_{50}$  of  $159.10 \mu\text{g/mL}$  (Fig. 2. 5B). Standard was suramin ( $IC_{50}$   $14.01 \mu\text{g/mL}$  ( $10.8 \mu\text{M}$ )).

### 2. 3. 3 SC fractions exhibited antioxidant and metal chelation potential

SCEC, SCEL and SCE showed better DPPH radical scavenging property compared to SCH and SCD (Table 2. 2).  $IC_{50}$  of gallic acid was  $6.5 \pm 0.73 \mu\text{g/mL}$ . Similarly SCEC, SCEL and SCE exhibited promising ABTS cation decolorization potential compared to SCH and SCD (Table 2. 2).  $IC_{50}$  of the standard trolox was  $5 \pm 0.51 \mu\text{g/mL}$ . SCEC, SCEL and SCE showed potent hydroxyl radical scavenging and metal chelation activity compared to SCH and SCD (Table 2).  $IC_{50}$  of catechin was  $9 \pm 0.86 \mu\text{g/mL}$  and that of EDTA was  $4.67 \pm 0.36 \mu\text{g/mL}$ .



**Figure 2.4A and B. SCE, SCD and SCEC exhibited alpha-glucosidase inhibitory property.** (A) Yeast alpha glucosidase inhibition. (B) Rat intestinal alpha glucosidase inhibition. Values are means  $\pm$  SD; n = 6. SCE, *S. cochinchinensis* (SC) ethanol extract; SCD, SC dichloromethane fraction and SCEC, SC ethyl acetate fraction.



**Figure 2. 5A and B. DPP-IV and PTP-1B inhibitory property of SCE and SCEC** (A) DPP-IV inhibition by SCE and SCEC; SCEC  $IC_{50}$ -  $87.63 \pm 1.88$   $\mu\text{g/mL}$  and SCE  $IC_{50}$ -  $269.98 \pm 2.95$   $\mu\text{g/mL}$ . Values are means  $\pm$  SD; n = 6. (B) PTP-1B inhibitory property of SCE and SCEC; SCEC  $IC_{50}$ -  $55.83 \pm 1.24$   $\mu\text{g/mL}$  and SCE  $IC_{50}$ -  $159.10 \pm 1.91$   $\mu\text{g/mL}$ . Values are means  $\pm$  SD; n = 6. SCE, *S. cochinchinensis* (SC) ethanol extract and SCEC, SC ethyl acetate fraction.

### 2. 3. 4 Antiglycation property was observed in SCE, SCEC and SCEL

AGEs derived from BSA was analysed using 2 methods; by relative fluorescence unit (RFU) measurements and SEM analysis. There were 22 groups under day1 and day7 experiments. In detail, 2 untreated control groups (one each with day1 and day7 experiments), 2 quercetin treated groups (day1 and day7 experiments) and 18 extract treated groups (3 doses- 100, 500 and 1000  $\mu\text{g/mL}$  of SCE, SCEC and SCEL under day1 and day7). Quercetin (100  $\mu\text{M}$ ) showed significant ( $p \leq 0.05$ ) antiglycation property in RFU measurement and also in SEM analysis (Fig. 2. 6A and B b). Significant decrease ( $p \leq 0.05$ ) in fluorescence in dose dependent manner was observed in day1 and day7 experiments at 500 and 1000  $\mu\text{g/mL}$  doses of three extracts, indicative of antiglycation property (Fig. 2. 6A). SEM analysis of the



microstructure of control group of day1 showed highly granular agglomeration with uneven pores and highly complex cross linking (Fig. 2. 6B a). 500 and 1000 µg/mL doses of SCE, SCEC and SCEL reduced highly complex microstructure to simple membranous structure without any cross linking in day1 experiment (Fig. 2. 6B c-h).

### 2. 3. 5 Protection from hyperglycemia-induced oxidative stress

High glucose treatment induced the generation of significant amount of ROS in HepG2 cells (64.23%; Fig 2. 7A and B, b), but co-treatment with SCE or SCEC significantly attenuated ROS in a dose dependent manner ( $p \leq 0.05$ ). SCE and SCEC were selected on the basis of their potent *in vitro* antioxidant property. Results showed that 41.94, 51.28 and 59.57% decrease of ROS level with 10, 50 and 100 µg/mL SCE respectively (Fig 2. 7B, d-f) compared to high glucose control group. Similarly SCEC caused 34.92, 45.79 and 56.72% decrease of ROS level with 10 and 50 and 100 µg/mL dose respectively (Fig 2. 7B, g-i). Quercetin (25µM) showed significant ( $p \leq 0.05$ ) decrease (60.04%) of ROS (Fig 2. 7B, c). All extracts were found to be absolutely safe up to 100 µg/mL in all cell lines; HepG2, L6 and 3T3-L1 (Fig 2. 8). There was no significant difference in viability of cells of treated groups and untreated control group (Fig 2. 8).

Samples	IC <sub>50</sub> values of DPPH radical scavenging assay in µg/mL	IC <sub>50</sub> values of ABTS radical scavenging assay in µg/mL	IC <sub>50</sub> values of hydroxyl radical scavenging assay in µg/mL	IC <sub>50</sub> values of metal chelation activity in µg/mL
SCE	133.20±2.45	54.95±1.12	34.74±1.06	89.31±1.82
SCH	402.62±3.41	321.12±2.94	364.23±3.17	295.21±4.67
SCD	541.65±3.61	96.29±1.90	164.37±2.56	211.38±3.61
SCEC	129.43±1.84	35.72±1.02	31.64±0.98	86.49±1.76
SCEL	130.04±1.92	36.47±1.21	42.81±1.52	94.38±2.04

**Table 2. 2.** IC<sub>50</sub> values of antioxidant (DPPH, ABTS and hydroxyl radical scavenging) and metal chelation assays. Values are means ± SD; n = 6.

### **2. 3. 6 Proliferation potential of SCE in pancreatic beta-cell lines: RIN-m5F, MIN-6, PANC-1 and P8 cell lines**

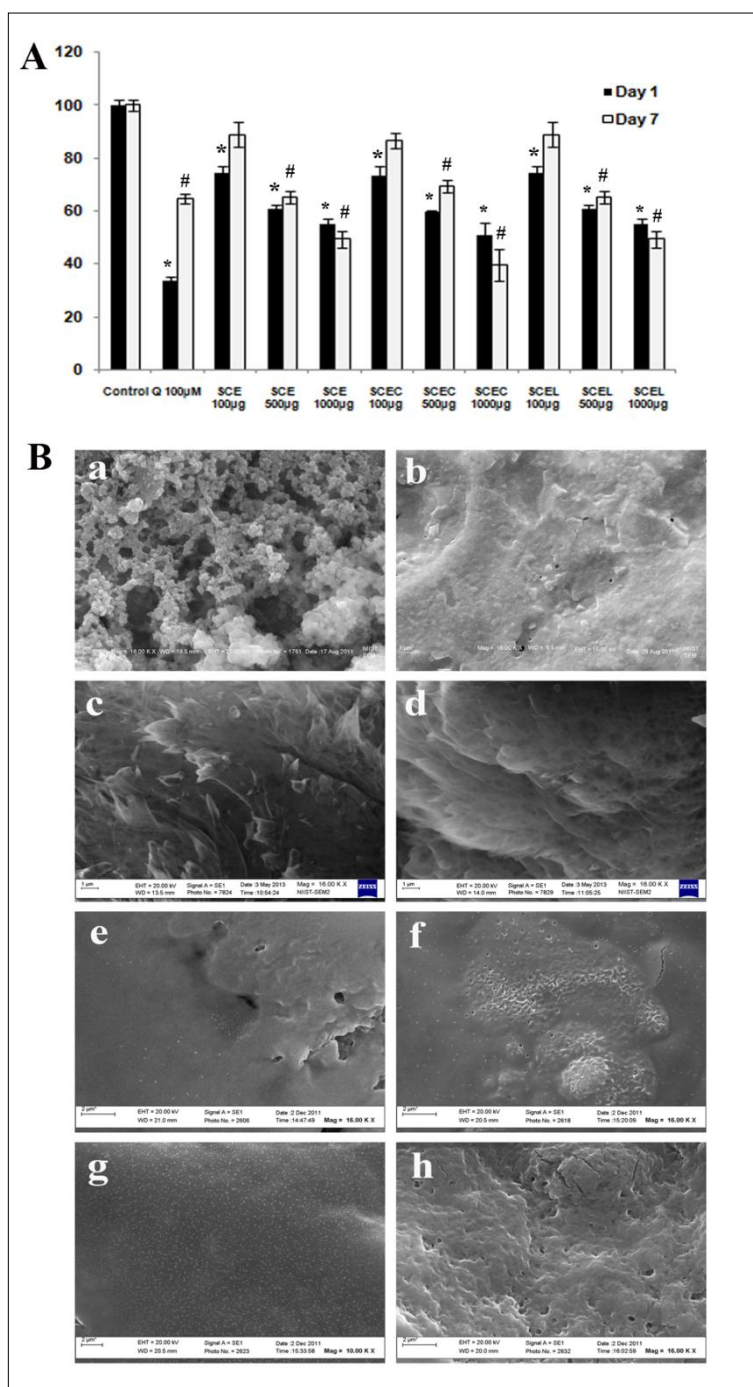
Treatment with SCE (1.25, 2.5, 5 and 10 µg/mL) induced significant cell proliferation in RIN-m5F, MIN-6 and P8 cells in a dose dependent manner. In details, µg/mL of SCE caused cell proliferation rate 10 3.5 fold, 1.5 fold and 1.29 fold respectively compared to control in RIN-m5F, MIN-6 and P8 cells (Fig. 2. 9A, B and D) and other extracts did not show any positive effect. But, in PANC-1 cells SCE caused significant cell proliferation only with lower doses (1.25 and 2.5 µg/mL) compared to control group (Fig. 2. 9C).

### **2. 3. 7 Enhancement of glucose uptake in L6 myotubes**

Pre-treatment of myotubes with SCE and its fractions at a dose of 100 µg/mL for 16 h with insulin (100 nM) resulted in increase of glucose uptake in an additive manner (Fig. 2. 10A,  $p \leq 0.05$ ). Among various extracts studied, both SCE and SCE-L exhibited better activity both in the absence and presence of insulin in a dose dependent manner (Fig. 2. 10B,  $p \leq 0.05$ ). Insulin alone showed a significant increase in glucose uptake (1.9 fold of basal,  $p \leq 0.05$ ) in L6 myotubes. Metformin and rosiglitazone were standards (Fig. 2. 10A).

### **2. 3. 8 Adipogenesis**

The treatment with SCE and its fractions (30 µg/mL) induced a moderate level of differentiation of 3T3-L1 preadipocytes to adipocytes, but less than rosiglitazone (Fig. 2. 10C). This was based on the morphological observation and quantification of triglycerides by oil red O staining (Fig. 2. 10C; Fig. 2. 11A). SCE at 50 µg/mL dose exhibited a significant decrease in GPDH activity compared to MDI positive group ( $p \leq 0.05$ , Fig. 2. 11B), at the same time 25 and 50 µg/mL doses of SCE exhibited a significant decrease in DGAT1 activity and TG content compared to MDI positive group ( $p \leq 0.05$ , Fig. 2. 11C and D). However, adiponectin level was significantly increased by SCE treatment (25 and 50 µg/mL) compared to MDI positive group ( $p \leq 0.05$ , Fig. 2. 11E). Rosiglitazone was the reference standard.



**Figure 2. 6A and B. Fluorescence quantification and scanning electron microscope (SEM) microstructure analysis of advanced glycation end products revealed the antiglycation property of SCE, SCEC and SCEL. (A) Quantification of fluorescence intensity of glycated products in presence of various concentrations of SCE, SCEC and SCEL (100, 500, 1000 µg/mL) under 2 different time intervals (day1 and day7) in terms of relative fluorescence units (RFU). Quercetin (100 µM) was used as reference compound. RFU are normalized to 100. Values are means  $\pm$  SD; n = 6. \* represents groups differ significantly from day 1 control group ( $p \leq 0.05$ ) and**

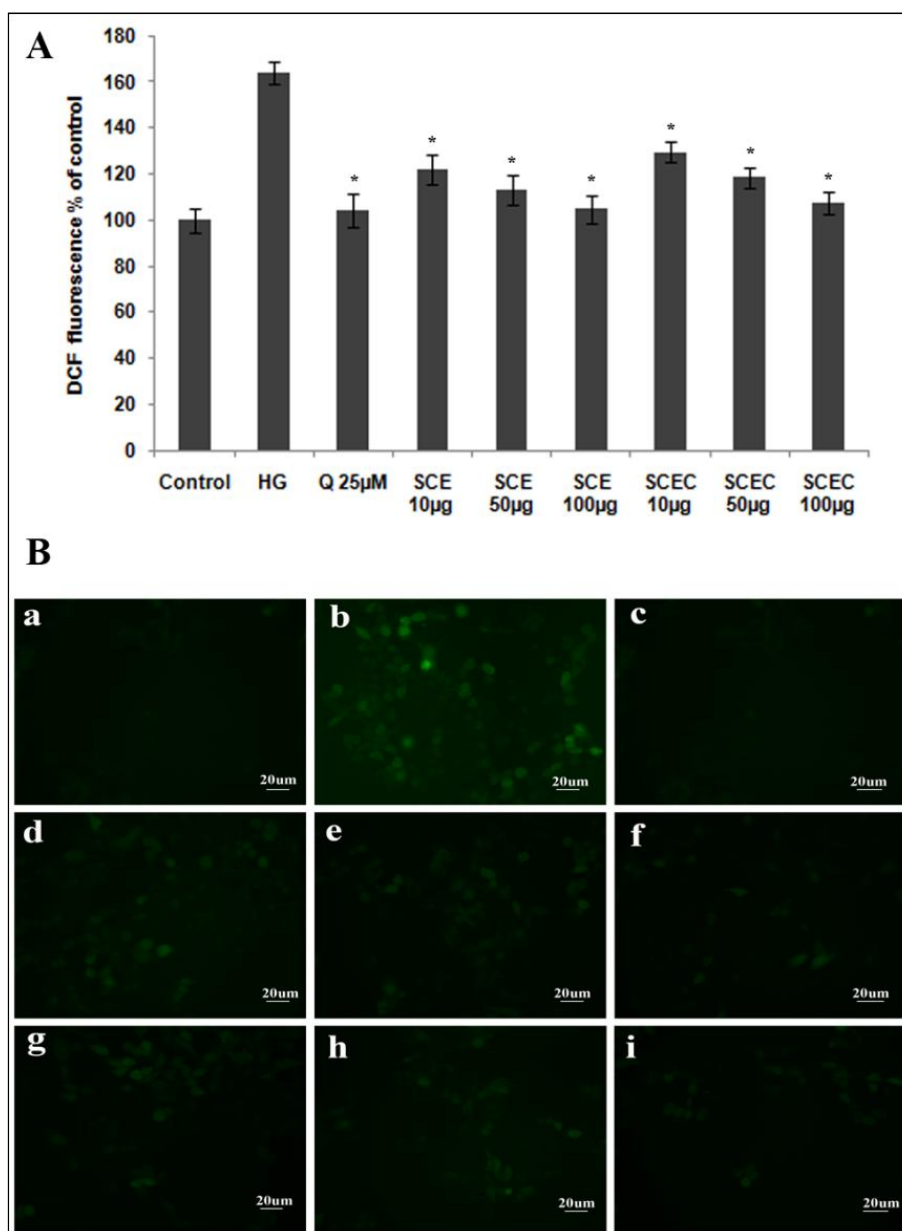
≠ represents groups differ significantly from day 7 control group ( $p \leq 0.05$ ). (B) Representative SEM microstructures of glycated products formed under various groups of day1 experiments (a-h), (a, control; b, quercetin 100  $\mu\text{M}$ ; c and d, SCE 100  $\mu\text{g/mL}$  and SCE 1000  $\mu\text{g/mL}$ ; e and f, SCEC 100  $\mu\text{g/mL}$  and SCEC 1000  $\mu\text{g/mL}$ ; g and h, SCEL 100  $\mu\text{g/mL}$  and SCEL 1000  $\mu\text{g/mL}$ . All samples were visualized at 16,000 $\times$  magnification.

### **2. 3. 9 Antihyperglycemic effect of SCE in normal and diabetic *in vivo* models**

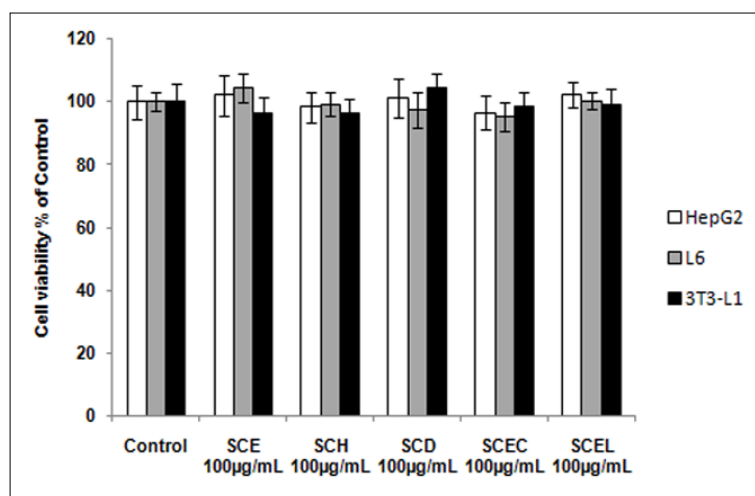
In acute toxicity study, SCE did not show any observable toxic effects in behaviour or physiology of animals up to 2 g/kg bw. In normal and SLM, the rise in BGL at 30 min of oral sucrose load was significantly reduced in SCE treated group compared to control group. SCE treatment at doses of 100, 250 and 500 mg/kg bw exhibited 7.56, 10.23 and 15.53% reduction respectively in plasma glucose in normal sucrose loaded rats and 18.18 and 20.42% by acarbose and metformin treatment (Fig. 2. 12A and B). In SLM, treatment with 500 mg/kg bw of SCE reduced the whole glycemic response by 12.88% while acarbose and metformin caused 15.73 and 17.12% reduction respectively (Fig. 2. 12C and D). SCE treatment (500 mg/kg bw) in STZ-S caused 23.48% improvement in blood glucose profile after 5 h of treatment and acarbose and metformin showed 30.27 and 33.18% respectively (Fig. 2. 12E and F).

### **2. 4 Discussion**

The pathogenesis of diabetes mellitus is complex and involves many mechanisms leading to several complications and demands a multiple therapeutic approach. Nowadays, medicinal plants have re-emerged as an effective source for the treatment of diabetes as it hold diverse group of compounds. Metformin exemplifies an efficacious oral glucose lowering agent derived from the research based on medicinal plants<sup>34</sup>. To date many antidiabetic medicinal plants have been reported although only a small number of these have received scientific evaluation to elucidate their mechanism of action. The World Health Organisation Expert Committee on diabetes has stressed the need of research on traditional medicine for future drugs<sup>35</sup>. In this study, phytochemically characterized SC was subjected to investigation on various biochemical targets relevant to diabetes like AG,



**Figure 2. 7A and B. SCE and SCEC fractions protected HepG2 cells against ROS generation during hyperglycemia.** Analysis of high glucose induced intracellular ROS levels in HepG2 cells by DCFDA method. Cultured HepG2 cells were treated with SCE or SCEC in the presence of high glucose (HG; 25 mM) for 24 h and then incubated with H<sub>2</sub>DCFDA. The results are shown as (A) the quantitative analysis of fluorescence from three independent experiments. Values are means  $\pm$  SD; n = 6. \* represents groups differ significantly from HG group ( $p \leq 0.05$ ). (B) Representative microscopic scans a - i (a, vehicle control; b, high glucose (HG); c, HG + Quercetin; d-f, HG + 10  $\mu$ g, 50  $\mu$ g and 100  $\mu$ g SCE; g-i, HG + 10  $\mu$ g, 50  $\mu$ g and 100  $\mu$ g SCEC). All samples were visualized at 20 $\times$  magnification.



**Figure 2. 8.** Cell viability assay by MTT method revealed all the extracts at 100 µg/mL concentration are not toxic to HepG2, L6 and 3T3-L1 cell lines. Results were normalised to 100 based on control readings. Values are means  $\pm$  SD; n = 6.

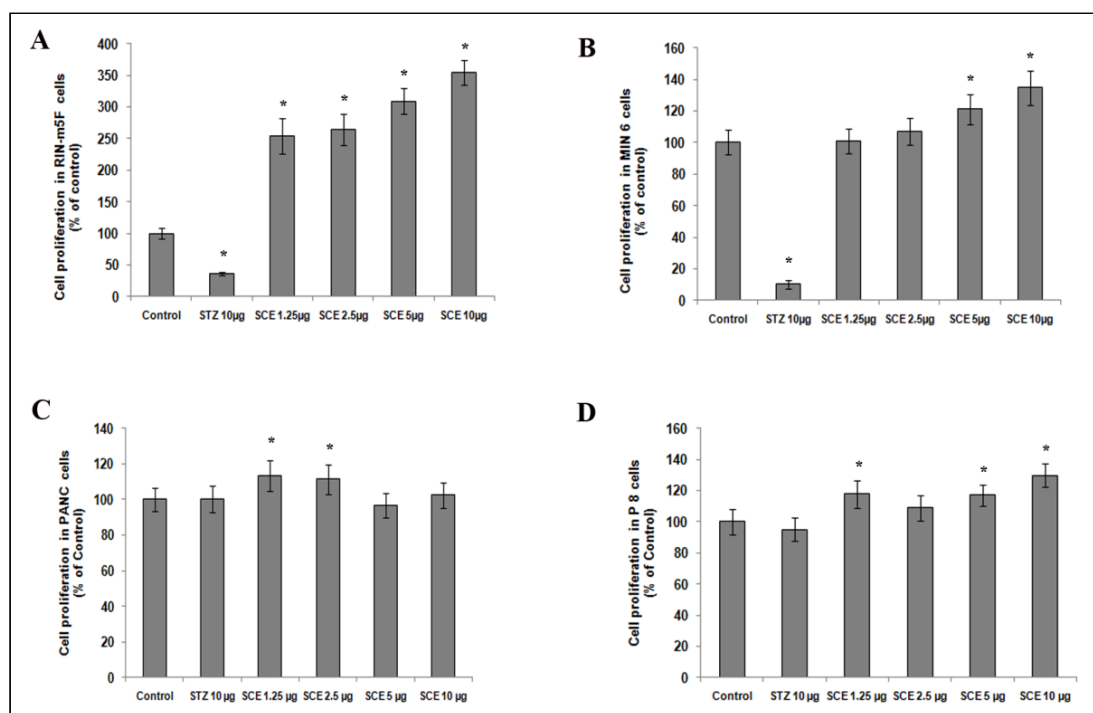
glycation, DPP-IV, PTP-1B and hyperglycemia induced oxidative stress along with pancreatic beta cell proliferation, insulin dependent glucose uptake and adipogenesis using *in vivo* and *in vitro* models. Cell line based *in vitro* models are very much important in diabetic research as it is helpful to determine the mechanism of action of a plant extract with traditional use and/or human or *in vivo* data to support the antidiabetic effect<sup>36</sup>. In addition, the cell line based model allows the use of less amount of test material with reduced variability in results<sup>36</sup>.

Oxidative stress due to hyperglycemia and dyslipidemia is one of the physiological parameter evident in diabetes<sup>37</sup>. Depletion of antioxidant level has been demonstrated in diabetic patients and extra administration of antioxidants to compensate the depletion, had helped to prevent diabetes complications<sup>38</sup>. Hyperglycemia induces accelerated hydroxyl radical generation and reactive oxygen species production which could represent the key event in the development of diabetic complications<sup>39, 40</sup>. So we had analysed the antioxidant potential and hydroxyl radical scavenging activity of various extracts and the ability of extracts to prevent ROS generation under hyperglycemia. The results revealed significant antioxidant potential of SCE, SCEC and SCEL in an *in vitro* cell free system (Table 2. 2) and protected HepG2 cells from hyperglycemia induced oxidative stress by preventing generation of ROS (Fig. 2. 7A and B,

$p \leq 0.05$ ). It has been suggested that during hyperglycemic conditions, a non-enzymatic reaction occur between proteins and monosaccharides (glycation) leading to the formation of pathologically significant AGEs<sup>41</sup>. Due to far reaching consequences of AGEs in the body, the estimation of glycated haemoglobin (% HbA1c) has been advised by clinicians in addition to glucose in diagnosing metabolic syndrome. Biologically AGEs alter enzyme activity, modify protein and are main culprit in diabetes induced cardiomyopathy, retinopathy and neuropathy. Moreover, AGEs induce oxidative stress and vice versa<sup>41</sup>. So there is a tremendous interest in antiglycation agents for diabetes therapy. But as of today no specific drug is available with antiglycation potential. Our study revealed significant antiglycation activity of SCE, SCEC and SCEL (Fig. 2. 6A and B) which could possibly one prominent mechanism of its known antidiabetic property. The two categories of antiglycation agents (AGE inhibitors and AGE breakers) act primarily as chelators by inhibiting metal-catalyzed oxidation reactions that catalyze AGE formation<sup>42</sup>. From the SEM microstructure analysis of AGEs, it is clear that SCE, SCEC and SCEL exhibited antiglycation via its AGE inhibitor property<sup>43</sup>. The *in vitro* method had shown potent metal chelation capacity of SC which may be the mechanism behind the better antiglycation potential of this plant.

PTP-1B is an abundant and widely expressed enzyme localized in endoplasmic reticulum. Theoretically, inhibition of action of PTP-1B that terminates insulin signalling would be expected to increase insulin sensitivity<sup>44</sup>. The broad use of PTP-1B inhibitors, although with potential benefits over the insulin signalling pathway, might exert undesirable effects on response to stressors of the immune system including the fine tuning of the pro-inflammatory and pro-resolution balance<sup>45</sup>. SCEC and SCE showed a moderate PTP-1B inhibitory property which may be beneficial in enhancing insulin sensitivity without exerting much undesirable effects (Fig. 2. 5B). DPP-IV is a serine exopeptidase which regulates the half- life of two key glucoregulatory incretin hormones like glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1)<sup>46</sup>. Inhibition of DPP-IV prolongs and enhances the activity of endogenous GIP and GLP-1, which serve as important prandial stimulators of insulin secretion in



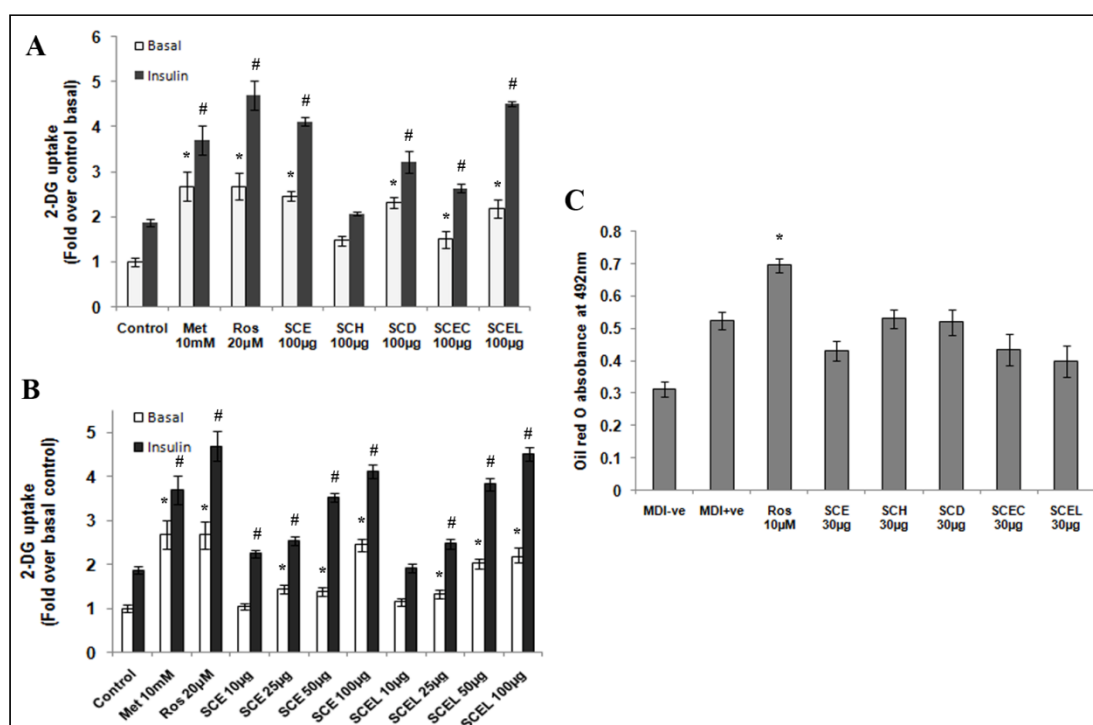


**Figure 2.9 A-D. SCE induce proliferation in various pancreatic beta cell lines like RIN-m5F, MIN-6, PANC and P8 cells.** (A) proliferation potential of SCE in RIN-m5F cells. (B) proliferation potential of SCE in MIN-6 cells. (C) proliferation potential of SCE in PANC cells. (D) proliferation potential of SCE in P8 cells. Results were normalised to 100 based on control readings. Values are means  $\pm$  SD; n = 6. \* represents groups differ significantly from control group ( $p \leq 0.05$ ).

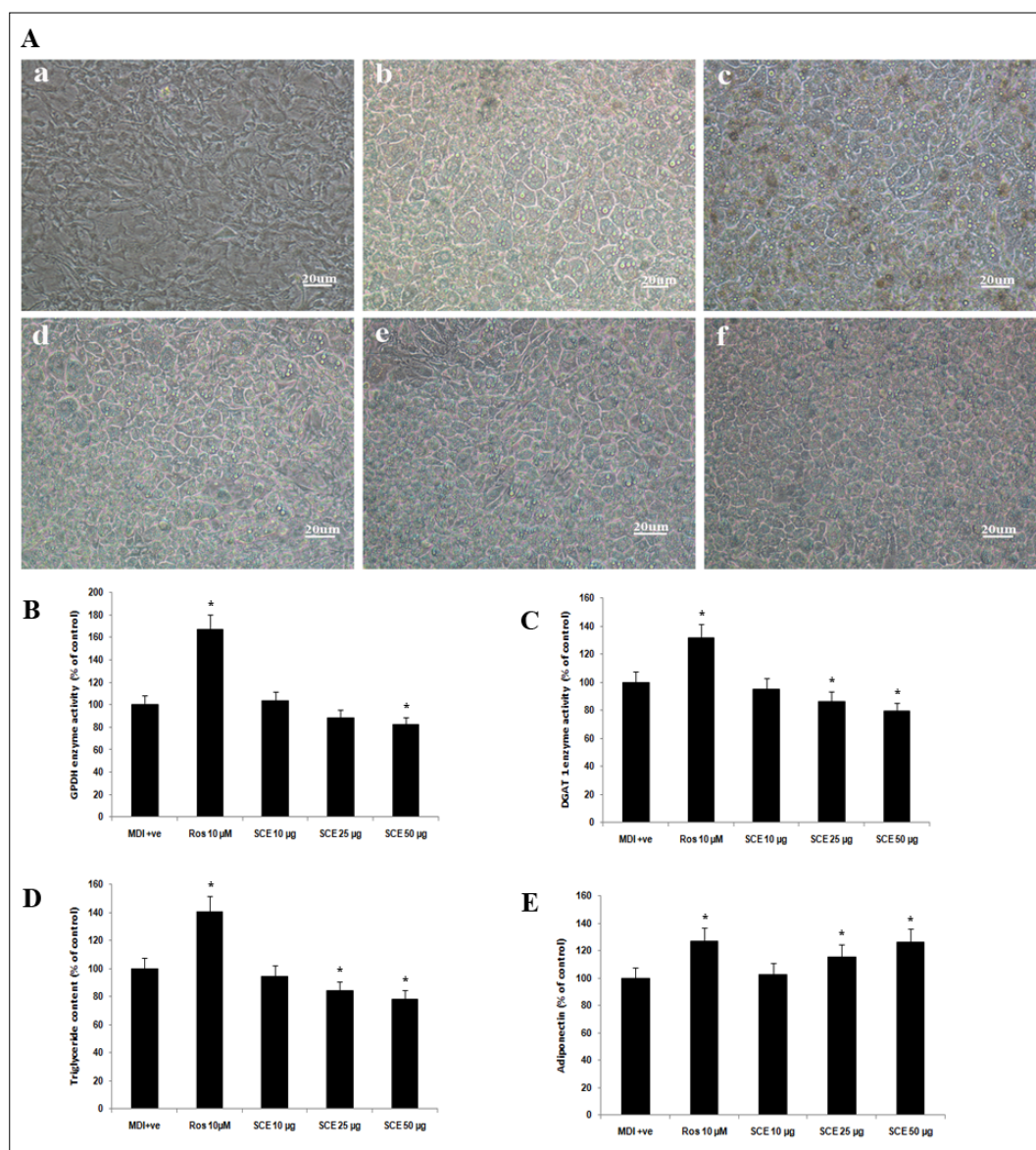
response to glucose and it reduces rate of glucagon secretion and conserves beta-cell mass<sup>46</sup>. SCEC and SCE exhibited moderate DPP-IV inhibitory potential (Fig. 2. 5A).

Significantly enhanced pancreatic beta cell proliferation was noticed in RIN-m5F, MIN-6 and P8 cells by SCE treatment (Fig. 2. 9A-D). This pancreatic beta cell proliferation potential of test material represents very useful criteria to evaluate antidiabetic activity, which could protect the beta cells from degeneration due to gluco-lipotoxicity during type 2 diabetes mellitus or protect from autoimmune mediated destruction as in the case of T1DM<sup>47</sup>. With this result, we strongly believe that this pancreatic beta cell protective property of SCE contribute significantly to its antidiabetic efficiency.





**Figure 2. 10A-C. Glucose uptake and adipocyte differentiation studies in all 5 extracts.** (A) 2-deoxy glucose uptake in L6 myotubes. Cells were incubated for 16 h with different extracts (100 µg/mL) or standards. After incubation myotubes were left untreated (white bars) or stimulated with 100 nM insulin (black bars) for 20 min, followed by the determination of 2-DG uptake. Results are expressed as fold stimulation over control basal. Metformin (10mM) and rosiglitazone (20µM) were the standards. Values are means  $\pm$  SD; n = 6. \* represents groups differ significantly from basal control group ( $p \leq 0.05$ ). # represents groups differ significantly from insulin control group ( $p \leq 0.05$ ). (B) The dose dependent (10, 25, 50 and 100 µg/mL) 2-deoxy glucose uptake of SCE and SCEL in L6 myotubes. (C) Quantification of triglyceride content in differentiating 3T3-L1 adipocytes treated with different extracts (30 µg/mL) or rosiglitazone (10 µM) for 8 days by oil red O staining. Data are expressed as the means  $\pm$  SD; n = 6; \* represents groups differ significantly from MDI positive group ( $p \leq 0.05$ ). # represents groups differ significantly from insulin control group ( $p \leq 0.05$ ). MDI-ve, media without 3-isobutyl-1-methylxanthine (IBMX), dexamethasone and insulin; MDI+ve, media with IBMX, dexamethasone and insulin. Ros 10 µM, rosiglitazone 10 µM.

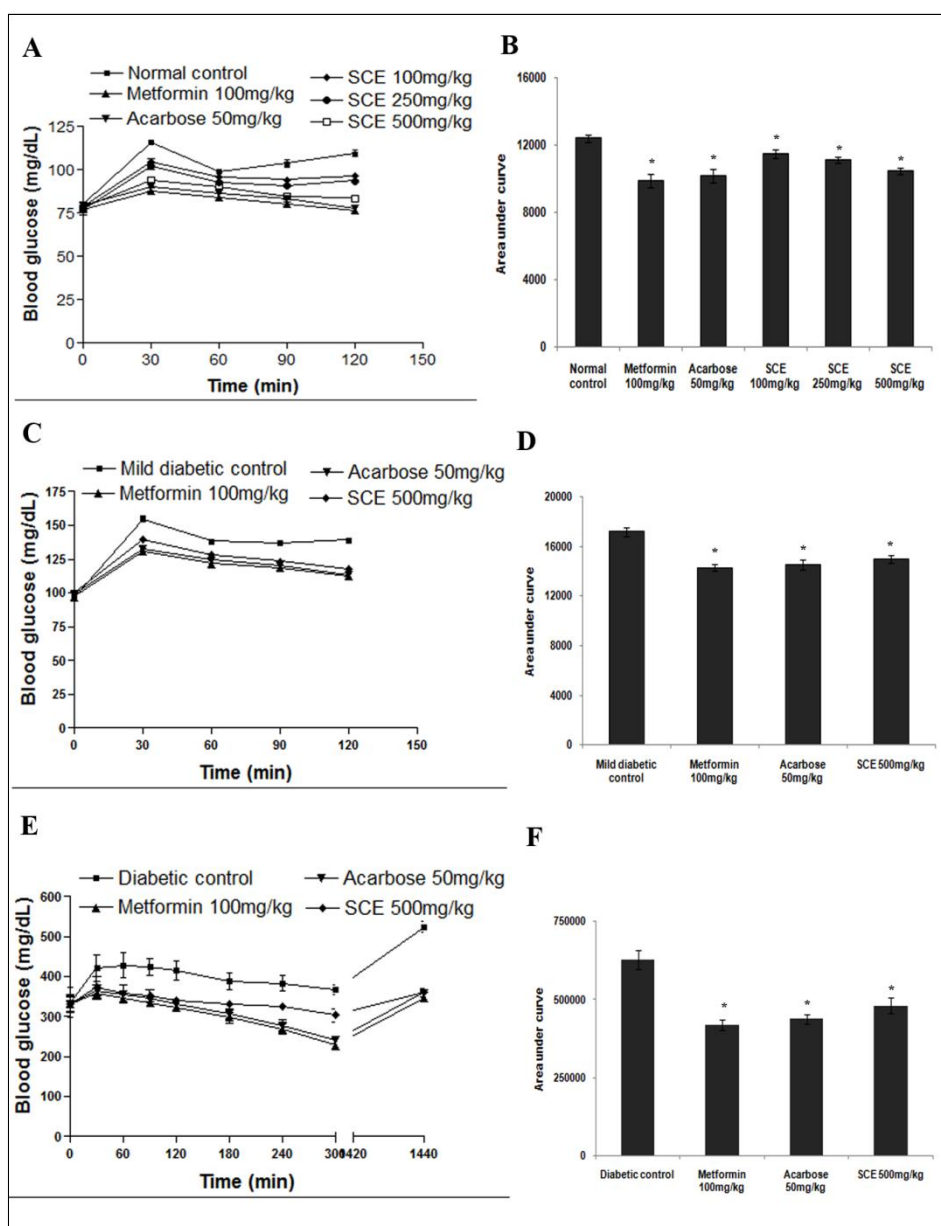


**Figure 2. 11A-E. Estimation of adipogenesis in SCE treatment.** (A) Cellular morphology. (Panels a–f) Micrographs ( $\times 10$ ) showing (a) MDI negative, (b) MDI positive (vehicle control) (c) differentiating 3T3-L1 adipocytes treated for 8 days with rosiglitazone (10  $\mu$ M), and (d–f) various concentrations of SCE (10, 25 and 50  $\mu$ g/mL, respectively). DMSO (0.1%, vehicle) in differentiation media served as the vehicle control group i.e MDI positive. (B) Glycerol-3-phosphate dehydrogenase activity in various groups (MDI positive, rosiglitazone at 10  $\mu$ M) and various concentrations of SCE (10, 25 and 50  $\mu$ g/mL, respectively). (C) Diacyl glycerol -3 phosphate activity in various groups (MDI positive, rosiglitazone at 10  $\mu$ M) and various concentrations of SCE (10, 25 and 50  $\mu$ g/mL, respectively). (D) The triglyceride content in various groups (MDI positive, rosiglitazone at 10  $\mu$ M) and various concentrations of SCE (10, 25 and 50  $\mu$ g/mL, respectively). (E) Adiponectin level in various groups (MDI positive, rosiglitazone at 10  $\mu$ M) and various concentrations of SCE (10, 25 and 50  $\mu$ g/mL, respectively). Results are

normalised to 100 based on control readings. Data are expressed as the means  $\pm$  SD; n = 6. \*Represents groups that differ significantly from the MDI positive (vehicle control) group ( $p \leq 0.05$ ).

Since insulin resistance is a major metabolic abnormality of T2DM, there has been considerable interest in insulin sensitizing agents to counteract insulin resistance for the treatment of this disease<sup>3</sup>. The result of the present study showed significant insulin dependent and independent glucose uptake proving insulin sensitizing property of SCE (Fig. 2. 10A and B,  $p \leq 0.05$ ). Further studies are required to find out the mechanism behind this effect. The peroxisome proliferator activated receptor (PPAR) gamma, the master regulator of adipogenesis is abundantly present in adipocytes which can maintain whole body insulin sensitivity and thiazolidinedione group of drugs (rosiglitazone and pioglitazone) act as PPAR modulators<sup>48, 49</sup>. Analysis of the effect of SCE treatment on various markers of adipogenesis such as diminished activity of GPDH and reduced TG content compared to rosiglitazone, the full PPAR gamma agonist allude partial PPAR gamma agonist property of SCE (Fig. 2. 11A, B and D). Adiponectin, solely secreted from adipocytes acts as a hormone with anti-inflammatory and insulin sensitizing properties<sup>50</sup>. There are reports to suggest the risk of T2DM appeared to decrease monotonically with increasing adiponectin level by several mechanisms<sup>51</sup>. So the potential of SCE to increase adiponectin level in 3T3-L1, suggest a role in its antidiabetic property and this is the first report in this regard (Fig. 2. 11E). But detailed study on transactivation is required to confirm this<sup>52</sup>. Rosiglitazone is effective insulin sensitizer<sup>48</sup>, act through its PPAR agonism. It enhances glucose uptake and adipocyte differentiation in a variety of insulin-resistant states<sup>53</sup>. So rosiglitazone has been taken as positive control for both glucose uptake and adipocyte differentiation studies.

Obesity is characterized by the accumulation of triacylglycerol in adipocytes and is an important risk factor for diabetes. Diacylglycerol acyltransferase (DGAT) catalyzes the final reaction of triacyl glycerol synthesis and has two isoforms- DGAT-1 and DGAT-2. DGAT-1 plays a role in VLDL synthesis; increased plasma VLDL concentrations may promote obesity and thus DGAT-1 is



**Figure 2. 12A-F. The antihyperglycemic effect of SCE in normal rats, mild diabetic rat model (SLM) and streptozotocin-induced diabetic rat model (STZ-S) after sucrose administration.** (A) The glycemic response curve and (B) incremental AUC<sub>0-120</sub> min in normal rats. (C) The glycemic response curve and (D) incremental AUC<sub>0-120</sub> min in SLM model. (E) The glycemic response curve and (F) incremental AUC<sub>0-1440</sub> min in STZ-S model. Data are expressed as the mean  $\pm$  SD, n = 6. \* represents groups differ significantly from control group ( $p \leq 0.05$ ). SCE, *S. cochinchinensis* (SC) ethanol extract.

considered as potential therapeutic target of obesity and associated complications<sup>54</sup>. Here, a decrease in the DGAT-1 activity by the treatment of SCE was observed in the study may attribute to its potential to reduce development of obesity as well hyperglycemia induced dis/hyperlipidemia (Fig. 2. 11C).

The postprandial hyperglycemia (PPH) became a relevant target clinically and scientifically due to the importance in cardiovascular diseases and other complications<sup>55</sup>. The enzyme AG, present in the intestinal brush border cells hydrolyses complex carbohydrates to simple sugars. Inhibition of AG modulate carbohydrate digestion rate and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting PPH and insulin levels<sup>56</sup>. Additional therapeutic properties of AG inhibitors include protection against pancreatic beta cell apoptosis, inhibition of attachment of macrophage to vascular endothelium and amelioration of development of atherosclerosis<sup>57</sup>. Initial *in vitro* screening using yeast AG is required to see whether the study material has some alpha glucosidase inhibitory property<sup>58</sup>. Our *in vitro* studies showed promising AG inhibitory activity against both yeast derived and rat intestinal enzymes by SCE and its fractions SCD and SCEC (Fig. 2. 4A and B). In sucrose loaded normal and SLM models, SCE prevented acute PPH effectively compared to normal control and mild diabetic control (Fig. 2. 12A-D,  $p \leq 0.05$ ). This reveals the efficacy of SCE to control sucrose induced PPH significantly. This antihyperglycemic activity of SCE at 500 mg/kg bw was comparable with the existing drugs like acarbose and metformin. So we selected only 500 mg/kg dose for SLM and STZ-S studies. In streptozotocin models of diabetes, due to the destruction of pancreatic beta-cells, insulin secretion has been impaired and cause blood glucose elevation<sup>55</sup>. SCE treatment in STZ model resulted in attenuation of PPH, whereas diabetic control animals showed elevated blood glucose even after 5 h of sucrose load (Fig. 2. 12E and F,  $p \leq 0.05$ ). From this it is clear that SCE negate PPH by inhibiting AG that modify sucrose breakdown rate in small intestine in normal and diabetic rats.

Deficiency of specific vitamins and minerals play important role in glucose metabolism and insulin signalling contribute to the development of diabetes<sup>59</sup>. In

the present investigation, SCE was found to have high amount of calcium, moderate amount of sodium, potassium and magnesium and traces of manganese and zinc. There are also reports to link the role of these minerals in ameliorating complications arising from diabetes<sup>59</sup>. In addition, our TPC and TFC measurement showed the presence of high content of phenolics and flavonoids (Table 2. 1). Accordingly, HPLC analysis revealed the presence of beta-sitosterol, phloretin 2'glucoside and oleanolic acid. All these compounds are reported to have beneficial role in diabetes as well as to attenuate diabetes induced complications via different ways: beta-sitosterol improves glucose uptake and lipid metabolism<sup>60, 61</sup> and alpha glucosidase inhibition<sup>62</sup>; phloretin 2'glucoside enhances glucose uptake<sup>63, 64</sup> and oleanolic acid improves insulin response<sup>65, 66</sup> and possesses alpha glucosidase inhibitory property<sup>67</sup>. The results exhibited by SC in the present study may be due to the synergistic action of these three compounds in addition to other polyphenolic components.

## **2.5 Conclusion**

Overall results reveal potent antihyperglycemic activity via inhibition of alpha glucosidase and enhanced insulin sensitivity with moderate antiglycation and antioxidant potential of SC which contribute significantly to its antidiabetic property. The presence of known insulin sensitizers and AG inhibitors like phloretin 2'glucoside, oleanolic acid and beta-sitosterol in SC play an important role in these multifaceted activities of SC with respect to diabetes.



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### **Beneficial role of *Symplocos cochinchinensis* against pathophysiological alterations of streptozotocin induced diabetes**

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#### **3.1 Introduction**

Diabetes is the chronic metabolic syndrome characterised by impaired insulin secretion or abnormal insulin utilization by peripheral tissues<sup>1</sup>. This may generate an array of disturbances in glucose and lipid homeostasis resulting in hyperglycemia or dyslipidemia; the key risk factors of diabetes<sup>2</sup>. Persistent hyperglycaemia (glucotoxicity) in diabetes causes increased production of oxygen free radicals from glucose autoxidation<sup>3</sup> and protein glycosylation<sup>4</sup> which leads to oxidative stress. This affects normal functioning of vital organs like pancreas, liver, kidney and eye leading to secondary complications like liver cirrhosis, retinopathy and microvascular complications<sup>5</sup>. So, there is increasing importance for therapeutics which protect these organs from deleterious effect of diabetes associated problems. In view of these, therapeutic agents that can bring about tight glycemic control as well as protection from oxidative stress are very much essential to lessen the diabetes complications<sup>6,7</sup>.

No detailed study on the effect of *Symplocos cochinchinensis* on hyperglycemia associated secondary complications like liver damage, retinopathy, glycation, microvascular complications, muscle damage etc. is available in the literature except the study of Sunil et al.<sup>8,9</sup>. Since diabetes is the multifactorial disease that affects the functioning of various vital organs leading to different dangerous complications mentioned above, it is essential to study the effect of test material on foresaid complications if we are aiming for the development of therapeutics in future. It is known that differential bioactivity of various compounds present in the plant extract may be helpful to protect the organs of different nature and function from the deleterious effects of hyperglycemia and oxidative stress<sup>10</sup>. Similarly bark of SC is known for its

composition of various bioactives<sup>11</sup>. We had seen the effect of SC on various druggable targets like alpha glucosidase inhibition, glucose uptake, adipogenic potential, oxidative stress, pancreatic beta cell proliferation, inhibition of protein glycation, protein tyrosine phosphatase-1B (PTP-1B) and dipeptidyl peptidase-IV (DPP-IV). We found SC is effective in alpha glucosidase inhibition insulin dependent glucose uptake in L6 myotubes, pancreatic beta cell regeneration in RIN-m5F and reduced triglyceride accumulation in 3T3-L1 cells, protection from hyperglycemia induced generation of reactive oxygen species in HepG2 cells. So herein, studies have been conducted to check whether SC is effective against diabetes induced secondary complications. There is high demand for the medication options in the form of drugs/nutraceuticals/phytoceuticals for diabetic retinopathy, neuropathy, nephropathy etc. Hence, hydroethanol (70% ethanol-water) extract of SC was evaluated against streptozotocin induced pathophysiological alterations of liver, kidney, pancreas, muscle and eye lens in Sprague Dawley rats with more emphasis on oxidative stress and glycation related parameters including histopathological changes in pancreas.

### **3. 2 Experimental details**

#### **3. 2. 1 Chemicals and reagents**

All the chemicals and biochemicals were from Sigma (St. Louis, MO, USA).

#### **3. 2. 2 Plant material**

The plant material was processed as described in Chapter 2 section 2. 2. 2. SCE has been selected for *in vivo* study due to its better activity with *in vitro* assays, increased yield of more bioactive molecules and less toxicity of the solvent<sup>12</sup>. Since its selective nature, 70% ethanol is most suitable solvent for *in vivo* pharmacological evaluation compared to other solvents; it will dissolve only the required bioactive constituents with minimum amount of the inert materials<sup>12</sup>.

#### **3. 2. 3 Induction of diabetes in animals and experimental design**

Male albino rats of Sprague Dawley (SD) strain (160±20 g), bred at animal facility of CSIR-CDRI, Lucknow were selected for this study. The maintenance of animals is outlined in Chapter 2 section 2. 2. 15. Animals were made diabetic by single

intraperitoneal injection of streptozotocin (60 mg/kg in 100 mM citrate buffer - pH 4.5) after overnight fasting. Animals showing fasting blood glucose level (BGL) >270 mg/dL after 72 h were selected, termed as diabetic. Normal animals received intraperitoneal injection of 100 mM citrate buffer (pH 4.5). Rats were randomly divided into 6 groups as given below:

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

Group 2: Normal animals treated with SCE 500 mg/kg body weight per day (mg/kg bwd) (N+SCE500 for toxicity evaluation of extract).

Group 3: Diabetic control animals (DC) treated with streptozotocin (60mg/kg).

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

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

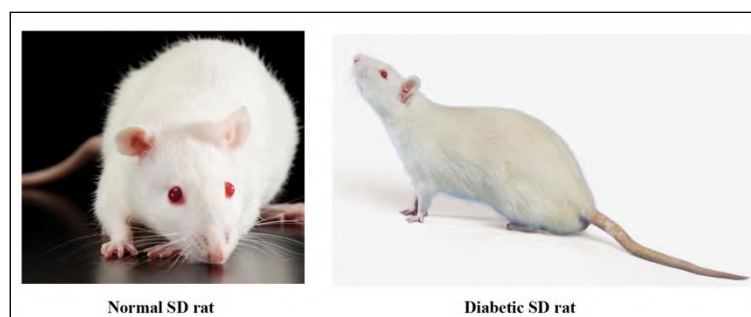
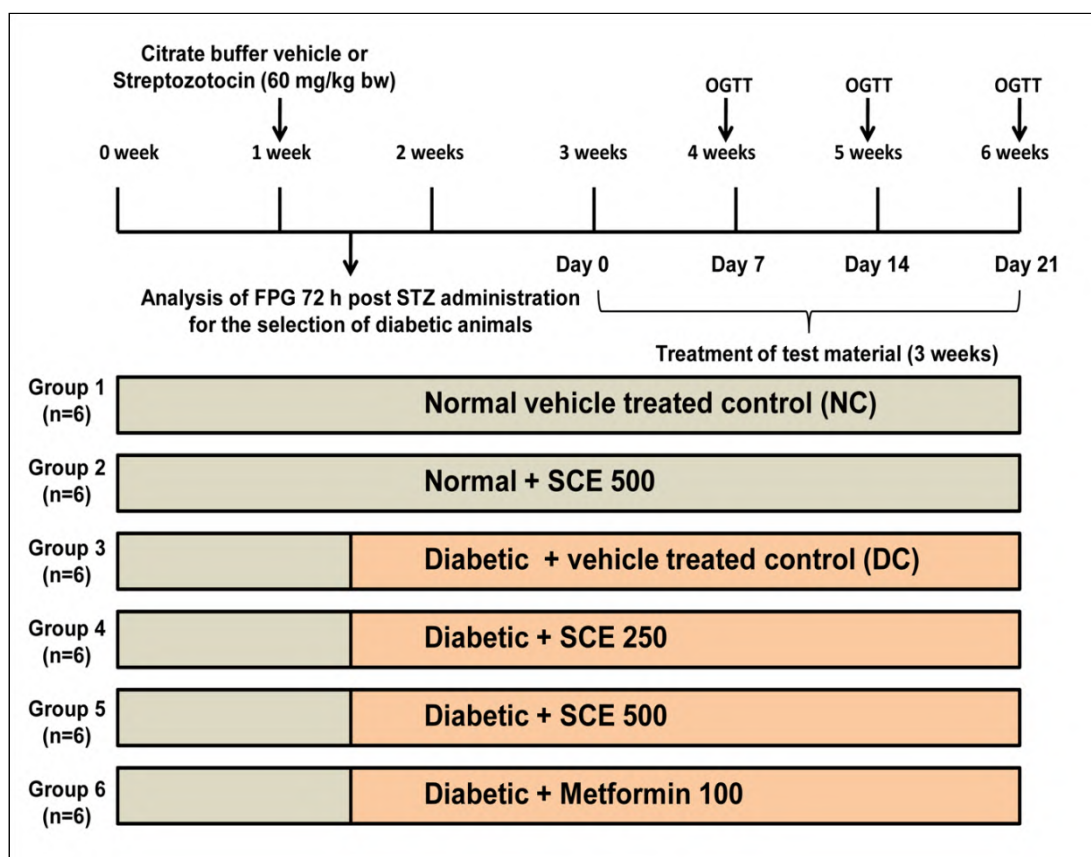
Group 6: Diabetic animals treated with metformin 100 mg/kg bwd (D+Met100).

Animals of experimental groups were orally administered with SCEs (250 or 500 mg/kg bwd) or metformin (100 mg/kg bwd) dissolved in 1.0% gum acacia for 21 days. Metformin is the widely used antidiabetic to treat the cardinal symptoms of diabetes like polyphagia, polydipsia and polyuria due to its pleiotropic effect via various targets and it shows wide tolerance and less toxicity compared to other antidiabetics<sup>13</sup>. Animals of control group were given an equal volume of 1.0% gum acacia. For all animal groups, food and water intake was determined daily; body weight was checked at every week. After the 21 days treatment, rats were sacrificed on day 22 by cervical dislocation under light ether anaesthesia. Schematic representation of experimental design is given as Fig. 3. 1.

### **3. 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 glucose<sup>14</sup>. OGTT was performed on day 7, 14 and 21.





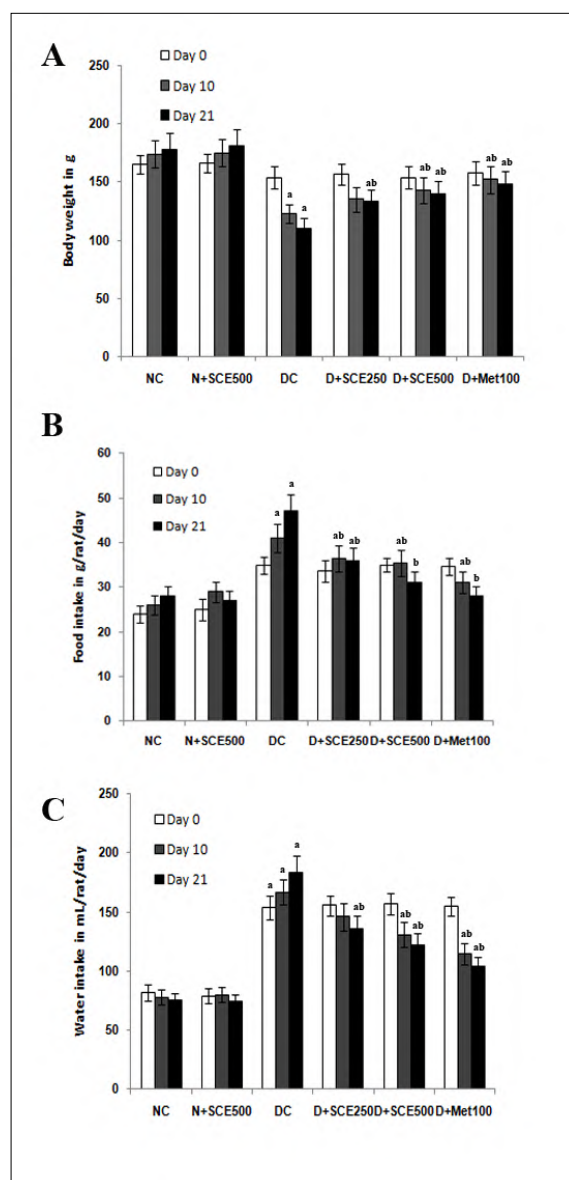
**Figure 3. 1.** Schematic representation of experimental design, Normal and diabetic SD rats. STZ, Streptozotocin; FPG, Fasting plasma glucose, SCE, *S. cochinchinensis* (SC) ethanolic extract.

### 3. 2. 5 Homeostatic model assessment-insulin resistance (HOMA-IR)

HOMA-IR was calculated using the formula;  $HOMA-IR = (\text{glucose} \times \text{insulin})/405$ , where the concentration of glucose expressed in mg/dL and that of insulin in mU/L.

### 3. 2. 6 Biochemical parameters

Blood samples were collected from retro-orbital plexus of each animal in heparin coated



**Figure 3. 2A-C. Effect of SCE on body weight, food and water intake.** (A) Body weight, (B) food intake and (C) water intake. Data are expressed as the mean  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from normal control group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from diabetic control group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both normal control and diabetic control group ( $p \leq 0.05$ ). SCE, *S. cochinchinensis* (SC) ethanolic extract; NC, normal control group; N+SCE 500, normal control group treated with SCE 500 mg/kg bwd; DC, diabetic control group; D+SCE 250, diabetic control group treated with SCE 250 mg/kg bwd; D+SCE 500, diabetic control group treated with SCE 500 mg/kg bwd and D+Met 100, diabetic control group treated with metformin 100mg/kg bwd.

tubes for the separation of plasma and further analytical procedures on day 0 and 21, during the experiment. Plasma was separated by centrifugation at  $3000 \times g$  for 20 min at  $4^{\circ}C$ , aliquots were made, one lot was used for the immediate analysis of triglyceride, total

cholesterol, HDL-C, LDL-C and the remaining portion was immediately frozen and stored at  $-80^{\circ}\text{C}$  for further analysis. Plasma level of insulin, % HbA1c, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, albumin and total protein were measured. Insulin level was assayed using an ELISA kit from Merckodia (Uppsala, Sweden). All other plasma parameters were quantified using kits from Agappe diagnostics (Knonauerstrasse, Switzerland).

### **3. 2. 7 Evaluation of oxidative stress markers and glycogen content**

Immediately after sacrifice of animal, liver, kidney and muscle tissues were excised, frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$  until further analysis. Hepatic and renal oxidative stress markers like reduced glutathione (GSH), total antioxidant activity, malondialdehyde (MDA), protein carbonyl content and antioxidant enzymes like superoxide dismutase (SOD, EC 1.15.1.1 ) and glutathione peroxidase (GPx, EC 1.11.1.9) were measured using kits from Cayman chemicals (Cayman, MI, USA). Liver and muscle glycogen content was measured using glycogen assay kit from Cayman chemicals.

### **3. 2. 8 Measurement of lens aldose reductase activity**

Eye ball was removed to take out lens from animal. The lenses were enucleated by posterior approach. 10% lens homogenate (w/v) was prepared in 0.1 M phosphate buffer saline (pH 7.4). After centrifugation at  $5000\times g$  for 20 min at  $4^{\circ}\text{C}$ , the supernatant was collected and used for the determination of aldose reductase enzyme (AR, EC 1.1.1.21) activity according to the method described by Hayman and Kinoshita<sup>15</sup>. In brief, reaction mixture contains 0.7 mL of sodium phosphate buffer (67 mM, pH 6.2), 0.1 mL of NADP ( $25\times 10^{-5}$  M) and 0.1 mL of lens homogenate, in a final volume of 1 mL. The enzyme reaction was started by the addition of the 0.1 mL substrate (DL±Glyceraldehyde, 1 mM) and absorbance was recorded at 340 nm for 3 min at 30 sec time interval. Enzyme activity was expressed as change in OD/min/mg protein ( $\Delta\text{OD}/\text{min}/\text{mg protein}$ ).

### **3. 2. 9 Histopathology**

Immediately after dissection, pancreas was taken out, cleaned and fixed in 10% neutral buffered formalin solution for the preparation of histopathological slides. After fixation, tissues were dehydrated in graded ethanol series, cleared in xylene and embedded in

paraffin wax. The solid sections were prepared at 5 mm thickness using a microtome, stained with haematoxylin-eosin (H&E). The sections were examined under light microscope and photomicrographs were taken.

### **3. 2. 10 Statistical analysis**

Quantitative glucose tolerance of each group was calculated by the area under the curve (AUC) method using GraphPad Prism software version 3 (Graphpad 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 were presented as mean  $\pm$  SD, n = 6.  $p \leq 0.05$  was considered to be significant.

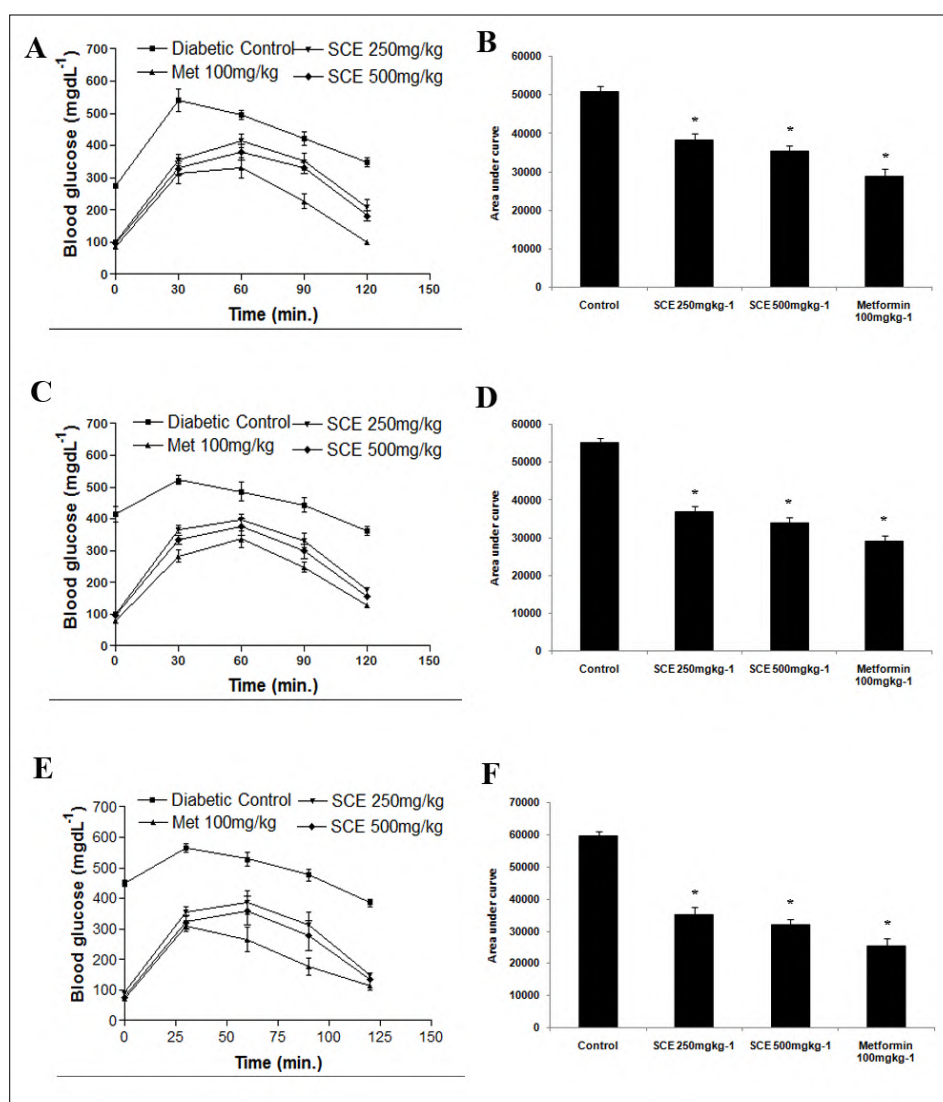
### **3. 3 Results**

#### **3. 3. 1 Fasting plasma glucose, body weight, food and water intake**

More than 90% of STZ injected animals had developed symptoms of diabetes. There was no change in fasting plasma glucose of normal control animals (NC) during the experiment period but the diabetic control animals (DC) showed a progressive and significant increase in fasting plasma glucose (from 274 to 449 mg/dL) during 21 days period of the experiment. The summary of body weight, food and water intake are given in Fig.3. 2.

#### **3. 3. 2 Amelioration of glucose intolerance by SCE**

The diabetic control animals showed severe glucose intolerance throughout the experiment period. Treatment with SCE 250 or 500 mg/kg bwd (SCE 250/500) and metformin 100 mg/kg bwd (Met 100) significantly ( $p \leq 0.05$ ) reduced blood glucose profile on day 7, 14 and 21. Day 7 OGTT exhibited 24.64%, 30.23% and 42.96% reduction in glycemic response in SCE 250/500 and Met 100 treated groups respectively (Fig. 3. 3A and B). Day 14 OGTT showed 32.93%, 38.41% and 47.30% reduction in glycemic response in SCE 250/500 and Met 100 groups respectively (Fig. 3. 3C and D), while day 21 OGTT exhibited further improvement of 40.76%, 46.28% and 57.53% reduction in glycemic response in SCE 250/500 and Met 100 groups respectively (Fig. 3. 3E and F). Both extracts and metformin showed dose and duration dependent reduction in glycemic responses.

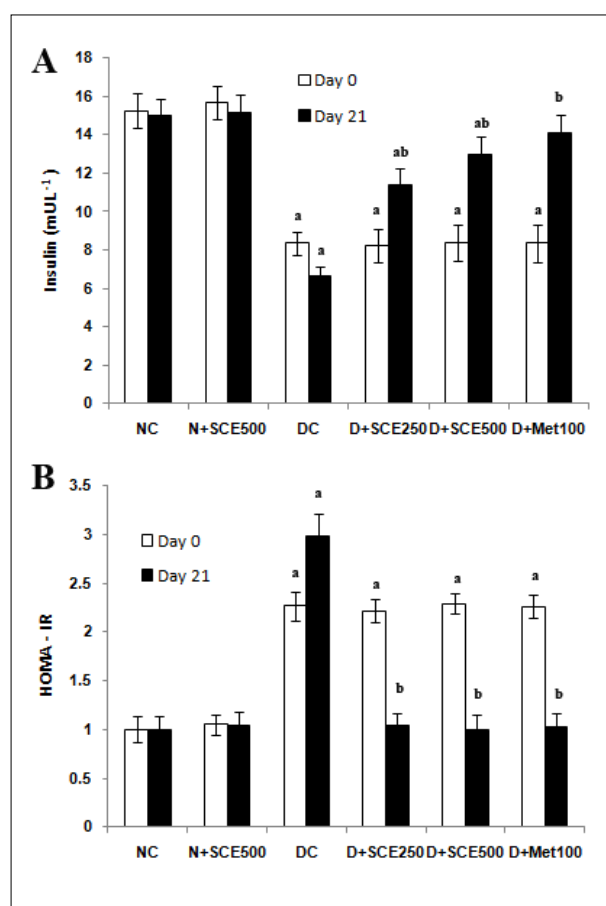


**Figure 3. 3A-F. The improved glucose tolerance exhibited by streptozotocin-induced diabetic rats by SCE treatment on day 7, 14 and 21.** (A) The glycemic response curve and (B) incremental AUC<sub>0-120</sub> min in normal rats, (C) The glycemic response curve and (D) incremental AUC<sub>0-120</sub> min in SLM model, (E) The glycemic response curve and (F) incremental AUC<sub>0-120</sub> min in STZ-S model. Data are expressed as the mean  $\pm$  SD, n = 6. \* represents groups differ significantly from control group (p $\leq$ 0.05). SCE, *S. cochinchinensis* (SC) ethanolic extract; DC, diabetic control group; D+SCE 250, diabetic control group treated with SCE 250 mg/kg bwd; D+SCE 500, diabetic control group treated with SCE 500 mg/kg bwd and D+Met 100, diabetic control group treated with metformin 100 mg/kg bwd .

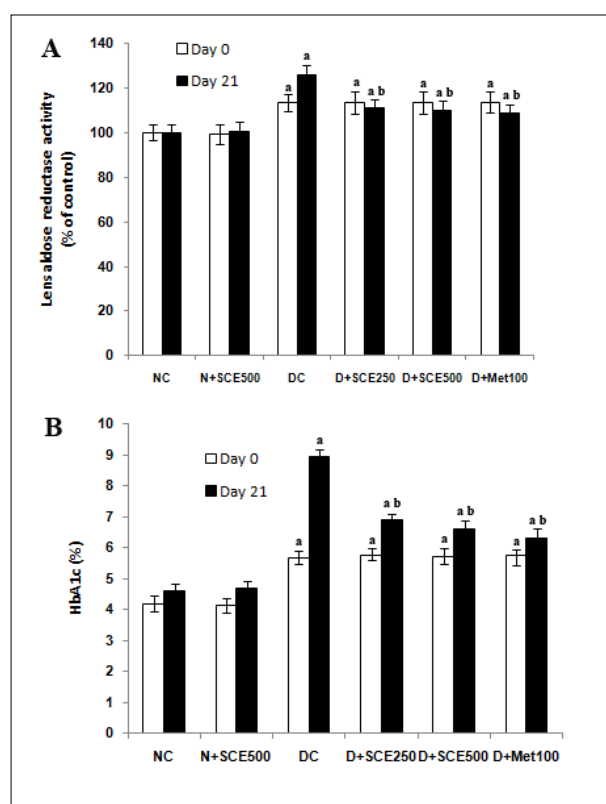
### 3. 3. 3 SCE improved plasma insulin level and insulin sensitivity

Plasma insulin level was depleted significantly (p $\leq$ 0.05) in STZ rats, while SCE and metformin treatment improved the insulin levels (Fig. 3. 4A). Insulin sensitivity was

determined using HOMA-IR based on fasting plasma glucose and insulin levels. The insulin resistance of the DC animals was found to be increased 2 fold when compared to normal group on day 0 (Fig. 3. 4B). The diabetic control group showed around 3 fold increase in the insulin resistance on day 21 while the SCEs and metformin treatment in diabetic groups improved the insulin sensitivity comparable to that of normal control animals (Fig. 3. 4B).



**Figure 3. 4A and B. SCE enhanced plasma insulin level and insulin sensitivity.** (A) Plasma insulin level and (B) Homeostatic model assessment-insulin resistance (HOMA-IR). Data are expressed as the mean  $\pm$  SD, n = 6. 'a' represents groups differ significantly from normal control group ( $p \leq 0.05$ ). 'b' represents groups differ significantly from diabetic control group ( $p \leq 0.05$ ). 'ab' represents groups differ significantly from both normal control and diabetic control group ( $p \leq 0.05$ ). SCE, *S. cochinchinensis* (SC) ethanolic extract; NC, normal control group; N+SCE 500, normal control group treated with SCE 500 mg/kg bwd; DC, diabetic control group; D+SCE 250, diabetic control group treated with SCE 250 mg/kg bwd; D+SCE 500, diabetic control group treated with SCE 500 mg/kg bwd and D+Met 100, diabetic control group treated with metformin 100mg/kg bwd .



**Figure 3. 5A and B. Effect of SCE in lens aldose reductase activity and % HbA1c level.** (A) Aldose reductase activity in the eye lens and (B) % HbA1c level of various groups. Data are expressed as the mean  $\pm$  SD, n = 6. 'a' represents groups differ significantly from normal control group ( $p \leq 0.05$ ). 'b' represents groups differ significantly from diabetic control group ( $p \leq 0.05$ ). 'ab' represents groups differ significantly from both normal control and diabetic control group ( $p \leq 0.05$ ). SCE, *S. cochinchinensis* (SC) ethanolic extract; NC, normal control group; N+SCE 500, normal control group treated with SCE 500 mg/kg bwd; DC, diabetic control group; D+SCE 250, diabetic control group treated with SCE 250 mg/kg bwd; D+SCE 500, diabetic control group treated with SCE 500 mg/kg bwd and D+Met 100, diabetic control group treated with metformin 100mg/kg bwd.

### 3. 3. 4 Effect of SCE in lens aldose reductase activity and % HbA1c level

The aldose reductase activity was elevated significantly ( $p \leq 0.05$ ) by 25.92% in the lens of diabetic animals after 21 days compared to the normal animals (Fig. 3. 5A). The lens aldose reductase activity was reduced by 14.66%, 15.99% and 17.32% respectively with SCE 250/500 and Met 100 for 21 days (Fig. 3. 5A). A significant elevation of % HbA1c was observed in diabetic control animals compared to the normal ones ( $p \leq 0.05$ ). %



HbA1c level of diabetic control animals was found to be 8.95 and that of normal control rats was 4.60 on day 21 (Fig. 3. 5B). SCE 250/500 or Met 100 treatment resulted in significant ( $p \leq 0.05$ ) improvement in % HbA1c; 6.92, 6.61 and 6.32 respectively after 21 days (Fig. 3. 5B).

### **3. 3. 5 SCE enhanced hepatic and renal function**

ALT, the specific biomarker for hepatic damage was significantly ( $p \leq 0.05$ ) enhanced (1.58 fold) in STZ diabetic rats compared to normal control (Fig. 3. 6A). The second important indicator of hepatic injury, AST was also significantly ( $p \leq 0.05$ ) elevated (1.59 fold) in diabetic rats compared to normal control (Fig. 3. 6B). The decreased plasma albumin level of diabetic animals (0.44 fold) indicated the diminished liver and kidney function (Fig. 3. 6C). The abnormal plasma concentration of total protein (0.40 fold decrease), urea (3.36 fold increase) and creatinine (2.46 fold increase) revealed the renal dysfunction in diabetic rats (Fig. 3. 6D-F). The diabetic animals treated with SCE 250/500 exhibited significant ( $p \leq 0.05$ ) improvement after 21 days in ALT, AST, albumin, urea, creatinine and total protein levels (Fig. 3. 6A-F).

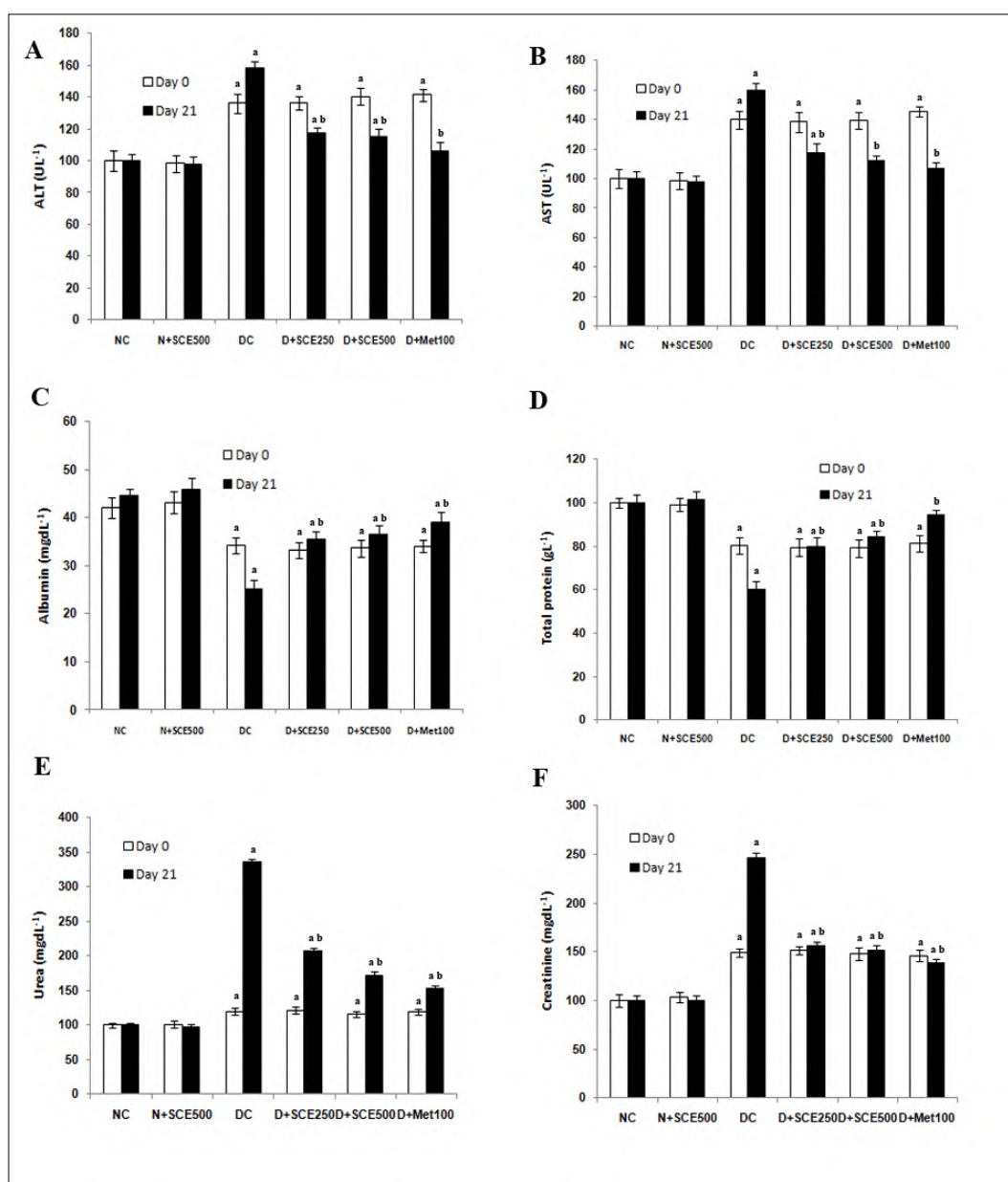
### **3. 3. 6 Beneficial effect of SCE in plasma lipid profile**

DC animals showed significantly ( $p \leq 0.05$ ) elevated plasma triglyceride (2.14 fold), total cholesterol (3.37 fold) and LDL-C (8.9 fold) concentration compared to normal control animals; 0.35 fold decrease in HDL-C level was also noticed in DC animals (Fig. 3. 7A-D). Administration of SCE 250/500 for 21 days resulted in a significant ( $p \leq 0.05$ ) decrease in the level of TG, TC and LDL (Fig. 3. 7A-D). Effect of SCE 500 on lipid profile was comparable to that of metformin. Plasma HDL concentration was elevated significantly ( $p \leq 0.05$ ) in both SCEs and metformin treated animals.

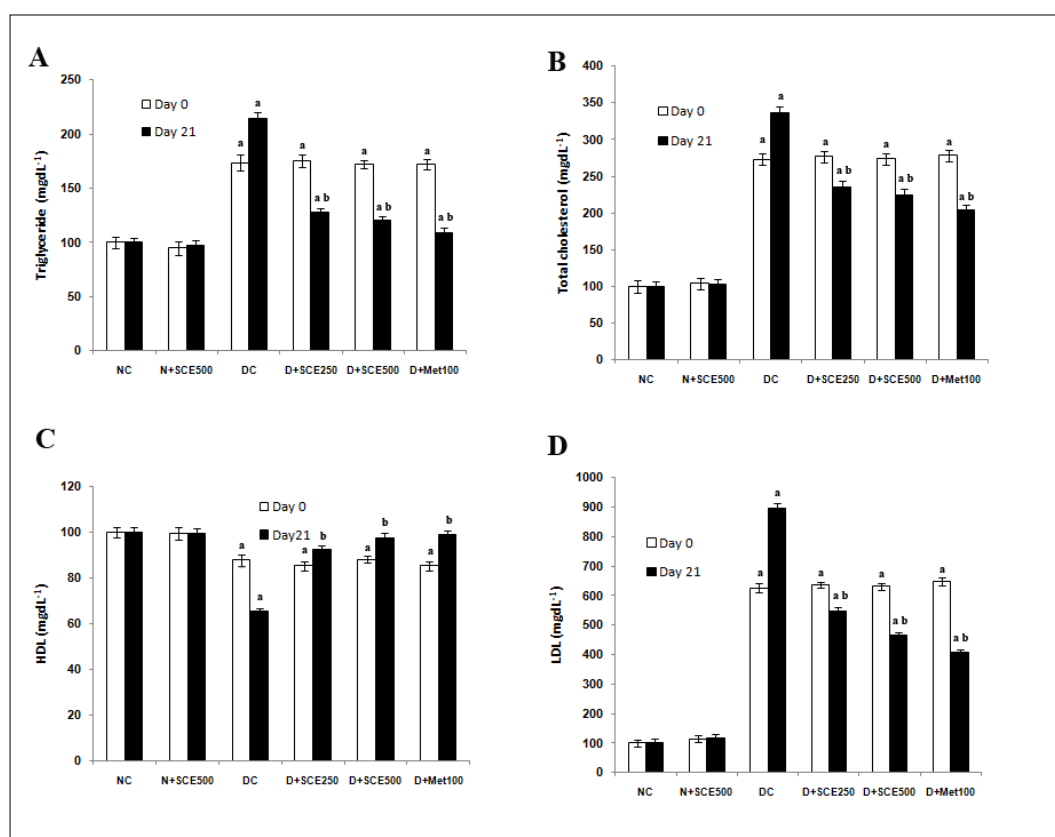
### **3. 3. 7 SCE showed protection against hepatic and renal oxidative stress**

A marked increase in the carbonyl content (2.64 fold), MDA level (1.65 fold), and antioxidant enzyme activities; SOD (1.46 fold) and GPx (1.72 fold) were detected in hepatic tissue of diabetic animals compared to normal controls (Fig. 3. 8C-F). Similarly, in renal tissue also there was a significant ( $p \leq 0.05$ ) increase in the carbonyl content (1.57 fold), MDA level (2 fold), and antioxidant enzyme activities; SOD (1.37 fold) and GPx

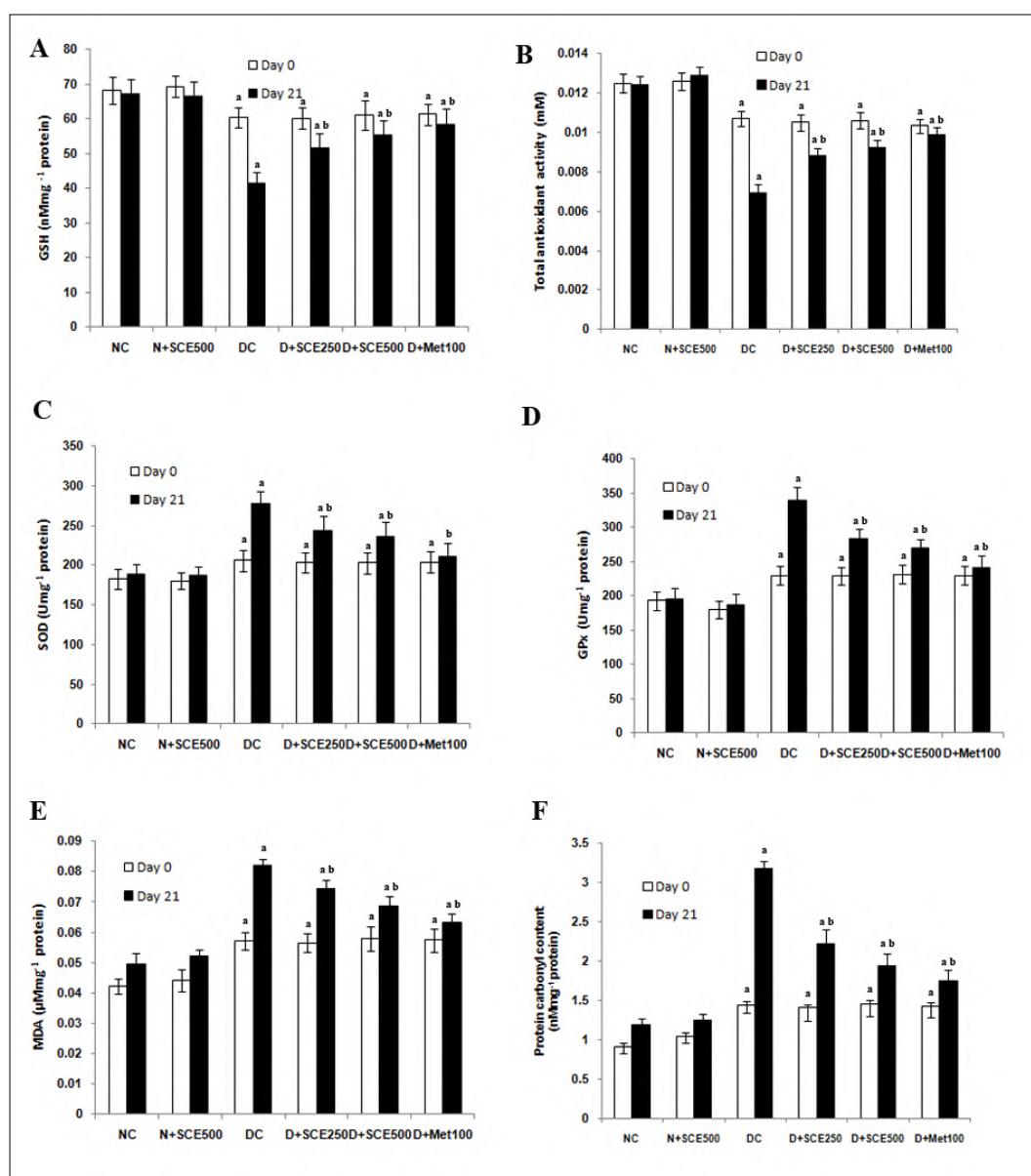




**Figure 3. 6A-F. SCE enhanced hepatic and kidney function.** Plasma concentration of (A) alanine aminotransferase (ALT), (B) aspartate aminotransferase (AST), (C) albumin, (D) total protein, (E) urea and (F) creatinine in various groups. Data are expressed as the mean  $\pm$  SD, n = 6. 'a' represents groups differ significantly from normal control group ( $p \leq 0.05$ ). 'b' represents groups differ significantly from diabetic control group ( $p \leq 0.05$ ). 'ab' represents groups differ significantly from both normal control and diabetic control group ( $p \leq 0.05$ ). SCE, *S. cochinchinensis* (SC) ethanolic extract; NC, normal control group; N+SCE 500, normal control group treated with SCE 500 mg/kg bwd; DC, diabetic control group; D+SCE 250, diabetic control group treated with SCE 250 mg/kg bwd; D+SCE 500, diabetic control group treated with SCE 500 mg/kg bwd and D+Met 100, diabetic control group treated with metformin 100mg/kg bwd .



**Figure 3. 7A-D. Beneficial effect of SCE in plasma lipid profile.** Plasma concentration of (A) Triglyceride (TG), (B) Total Cholesterol (TC), (C) High density lipoprotein cholesterol (HDL-C), and (D) Low density lipoprotein cholesterol (LDL-C) in various groups. Data are expressed as the mean  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from normal control group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from diabetic control group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both normal control and diabetic control group ( $p \leq 0.05$ ). SCE, *S. cochinchinensis* (SC) ethanolic extract; NC, normal control group; N+SCE 500, normal control group treated with SCE 500 mg/kg bwd; DC, diabetic control group; D+SCE 250, diabetic control group treated with SCE 250 mg/kg bwd; D+SCE 500, diabetic control group treated with SCE 500 mg/kg bwd and D+Met 100, diabetic control group treated with metformin 100mg/kg bwd .



**Figure 3. 8A-F. SCE showed protection against hepatic oxidative stress.** Hepatic (A) reduced glutathione (GSH) level, (B) total antioxidant activity, (C) super oxide dismutase (SOD) activity, (D) glutathione peroxidase (GPx) activity, (E) malondialdehyde (MDA) level and (F) protein carbonyl content. Data are expressed as the mean  $\pm$  SD, n = 6. 'a' represents groups differ significantly from normal control group ( $p \leq 0.05$ ). 'b' represents groups differ significantly from diabetic control group ( $p \leq 0.05$ ). 'ab' represents groups differ significantly from both normal control and diabetic control group ( $p \leq 0.05$ ). SCE, *S. cochinchinensis* (SC) ethanolic extract; NC, normal control group; N+SCE 500, normal control group treated with SCE 500 mg/kg bwd; DC, diabetic control group; D+SCE 250, diabetic control group treated with SCE 250 mg/kg bwd; D+SCE 500, diabetic control group treated with SCE 500 mg/kg bwd and D+Met 100, diabetic control group treated with metformin 100mg/kg bwd.

(1.63 fold) (Fig. 3. 9C-F) and carbonyl content (1.58 fold) in muscle (Fig. 3. 10C) in diabetic animals compared to normal control animals. But, GSH and total antioxidant levels in hepatic and renal tissues were found to be decreased (Fig. 3. 8A-B and 3. 9A-B). Both the doses of SCE and metformin treatment reduced renal and hepatic carbonyl content, MDA level, SOD, GPx enzyme activities (Fig. 3. 8C-F and 3. 9C-F) and muscle carbonyl content (Fig. 3. 10C) which is comparable to normal range. SCE and metformin treatment also improved the antioxidant status of hepatic and renal tissues by protecting GSH and total antioxidant levels (Fig. 3. 8A-B and 3. 9A-B).

### **3. 3. 8 SCE promoted elevation in glycogen content**

There was a significant depletion in glycogen content of liver (71%) and muscle (59%) of DC animals (Fig. 3. 10A and B) compared to normal control group ( $p \leq 0.05$ ). Administration of SCE 250/500 or Met 100 for 21 days improved significantly the glycogen content in liver by 27.30, 36.58 and 43.83% respectively compared to diabetic animals (Fig. 3. 10A) and in muscle by 19.90%, 31.11% and 42.45% respectively (Fig. 3. 10B).

### **3. 3. 9 Protection of pancreatic islets integrity by SCE**

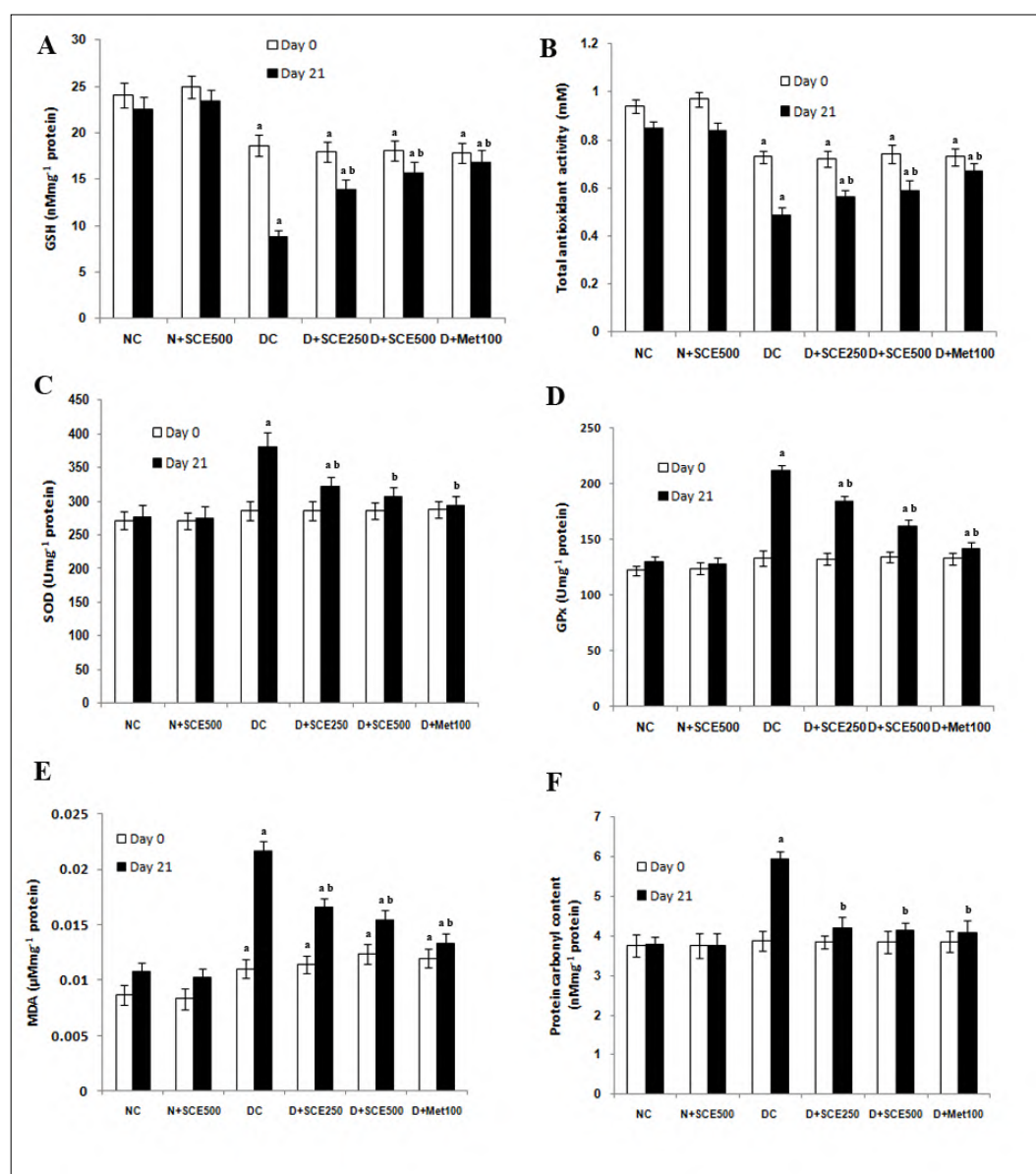
Histological analysis of pancreas from normal control rat revealed the presence of well defined islets of Langerhans surrounded by acinar cells of exocrine pancreas (Fig. 3. 11a). The pancreas from normal control rat treated with SCE 500 exhibited more or less similar morphology to that of normal control animal, confirming the safety of SCE at higher dose (Fig. 3. 11b). But histopathology of pancreas of DC displayed pancreatic islets damage and presence of inflammatory and necrotic cells (Fig. 3. 11c). However, administration of SCE 250/500 for 21 days prevented the histopathological alterations in DC animals; endocrine cells were found to be in normal morphology (Fig. 3. 11e 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. 3. 11d).

## **3. 4 Discussion**

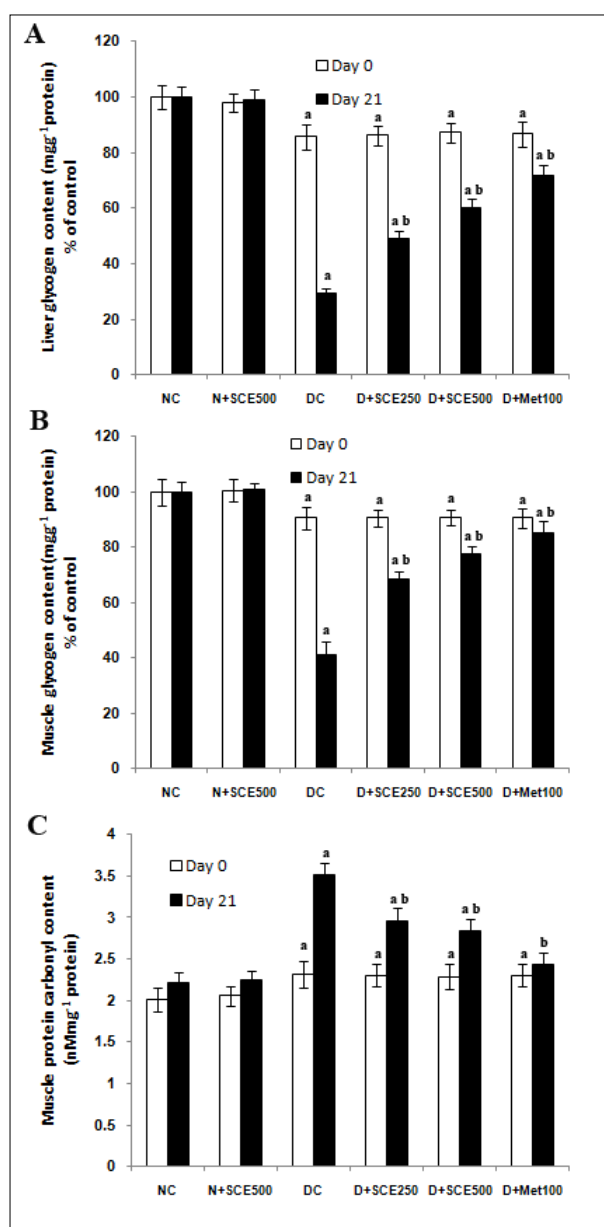
Diabetes is a syndrome of metabolic derangement with diminished production of insulin (type 1) or impaired response to insulin and pancreatic beta-cell dysfunction (type 2) <sup>1</sup>.

Both are characterised by hyperglycemia which leads to excessive urine production, compensatory thirst, increased fluid intake, unexplained weight loss, changes in energy metabolism, retinopathy, neuropathy, nephropathy and vascular complications<sup>5</sup>. The incidence of free radical-mediated toxicity is well documented in clinical diabetes<sup>16</sup> and STZ-diabetic rats<sup>17</sup>. The elevated levels of toxic oxidants in diabetic animals are due to processes such as glucose autoxidation and lipid peroxidation<sup>18</sup>. In order to minimise the oxidative stress derived diabetic complications, a strict and careful glycemic control by the use of safe and efficacious therapeutic agents with antihyperglycemic and antioxidant potential is strongly recommended. Since hyperglycemia alter the functions of various vital organs via. depletion or imbalance of redox status, detailed study on various organs with respect to innate antioxidant and associated activity is very much essential for the evaluation of efficacy of test material.

From ancient times, diabetes has been treated with herbal medicines with the emphasis on maintaining normoglycemia and protection from damaging effects of this disease. In this regard, we studied the effect of SCE in the level of antioxidant enzymes (SOD and Gpx), oxidative stress markers like GSH, MDA, protein carbonyls etc. in the liver and kidney tissues of STZ diabetic rat model. Further we checked the effect of SCE in the serum % HbA1c level, lens aldose reductase activity and glycogen and protein carbonyl content in muscle. The present study also evaluated the extent of pancreatic islets damage and functional alteration and the protective property of SCE by histopathology and HOMA-IR analysis. This is the first detailed report on effect of SCE on hyperglycemia induced secondary complications of liver, kidney, eye and muscle. In this study, we selected STZ as the diabetogenic agent, as it specifically targets pancreatic beta cells by its alkylating property without direct damage to other tissues<sup>19</sup>. The selectivity for beta cells is associated with preferential accumulation of streptozotocin in beta cells after entry through the GLUT-2 glucose transporter receptor due to the chemical structural similarity with glucose<sup>20</sup>. Animals exhibited fasting hyperglycemia (>270 mg/dL) by a single intraperitoneal injection of streptozotocin after 72 h. Animals also displayed diabetes symptoms like increased food and water intake and loss of body weight within the first week itself. SCE administration for 21 days modulated the feeding and water intake pattern of diabetic animals in a similar way as noticed in metformin treated group (Fig. 3. 2B and C). The weight loss of diabetic animals was also limited to an extent by SCE and



**Figure 3. 9A-F.** SCE showed protection against renal oxidative stress. Renal (A) reduced glutathione (GSH) level, (B) total antioxidant activity, (C) super oxide dismutase (SOD) activity, (D) glutathione peroxidase (GPx) activity, (E) malondialdehyde (MDA) level and (F) protein carbonyl content. Data are expressed as the mean  $\pm$  SD, n = 6. 'a' represents groups differ significantly from normal control group ( $p \leq 0.05$ ). 'b' represents groups differ significantly from diabetic control group ( $p \leq 0.05$ ). 'ab' represents groups differ significantly from both normal control and diabetic control group ( $p \leq 0.05$ ). SCE, *S. cochinchinensis* (SC) ethanolic extract; NC, normal control group; N+SCE 500, normal control group treated with SCE 500 mg/kg bwd; DC, diabetic control group; D+SCE 250, diabetic control group treated with SCE 250 mg/kg bwd; D+SCE 500, diabetic control group treated with SCE 500 mg/kg bwd and D+Met 100, diabetic control group treated with metformin 100mg/kg bwd.



**Figure 3. 10A-C. SCE promoted improvement in glycogen content.** (A) liver glycogen content, (B) muscle glycogen content (C) muscle protein carbonyl content. Data are expressed as the mean  $\pm$  SD,  $n = 6$ . 'a' represents groups differ significantly from normal control group ( $p \leq 0.05$ ). 'b' represents groups differ significantly from diabetic control group ( $p \leq 0.05$ ). 'ab' represents groups differ significantly from both normal control and diabetic control group ( $p \leq 0.05$ ). SCE, *S. cochinchinensis* (SC) ethanolic extract; NC, normal control group; N+SCE 500, normal control group treated with SCE 500 mg/kg bwd; DC, diabetic control group; D+SCE 250, diabetic control group treated with SCE 250 mg/kg bwd; D+SCE 500, diabetic control group treated with SCE 500 mg/kg bwd and D+Met 100, diabetic control group treated with metformin 100mg/kg bwd.



metformin treatment (Fig. 3. 2A). 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. 3. 3A-F).

Insulin production becomes impaired in STZ-induced diabetic rats<sup>21</sup>. Our animals in the diabetic group showed impaired plasma insulin level (Fig. 3. 4A). But with SCE treatment plasma insulin level was elevated significantly. HOMA-IR analysis based on fasting measurements of glucose and insulin serves as reasonable and reliable method to measure the insulin sensitivity and beta cell function of rodent models as well as human subjects<sup>22</sup>. From the HOMA-IR results it is clear that the degree of insulin resistance is higher in diabetic group during the experiment period from day 0 to day 21. Both doses of SCE and metformin assisted in protecting the pancreas from its structural as well as functional alterations (Fig. 3. 4 and 3. 11a-f). This may be due to the insulinotropic effect of this extract. This improved pancreatic function in SCE treated STZ diabetic animals is in line with the beneficial effect of SCE in the pancreatic beta cell proliferation in cell line models that we have noticed in Chapter 2.

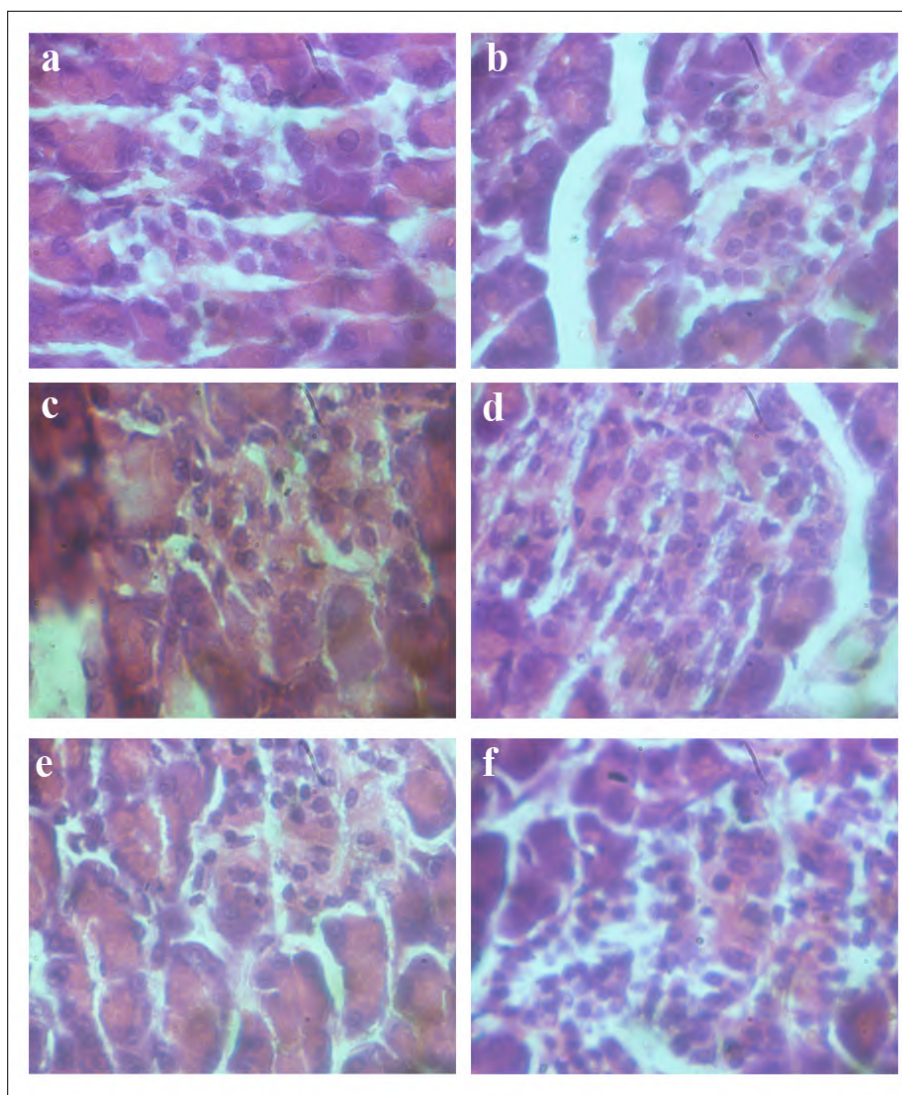
The excess prevalence of liver disease in diabetic population has stimulated interest in studying pathological changes in liver during hyperglycemia for control and management of diabetic complications<sup>23</sup>. AST and ALT levels were increased in diabetic control animals while treatment with SCE or metformin reduced plasma AST and ALT levels (Fig. 3. 6A and B). This indicates the hepatoprotective property of SCE in STZ diabetes. The enzymes activities of AST and ALT provide the link between carbohydrate and protein metabolism by the inter-conversion of the intermediates of metabolic pathways<sup>24</sup>. AST catalyzes the interconversion of aspartic and alpha-ketoglutaric acids to oxaloacetic and glutamic acids, while ALT catalyzes the interconversion of alanine and alpha-ketoglutaric acid to pyruvic and glutamic acids<sup>25</sup>. The ultimate result is the addition of keto acids into the Krebs cycle. The increased levels of AST and ALT activity can be regarded as a signal for enhanced gluconeogenesis<sup>26, 27</sup>. Because SCE decreases AST and ALT levels, it is acceptable that SCE reduces gluconeogenesis and can be further investigated for the treatment of diabetes mellitus. Negative nitrogen balance with elevated tissue proteolysis and declined protein synthesis can contribute to increased serum urea and creatinine levels, indicating impaired renal functions in diabetic animals<sup>28</sup>.



In the present study the diabetic control rats exhibited significantly higher plasma urea and creatinine levels compared to the normal control group. But SCE administration resulted in a lower plasma level of urea and creatinine in treated groups compared to the diabetic control group (Fig. 3. 6E and F). This shows protective property of SCE against renal damage.

In the pathogenesis of diabetic complications important risk factors include not only duration of hyperglycemia, but also dyslipidemia<sup>29</sup>. Hyper-triglyceridemia and low level of HDL are the most common lipid abnormalities related to diabetes mellitus<sup>30</sup>. Here also, the diabetic animals exhibited hyper-triglyceridemia, hypercholesterolemia, elevated LDL and low HDL level compared to the normal control group (Fig. 3. 7A-D). But in comparison with the diabetic control group, SCE treated groups showed a good TG-HDL ratio and lower TC and LDL levels indicating therapeutic potential of SC against lipid abnormalities associated with hyperglycemia.

The studies on oxidative stress and diabetic complications have shown that oxidative stress accelerates the development of pathological lesions linked to the state of hyperglycemia<sup>31</sup>. GSH and antioxidant enzymes are the key agents which protect tissues from oxidative damage. GSH can participate in the elimination of reactive intermediates by reducing hydroperoxides in the presence of glutathione peroxidase<sup>32</sup>. GSH is essential for the GSH redox cycle, which maintains adequate levels of reduced cellular GSH and a high GSH/GSSG ratio is essential for protection against oxidative stress<sup>33</sup>. There was significant depletion in GSH and total antioxidant level in the hepatic and renal tissues of diabetic rats compared to the normal control group. The decrease in GSH level also represents its increased utilization due to oxidative stress<sup>34</sup>. However, SCE treatment elevated the cellular antioxidant status as well as the GSH levels. ROS scavenging enzymes can respond to conditions of increased oxidative stress with a compensatory mechanism that increases the enzyme activity in diabetic rats<sup>35</sup>. SOD, a prominent member of innate antioxidant system catalyses dismutation of superoxide ( $O_2^-$ ) to oxygen and  $H_2O_2$ . Likewise glutathione peroxidase is involved in the reduction of deleterious lipid hydroperoxides to their corresponding alcohols and  $H_2O_2$  then to water. Both SOD and GPx activities is found to be increased in diabetic conditions<sup>35</sup>. In our study, also diabetic control showed elevated level of both hepatic and renal SOD and GPx activities.



**Figure 3. 11a-f. SCE showed protection of pancreas islets integrity.** Representative microscopic scans of rat pancreas stained by Hematoxylin and Eosin (H and E 400×), (a) normal control group, (b) normal control group treated with SCE 500 mg/kg bwd, (c) diabetic control group, (d) diabetic control group treated with SCE 250 mg/kg bwd, (e) diabetic control group treated with SCE 500 mg/kg bwd and (f) diabetic control group treated with metformin 100mg/kg bwd. SCE, *S. cochinchinensis* (SC) ethanolic extract.

But administration of SCE was able to bring down these enzyme activities and was comparable with metformin in hepatic (Fig. 3. 8C and D) and renal tissues (Fig. 3. 9C and D). This result along with other parameters related to oxidative stress like % HbA1c, carbonyl content, pancreas damage etc. reveal the significant potential of SC to protect

the innate antioxidant system of body. Moreover, SCE exhibited significant antioxidant potential in *in vitro* cell free assays as well as it could protect cells from hyperglycemia induced oxidative stress in HepG2 high glucose model. Thus, the *in vitro* radical scavenging property of SCE was found to be effectively translated to these *in vivo* results also.

The protein carbonyls like advanced oxidation protein products, AGEs, % HbA1c etc act as "metabolic memory" markers resulting from the hyperglycemia and hyperlipidemia-induced oxidative stress<sup>36</sup>. These increased chemical modification of proteins by carbohydrates and lipids in diabetes may bring about overload on metabolic pathways involved in detoxification of reactive carbonyl species. This increases the steady-state levels of reactive carbonyl compounds formed by both oxidative and non-oxidative reactions<sup>37</sup>. The protein carbonyl content of diabetic hepatic (Fig. 3. 8F), renal (Fig. 3. 9F) and muscle tissue (Fig. 3. 10C) were found to be significantly higher compared to the normal animal. In addition an increase in lipid peroxide concentration in the tissues of diabetic animals has been observed<sup>38</sup>. In line with this, the content of MDA in the diabetic liver (Fig. 3. 8E) and kidney (Fig. 3. 9E) was elevated than the normal control group in this study. Both protein carbonyl accumulation and lipid peroxidation in renal and hepatic tissues of diabetic rats was ameliorated by SCE treatment revealing potential capability of SCE against lipid peroxidation.

Diabetic subjects are more likely to be blind than non-diabetics of a similar age<sup>39</sup>. Retinopathy is the commonest complication of diabetes and approximately 10% of diabetics at any one time will have sight-threatening retinopathy requiring specialist ophthalmological management<sup>40</sup>. Although the complete etiology of diabetic retinopathy remains unknown, the polyol pathway has been associated with this. The organs like kidney, eye etc. that are susceptible to diabetic complications, exhibit insulin independent glucose uptake and possess the glucose metabolizing enzyme aldose reductase<sup>41</sup>. Under hyperglycemia, increased glucose flux occurs in these tissues; through polyol pathway, aldose reductase catalyzes the conversion of glucose to sorbitol and that may cause accumulation of sorbitol<sup>42</sup>. This may result in the development of hyperosmotic stress to the cells of retina which leads to the development of diabetic complications<sup>43, 44</sup>. The elevated % HbA1c level is considered as one of the important risk factor of proliferative

diabetic retinopathy<sup>45</sup>. The increased level of advanced glycation end products (AGEs) and glycated hemoglobin are the dangerous consequences related to hyperglycemia. AGEs damage the cells in different ways<sup>46</sup>, like by modifying intracellular proteins of endothelial cells or extracellular matrix proteins, or by spreading the precursors outside the cell and their complexes with serum albumins<sup>47</sup>. SCE treatment reduced both lens aldose reductase activity (Fig. 3. 5A) as well as elevated % HbA1c level (Fig. 3. 5B) of diabetic animals revealing its beneficial effects against major secondary complications. In our *in vitro* cell free assays, SCE showed a moderate antiglycation and metal chelation properties which may contribute partly for reduction in % HbA1c level of SCE administered STZ animals.

The liver preserves normal blood glucose concentrations by storing glucose as glycogen and by generating glucose from glycogen breakdown or from gluconeogenic precursors<sup>48</sup>. The liver equilibrates the uptake and storage of glucose via glycogenesis and regulates the release of glucose by activating glycogenolysis and gluconeogenesis<sup>49</sup>. The major storage tissues such as liver, kidney, and skeletal muscle depend on insulin for glucose access<sup>50</sup>. Glycogen deposition from glucose is altered in experimentally induced diabetic animals. Here also diabetic animals showed depletion in the amount of liver (Fig. 3. 10A) and muscle (Fig. 3. 10B) glycogen compared to the normal control. Considerable amount of glycogen was replenished in diabetic animals by SCE treatment.

Oxidative stress produced under diabetic conditions is likely involved in progression of pancreatic beta-cell dysfunction found in diabetes. Pancreatic beta-cells are vulnerable to damage induced by oxidative stress, possibly due to the low levels of antioxidant enzyme expressions<sup>51</sup>. Integrity of pancreas is very much essential for the normal insulin secretion. These symptoms are clear in our study from the histopathology details of pancreas of DC (Fig. 3. 11c). But, both the doses of SCE repaired most of the histopathological alterations in pancreas (Fig. 3. 11e and f). The antioxidant potential of SCE may have protected the pancreatic beta-cells from the toxic effects of hyperglycemia in these cells. It is worth to mention that metformin, the positive control employed in this study protected all the vital organs from the deleterious effects of hyperglycemia in STZ diabetic rats.

### **3. 5 Conclusion**

Overall results show that SCE can exert beneficial effects on liver, kidney, pancreas, eye lens and muscle against hyperglycemia induced secondary complications. Furthermore, SCE does not affect these cited parameters in normal control rats. These findings indicate that SCE only affects these markers in diseased conditions and suggests that this herb is safe for consumption by healthy subjects. On the basis of this, SC is recommended for further detailed research for the development of new chemical entities for the management of secondary complications of diabetes.

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

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#### **4. 1 Introduction**

The prevalence of obesity is escalating worldwide and is associated with an increased risk of developing insulin resistance and T2DM<sup>1</sup>. Many reports propose that obesity may be responsible for high incidence of T2DM due to the typical association of diabetes with obesity<sup>2</sup>. In the present clinical settings, only the chronic cases of obesity have received much attention<sup>3</sup>. 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<sup>4, 5</sup>. The general urbanization of the world's population with diminished physical activity resulted in the increased prevalence of obesity<sup>4</sup>. 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, hyperinsulinaemia, insulin resistance, hypertension, hypertriacylglycerolaemia and chronic metabolic disorders<sup>6, 7</sup>. 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<sup>8</sup>.

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<sup>9</sup>. 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<sup>9</sup>. 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<sup>9, 10</sup>. Besides this property, fructose is more lipogenic and do not induce thermogenesis<sup>9, 11</sup>. 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<sup>12, 13</sup>.

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 *Symplocos 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.

## **4.2 Experimental details**

### **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).

### **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 *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<sup>0</sup>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<sup>14, 15</sup>. 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<sup>th</sup> day to 78<sup>th</sup> day. The 57<sup>th</sup>, 64<sup>th</sup>, 71<sup>st</sup>, 77<sup>th</sup> and 78<sup>th</sup> 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).

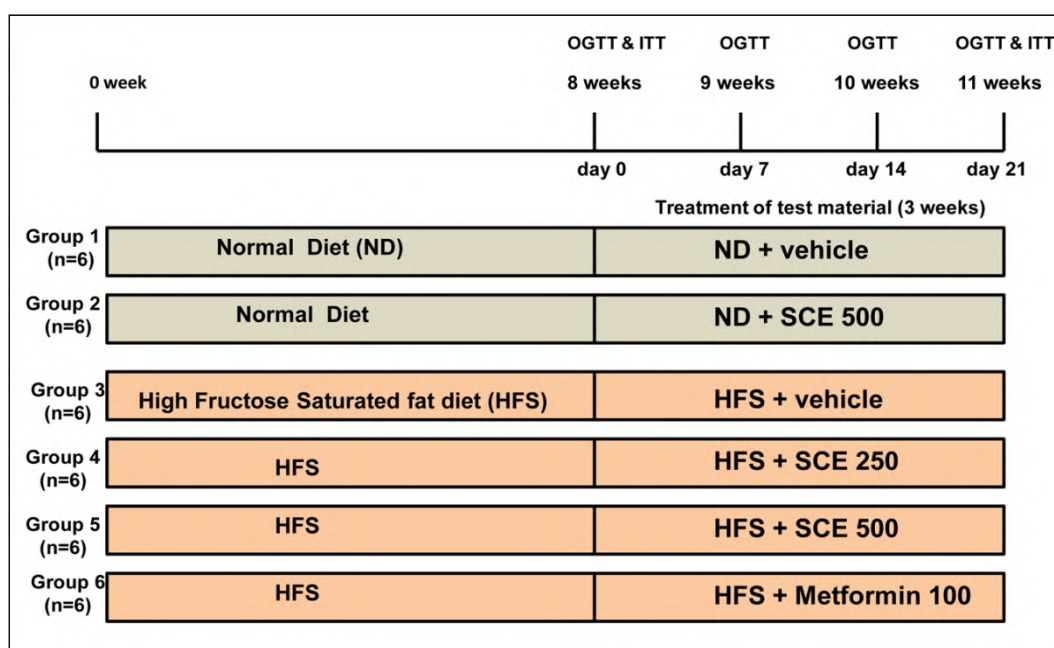
Group 3: High fructose saturated fat diet control animals (HFS) treated with vehicle (1.0% gum acacia) alone.

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



**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 79<sup>th</sup> 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;  $HOMA-IR = (\text{glucose} \times \text{insulin})/405$ , where the concentration of glucose was expressed in mg/dL and that of insulin in mU/L.

Sl. No.	Diet components (g/kg)	Normal diet (ND)	High fructose saturated fat (HFS) diet
1	Corn starch	36.5	-
2	Whole wheat	23.5	-
3	Fructose	-	<b>60.0</b>
4	Ground nut oil	5.0	-
5	Saturated fat	-	<b>12.0</b>
6	Casein (fat free)	20.0	20.0
7	Methionine	0.7	0.7
8	Cholesterol	-	<b>0.5</b>
9	Vitamin mixture	0.2	0.2
10	Mineral mixture	3.5	3.5
11	Wheat bran	10.6	3.1

**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<sup>0</sup>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<sup>0</sup>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<sup>16-18</sup>. 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)<sup>19</sup>. 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<sup>20</sup> and the protein concentration was determined using BCA kit (Pierce BCA



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<sup>21-23</sup>. For glucokinase activity assay<sup>21</sup>, 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 MgCl<sub>2</sub>, 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 MgCl<sub>2</sub>, 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<sup>22</sup>. The PEPCK activity was determined in the direction of oxaloacetate formation using the spectrophotometric assay<sup>23</sup>. 1 mL of the reaction mixture (pH 7.0) contained 77 mM sodium HEPES, 1 mM inosine 5'-diphosphate, 1 mM MnCl<sub>2</sub>, 1 mM DTT, 0.25 mM NADH, 2 mM phosphoenol pyruvate, 50 mM NaHCO<sub>3</sub>, 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<sup>24</sup>. 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<sup>25</sup> containing 100 mM dithiothreitol, and heated at 90-100<sup>0</sup>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.<sup>26</sup> 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 <sup>0</sup>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<sup>TM</sup> 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<sup>27</sup>. Total RNA was extracted from tissue samples, by guanidine isothiocyanate-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® 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 µg of RNA using the Super Script® 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.

mRNA	Accession no.	Amplicon	Forward primer (5' to 3')	Reverse primer (5' to 3')
Glut2/Slc2a2	NM_012879.2	194	GGCTAATTTTCAGGACTGGTTC	TTTCTTTGCCCTGACTTCCT
Glut4/Slc2a4	NM_012751.1	196	CCTCCTTTCTCATTGGCATC	ACATACATAGGCACCAACCC
GK/Gck	NM_001270849.1	195	AGCATCAGATGAAGCACAAG	CCACCACATCCATCTCAAAG
PEPCK/Pck1	NM_198780.3	199	AGAAATGCTTTGCGCTGC	CCCACACATTCAACTTTCCAC
G6Pase/G6pc	NM_013098.2	189	TCCAGCATGTACC GCAAGAG	ACTGACAGATGCAAAGGGGAC
Akt 2	NM_017093.1	192	GCCGAGTCCTACAGAATACC	ACTCCAGAGCTGACACAATC
SREBP1c/ Srebf1	NM_001276707.1	191	TTACACACCCAGGTCCAGAG	ATGCCTGCGGTCTTCATTG
Ppar $\gamma$ 2	NM_001145366.1	184	ACTATGGAGTCCATGCTTGTG	CCAAACCTGATGGCATTGTG
Ppar $\alpha$	NM_013196.1	190	TCCACGAAGCCTACCTGAAG	AAGAATCGGACCTCTGCCTC
Scd1	NM_139192.2	189	TTCCTCATCATTGCCAACAC	CTTTCCGCCCTTCTCTTTG
MCPI1/Ccl2	NM_031530.1	193	AGCTCAAGAGAGAGATCTGTG	GTGGAAAAGAGAGTGGATGC
FAS/Fasn	NM_017332.1	198	GGTGAAGTGTCTCCGAAAAG	TCCTGCTCTAACTGGAAGTG
PTP-1B/ Ptpn11	NM_001177593.1	136	GACCTGGTGGAGCATTACA	CGGCTAGCTTGCTTAACTCC
Sirt1	XM_003751934.1	185	TGAAAGTGATGACGATGACAG	GCCACAGTGTATATCATCC
beta-actin/ Actb	NM_031144.3	198	ACTGGGACGATATGGAGAAG	CATACAGGGACAACACAGC

**Table 4. 2.** Nucleotide sequence of qRT-PCR primers.

#### 4. 2. 14 Statistical analysis

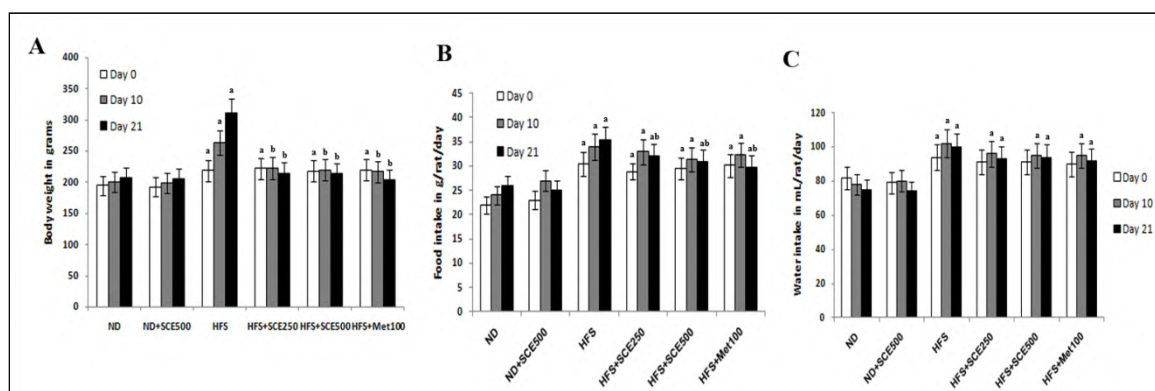
Quantitative glucose tolerance of each group was calculated by the area under the curve (AUC) method using Graph Pad Prism software version 3 (Graph Pad Software Inc., La Jolla, CA, USA). All other results were analyzed using a statistical program SPSS/PC+, version 11.0 (SPSS Inc., Chicago, IL, USA).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



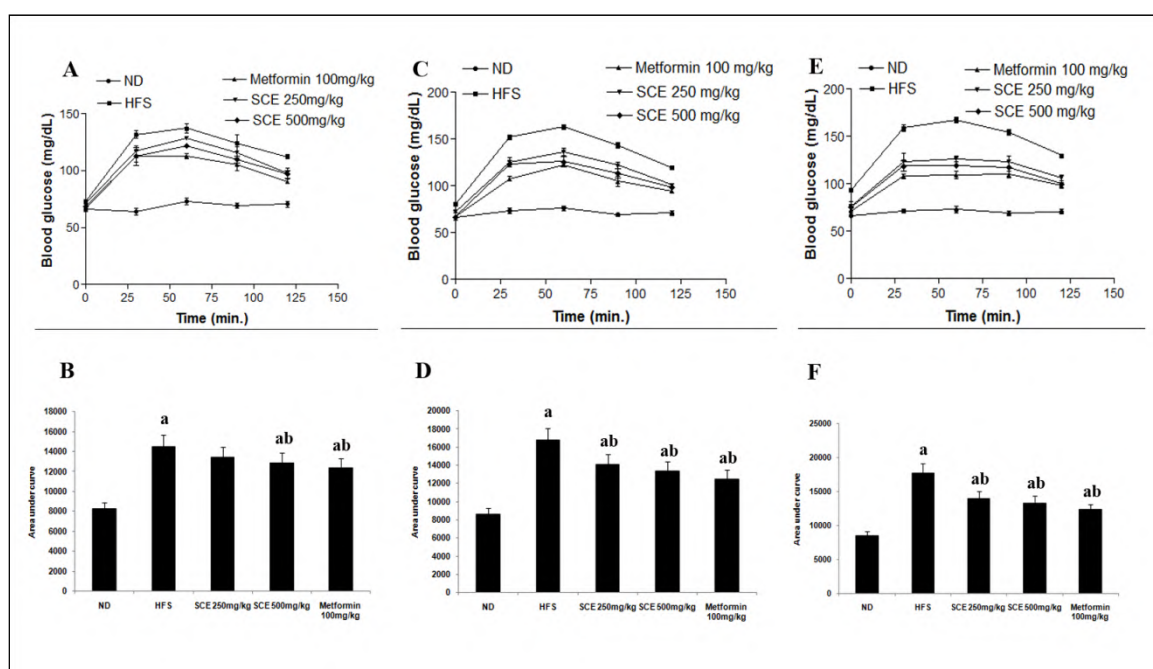
**Figure 4. 2A-C.** (A)Body weight, (B)food intake, (C)water intake at 3 time points, on day 0, 10 and 21in HFS fed SD rats. Data are expressed as the mean  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from HFS group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ). 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 .

#### **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 ( $p \leq 0.05$ , 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 ( $p \leq 0.05$ , 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 ( $p \leq 0.05$ , 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 ( $p \leq 0.05$ , 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).



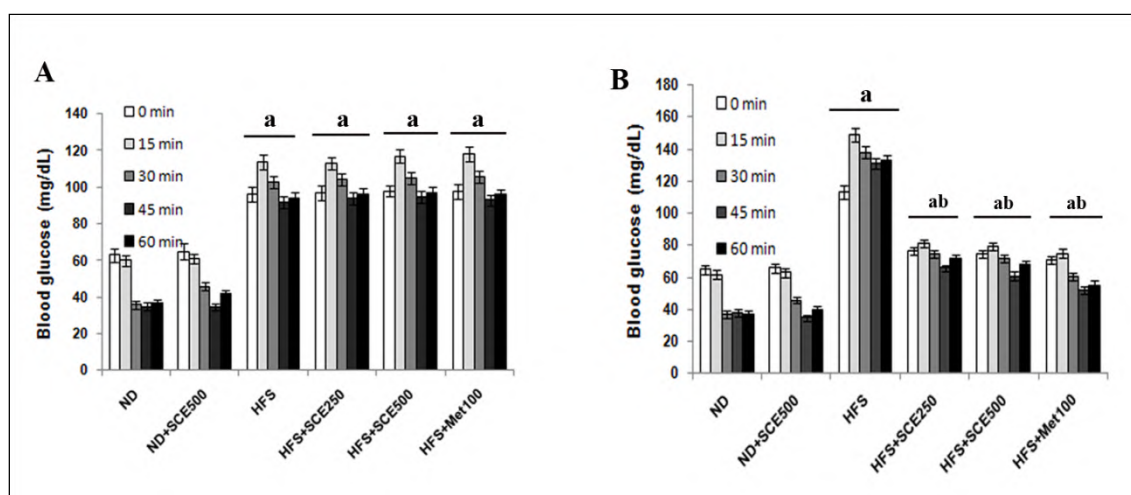
**Figure 4. 3A-F.** The improved glucose tolerance exhibited by HFS rats after 7, 14 and 21 days of SCE treatment (A) The glycemic response curve and (B) incremental AUC<sub>0-120</sub> min on day 7, (C) The glycemic response curve and (D) incremental AUC<sub>0-120</sub> min on day 14, (E) The glycemic response curve and (F) incremental AUC<sub>0-120</sub> min on day 21. Data are expressed as the mean  $\pm$  SD, n = 6. 'a' represents groups differ significantly from ND group ( $p \leq 0.05$ ). 'b' represents groups differ significantly from HFS group ( $p \leq 0.05$ ). 'ab' represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ). SCE, *S. cochinchinensis* (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 ( $p \leq 0.05$ , Fig. 4. 6A and B). Total cholesterol in plasma (4 fold) and LDL-C (5.5 fold) were also significantly ( $p \leq 0.05$ ) 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 ( $p \leq 0.05$ , Fig. 4. 6A-E).

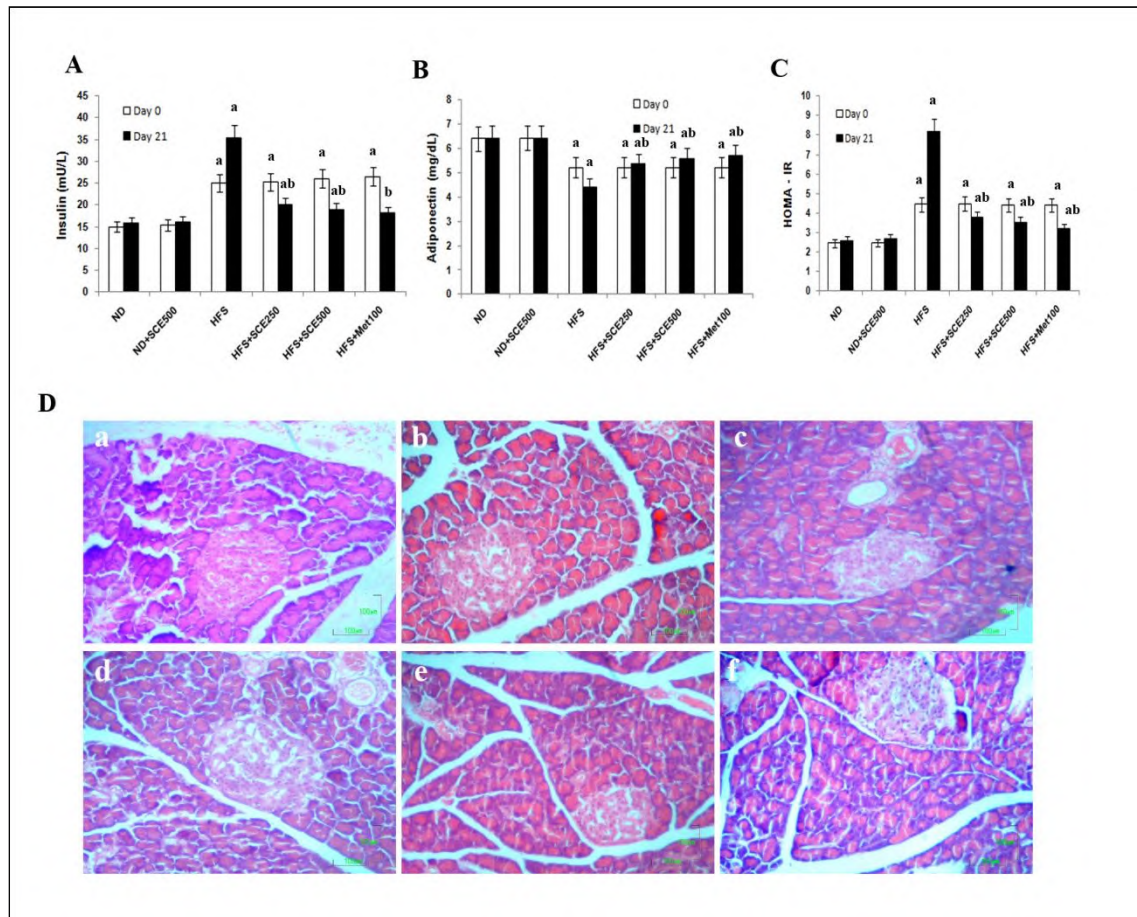
### 4. 3. 5 Renal and hepatic renal function and morphology was improved by SCE administration

The decrease in plasma albumin level of HFS animals (0. 30 fold) indicated the diminished liver and kidney function (Fig. 4. 7A).The significantly ( $p \leq 0.05$ ) 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  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ). 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 .

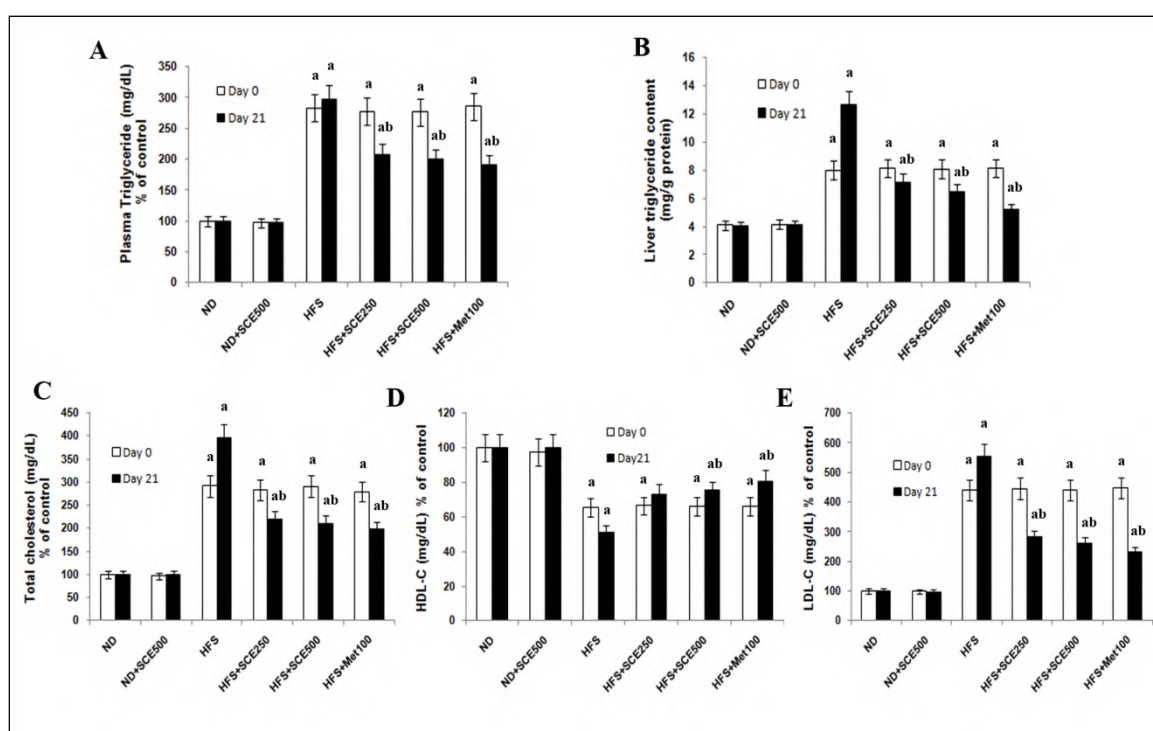




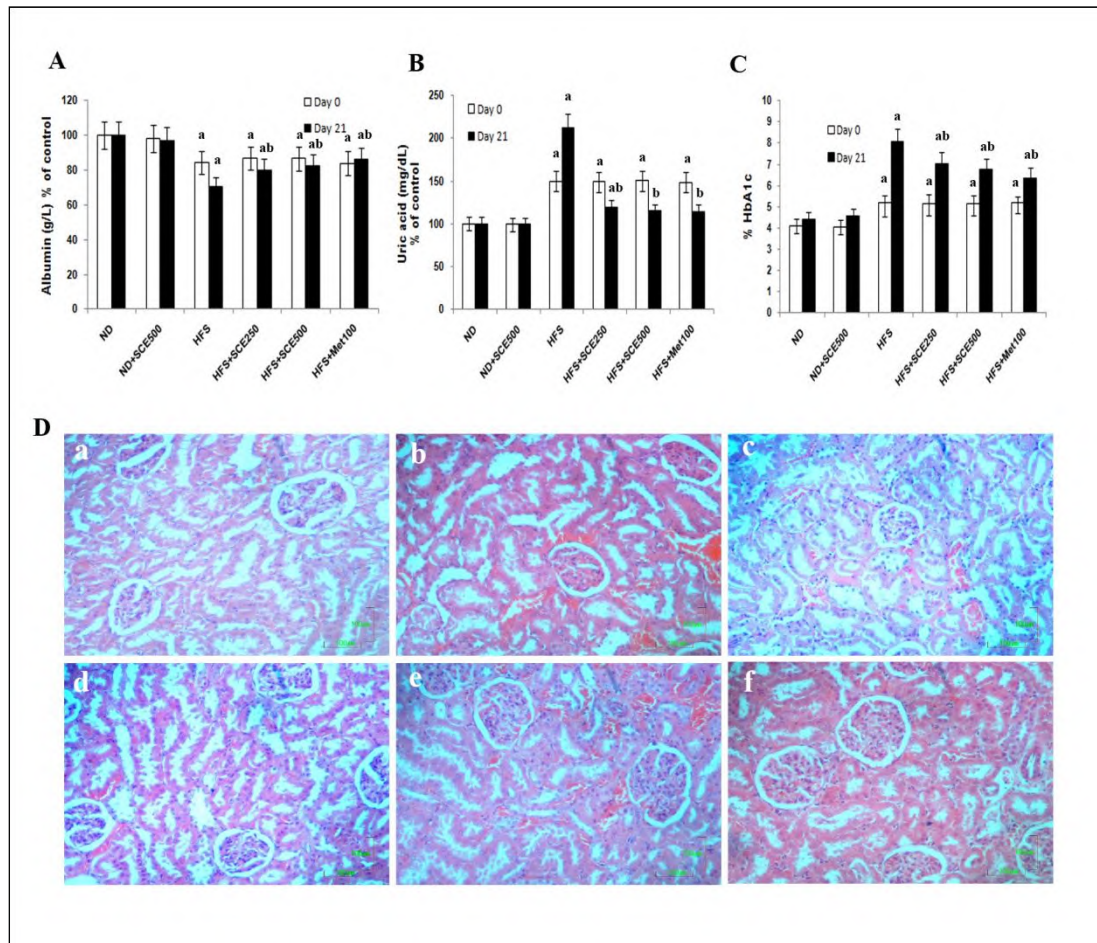
**Figure 4. 5A-D. Plasma insulin, adiponectin levels, insulin sensitivity on day 0, 21 and histopathology of pancreas after SCE treatment.** (A) Plasma insulin level, (B) Plasma adiponectin level and (C) Homeostatic model assessment-insulin resistance (HOMA-IR). Data are expressed as the mean  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from HFS group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ). (D) Representative microscopic scans of pancreas stained by Hematoxylin and Eosin (H and E 400 $\times$ ) (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.



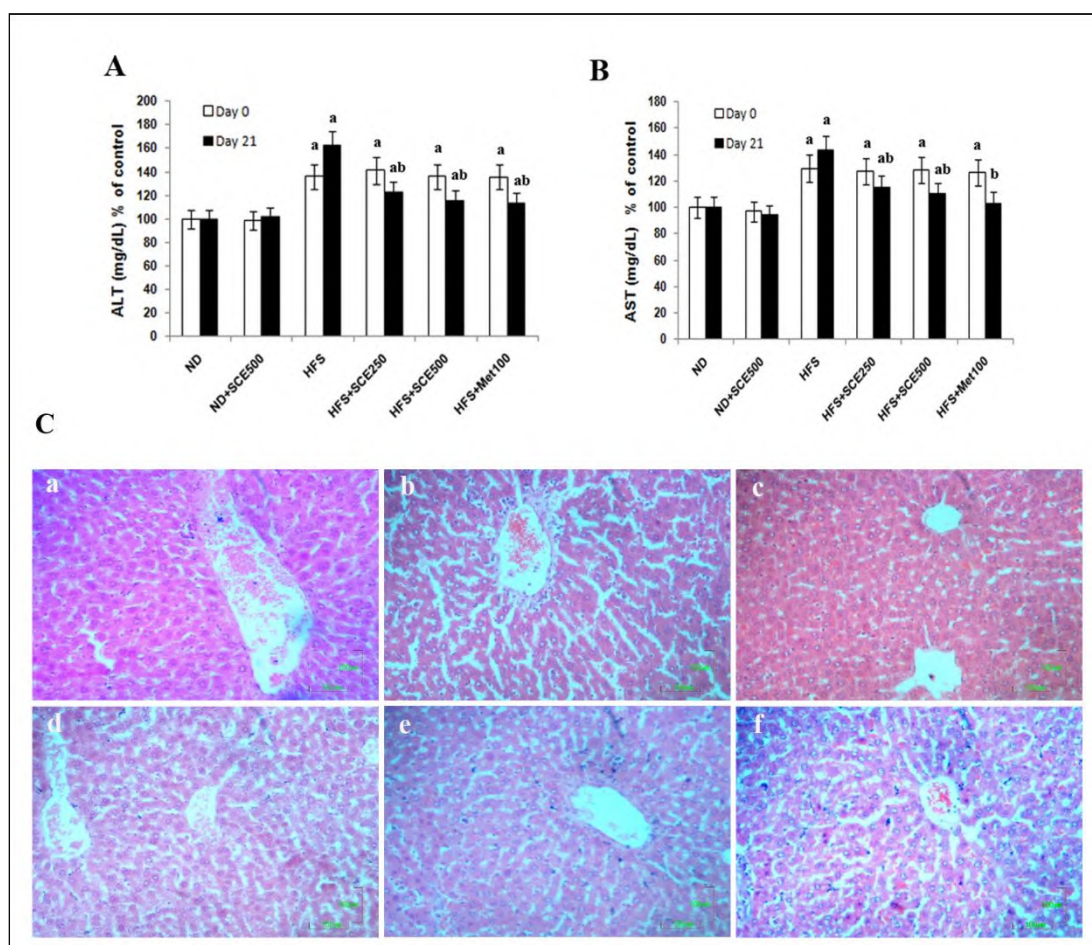
The HFS diet also modified renal tissue architecture and caused mesangial matrix expansion and inflammatory infiltration in the renal tissue of HFS animals. The renal tissue integrity was found to be maintained by the treatment with SCE 250/500 or Met 100 compared to that of HFS vehicle control (Fig. 4. 7D a-f). ALT, the specific biomarker for hepatic damage was significantly ( $p \leq 0.05$ ) 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 ( $p \leq 0.05$ ) 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  $\pm$  SD,  $n = 6$ . ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from HFS group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ). 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 .

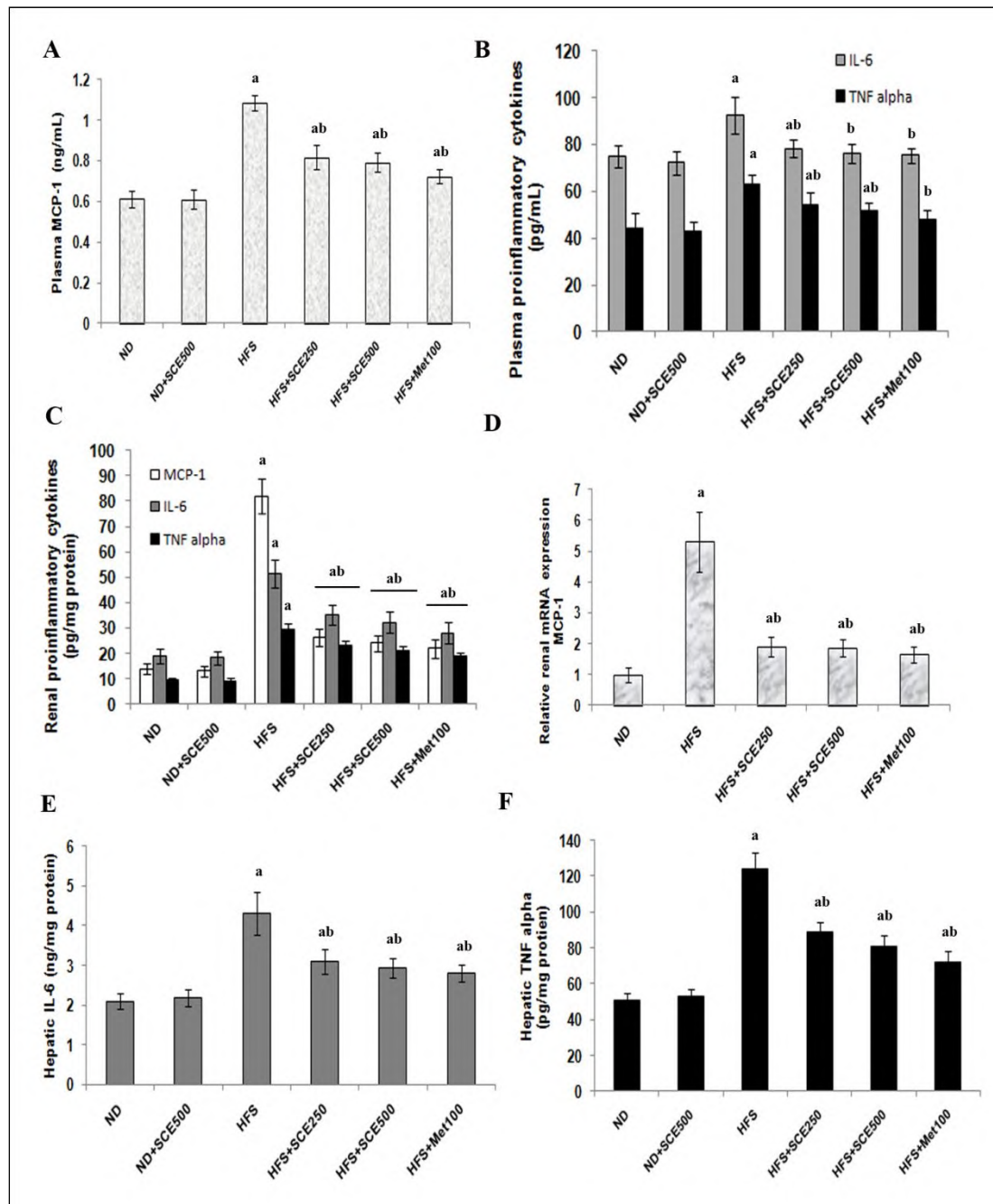


**Figure 4. 7A-D. Plasma albumin, uric acid, % HbA1c concentration and histopathology of kidney after SCE administration** (A) Plasma albumin level, (B) Plasma uric acid level and (C) %HbA1c. Data are expressed as the mean  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from HFS group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ). (D) Representative microscopic scans of kidney stained by Hematoxylin and Eosin (H and E, 400 $\times$ ) (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  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from HFS group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ). (C) Representative microscopic scans of liver stained by Hematoxylin and Eosin (H and E, 400 $\times$ ) (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.

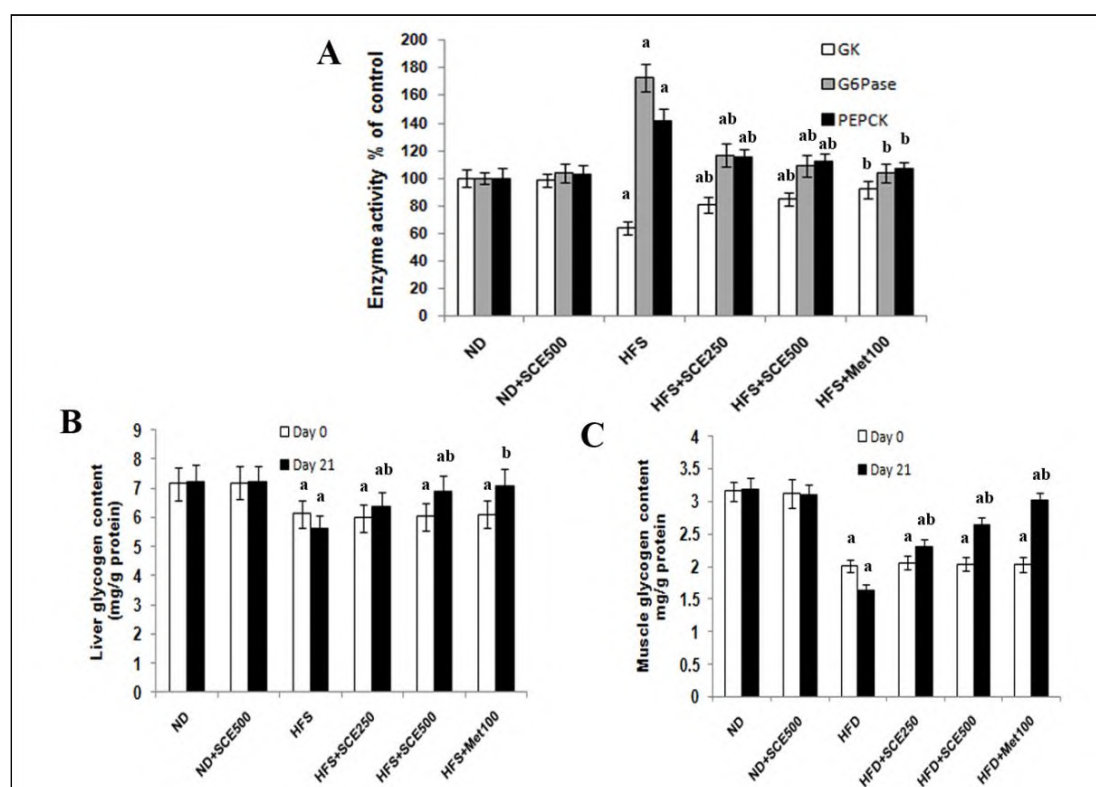




**Figure 4. 9A-F. Effect SCE administration on proinflammatory cytokines in plasma, kidney and liver after 21 days of treatment.** (A) Plasma concentration of MCP-1 (B) Plasma concentration of IL-6 and TNF-alpha (C) MCP-1, IL-6 and TNF-alpha in kidney tissue (D) mRNA level of MCP-1 in renal tissue (E) IL-6 in liver tissue (F) TNF-alpha in liver tissue. Data are expressed as the mean  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from HFS group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ). 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 ( $p \leq 0.05$ ) 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 ( $p \leq 0.05$ ) 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 ( $p \leq 0.05$ ) 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).



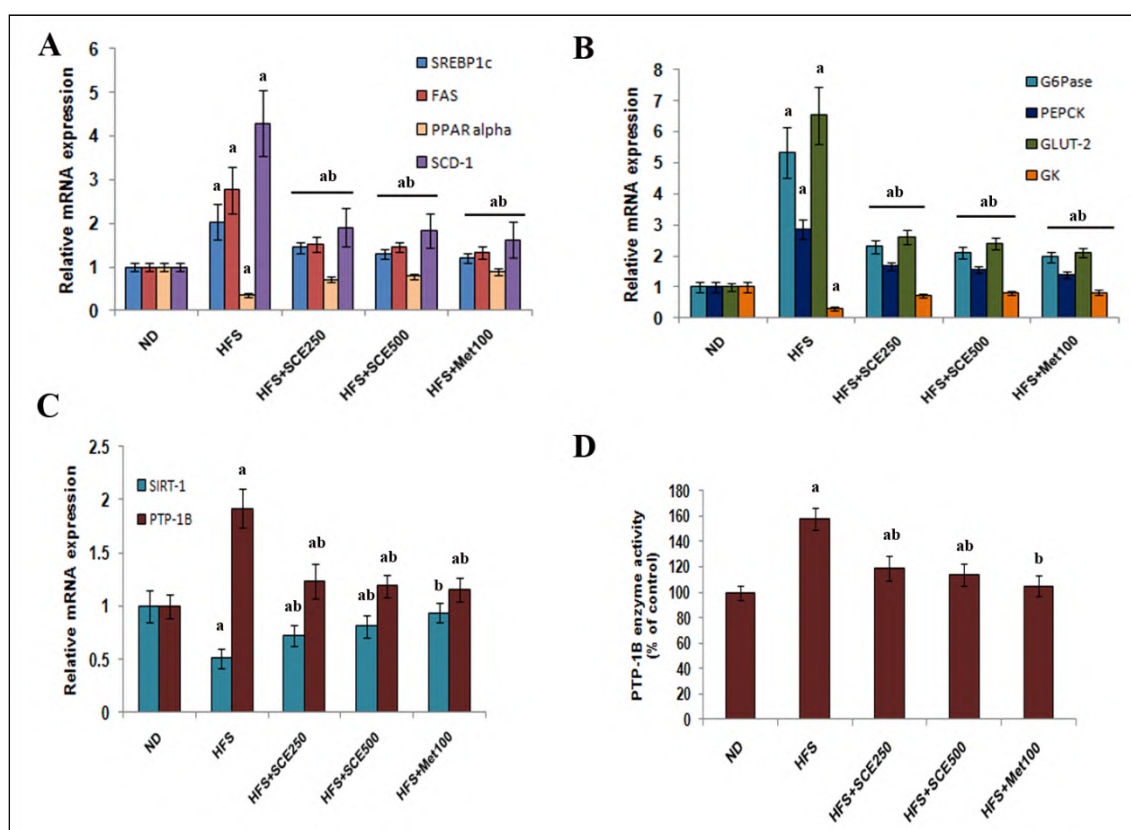
**Figure 4. 10A-C. Liver glycolytic and gluconeogenic enzyme activities, liver and muscle glycogen content after 21 days of SCE treatment** (A) Activities of Glucokinase (GK), Glucose 6 phosphate dehydrogenase (G6Pase) and Phosphoenol pyruvate carboxy kinase (PEPCK) in liver tissue (B) Liver glycogen content (C) Muscle glycogen content. Data are expressed as the mean  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from HFS group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ).

#### **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 ( $p \leq 0.05$ , 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 ( $p \leq 0.05$ ) 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 ( $p \leq 0.05$ ) 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), PEPCCK (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 ( $p \leq 0.05$ ) 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, PEPCCK, 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



**Figure 4. 11A-D.** Effect of SCE in gene expression in liver tissue isolated from SD rats after 21 days of extract treatment (A) Relative mRNA level of lipogenic genes: SREBP1c, FAS, PPAR alpha and SCD-1 (B) Relative mRNA level of glucogenic genes: G6Pase, PEPCK, GLUT-2 and GK (C) Relative mRNA level of genes SIRT-1 and PTP-1B. Data are expressed as the mean  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from HFS group (v). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ).

and skeletal muscle by SCE dosage in comparison with HFS control (Fig. 4. 13A and 14A). The expression of PPAR gamma 2 in adipose and SCD-1 in skeletal muscle was also significantly reinstated by SCE 250/500 treatment compared to the vehicle treated HFS group (Fig. 4. 13A and 14A).

#### 4. 3. 9 Effect of SCE on the expression of insulin signalling markers and transcription factors

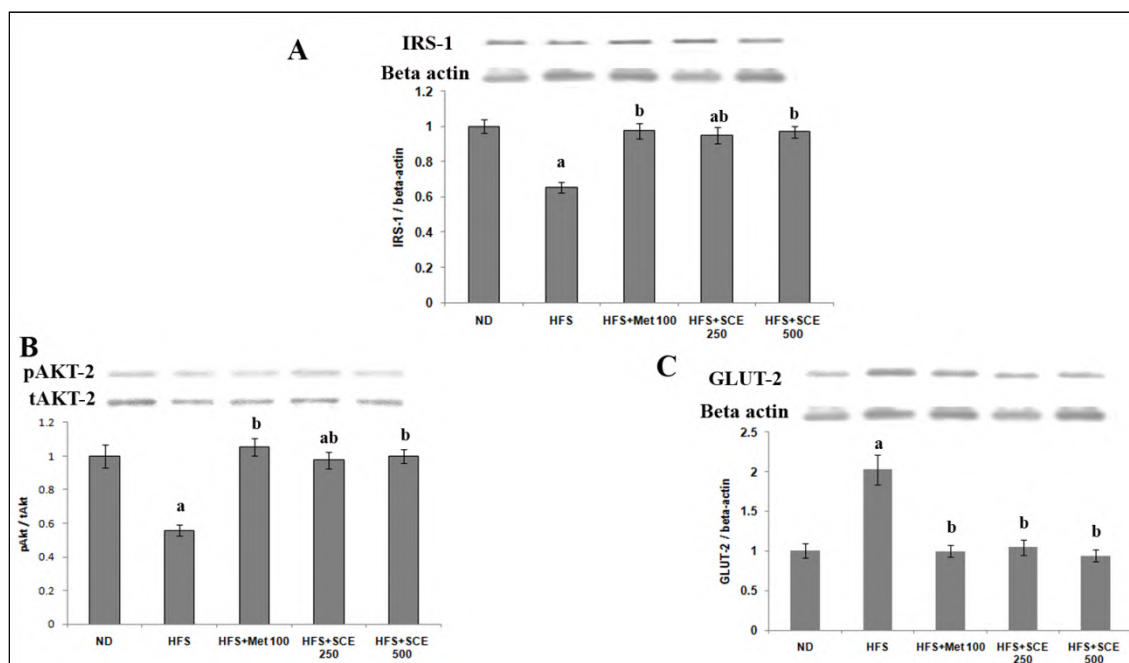
The expression of pattern of various proteins in the insulin signalling pathway like IRS-1, total AKT (tAKT), phosphorylated AKT (pAKT, Ser-473) and GLUT-2 of the liver tissue were analysed using western blotting (Fig. 4. 12A-C). We have also assessed the protein expression of IRS-2, pAKT, and GLUT-4 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 ( $p \leq 0.05$ ) 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<sup>10</sup>. 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<sup>13, 28</sup>. 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 *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<sup>9, 29</sup>. HFS induced metabolic derangement subsequently developed

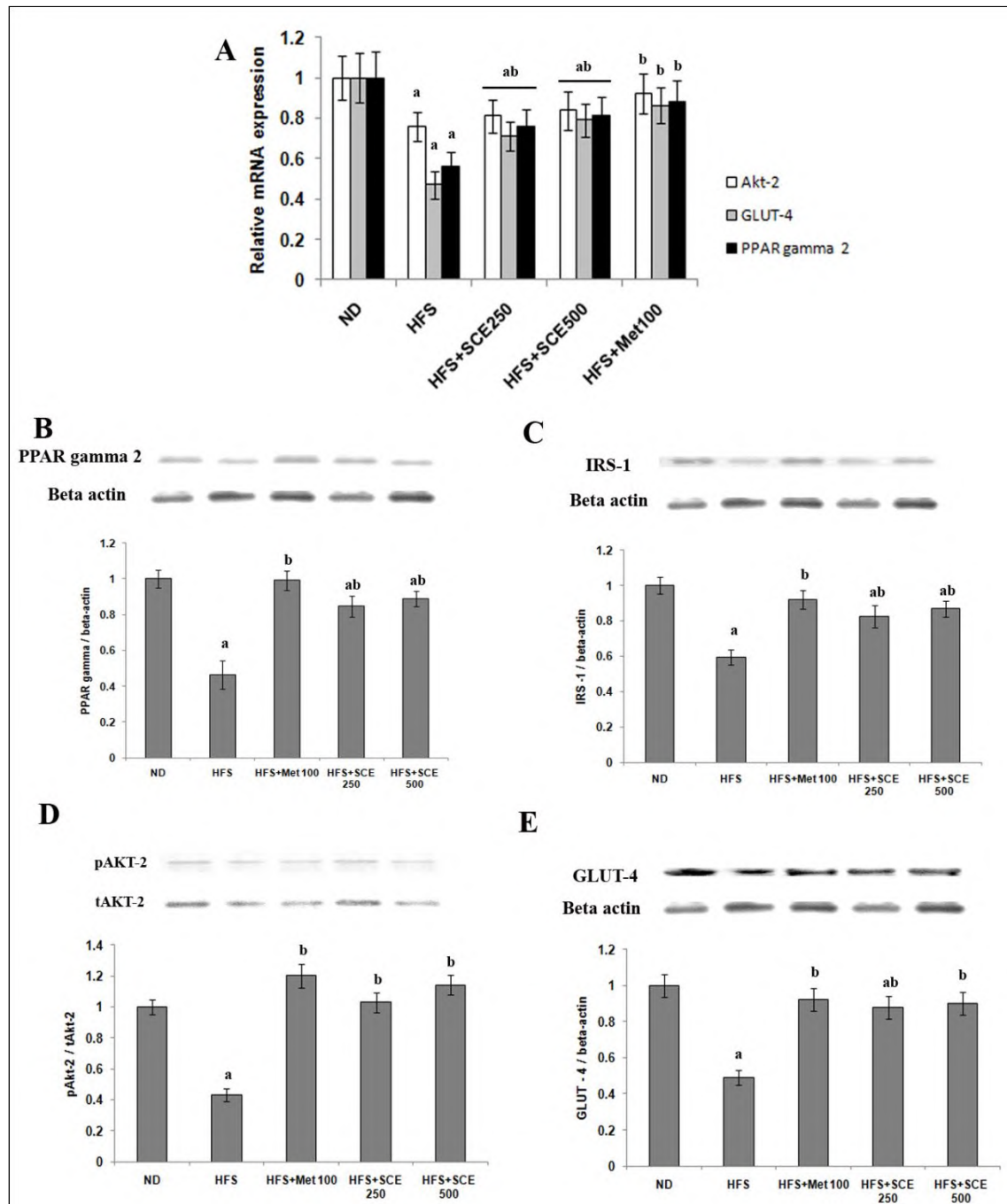




**Figure 4. 12A-C. Effect of SCE on the protein expression in liver tissue isolated from HFS fed SD rats after 21 days of extract treatment.** (A) insulin receptor substrate- 1 (IRS-1), (B) pAKT-2 (C) GLUT-2. Data are expressed as the mean  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from HFS group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ).

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 ( $p \leq 0.05$ ) 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 gain<sup>9, 30</sup>. 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 resistance<sup>31</sup>. 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 diet<sup>32</sup>. In the present study, OGTT patterns of HFS rats show the impaired glucose tolerance



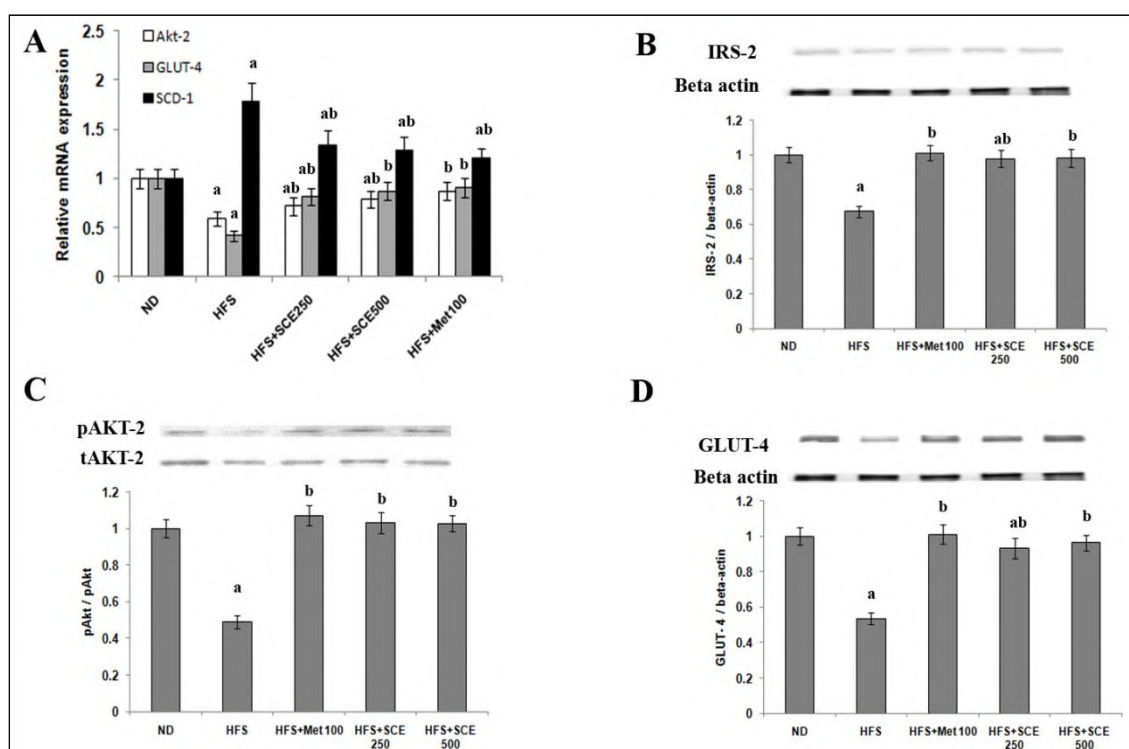
**Figure 4. 13A-E.** Effect of SCE on the gene and protein expression in adipose tissue isolated from HFS fed SD rats after 21 days of treatment (A) Relative mRNA level of genes: PPAR gamma, Akt-2 and GLUT-4 (B) Protein expression of PPAR gamma (C) insulin receptor substrate- 1 (IRS-1), (D) pAKT-2 (E) GLUT-4. Data are expressed as the mean  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from HFS group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ).

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<sup>33,34</sup>. 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<sup>35-37</sup>. The animals in the HFS group showed hyperinsulinemia (Fig. 4. 5A). However, SCE treatment significantly ( $p \leq 0.05$ ) 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<sup>38</sup>. 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<sup>39</sup>. Also, plasma adiponectin levels negatively correlate with visceral fat accumulation and insulin resistance<sup>40,41</sup>. Chronic fructose consumption reduces adiponectin responses, contributing to insulin resistance<sup>42</sup>. Adiponectin plays a protective role against insulin resistance by regulating glucose and lipid metabolism<sup>43</sup>. 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 *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<sup>44</sup>. 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<sup>45</sup>. This is due to the accelerated production of pyruvate and glycerol-3-phosphate which promote the elevated hepatic *de novo* lipogenesis and upregulated VLDL production and secretion<sup>45</sup>. According to Thorburn et al. and others relatively mild, short term elevation of triglyceride is sufficient to impair insulin action in peripheral tissues<sup>29, 46</sup>. 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<sup>47</sup>. 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<sup>48</sup>. 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<sup>49, 50</sup>. Uric acid has potent effects on proximal tubular cells stimulating MCP-1 production and mediates renal inflammatory responses<sup>51</sup>. 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<sup>52, 53</sup>. 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 ( $p \leq 0.05$ ) 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  $\pm$  SD, n = 6. ‘a’ represents groups differ significantly from ND group ( $p \leq 0.05$ ). ‘b’ represents groups differ significantly from HFS group ( $p \leq 0.05$ ). ‘ab’ represents groups differ significantly from both HFS and ND group ( $p \leq 0.05$ ).

proinflammatory cytokines like MCP-1 and TNF alpha<sup>54</sup>. MCP-1 plays a crucial role in inflammation mediated renal damage via promoting IL-6 release<sup>55</sup>. Expression level of IL-6 in the renal tissue correlates with the degree of mesangial hyperproliferation, tubular atrophy, and the intensity of interstitial infiltrates<sup>56, 57</sup>. 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<sup>58</sup>. Rats consuming

a diet rich in fructose and saturated fat showed significantly higher hepatic expressions of IL-6 protein and TNF alpha protein<sup>59</sup>. 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<sup>60</sup> 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<sup>21</sup>. The cytosolic PEPCK is a major flux generating enzyme for gluconeogenesis<sup>61</sup>. 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<sup>62, 63</sup>. 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<sup>64</sup>. 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<sup>65</sup>. 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.<sup>65</sup> 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<sup>65, 66</sup>. 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<sup>67</sup>. 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<sup>68, 69</sup>. High fructose and saturated fat feeding caused marked increase in hepatic lipid accumulation or steatosis<sup>70</sup>. Reports suggest that hepatic triglyceride accumulation is a major mediator of hepatic insulin resistance<sup>71</sup>. The impaired insulin signalling in the liver leads to decreased glycogen synthesis, and increased glycogenolysis and gluconeogenesis<sup>45</sup>. Insulin secretion increases as a compensatory response towards the elevated hepatic glucose output<sup>72</sup>. Hyperinsulinemia leads to increased de novo lipogenesis due to insulin mediated activation of the master regulator of the hepatic lipogenesis, SREBP-1c<sup>73</sup>. 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<sup>74, 75</sup>. According to Miyazaki et al.<sup>76</sup> 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<sup>73, 76</sup>. Fructose also increases the stability of FAS mRNA which is an important downstream component of lipid synthesis<sup>77</sup>. 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<sup>78</sup>. 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<sup>79</sup>. 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<sup>80</sup>. In our study, the expression level of PPAR alpha in HFS animals was found to be down regulated significantly ( $p \leq 0.05$ ). 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)<sup>70, 80</sup>.

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-1<sup>69, 81</sup>. The inhibitory phosphorylation of IRS blocks the IRS-mediated activation of its downstream signalling target, such as AKT<sup>82</sup>. Moreover, elevated expression of PTP-1B, usually associated with impaired insulin signalling, also leads to increased mRNA and promoter activity of SREBP-1c, and subsequent increases in the expression of FAS<sup>80, 83</sup>. 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<sup>84</sup>. 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



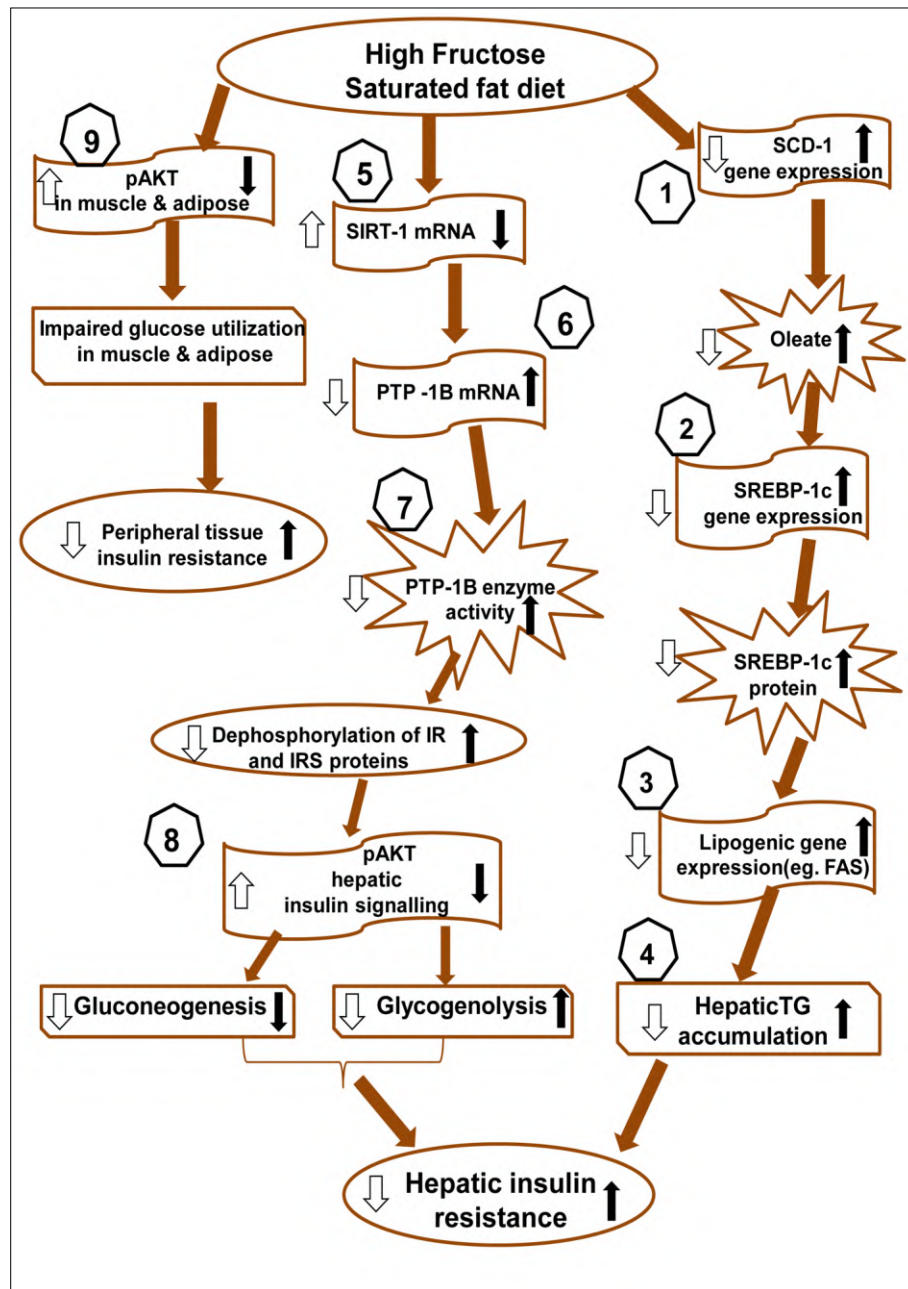
silencing<sup>85-88</sup>. Moderate upregulation of SIRT-1 expression might protect against metabolic disorders and hepatic steatosis induced by a high fat diet<sup>89</sup>. Sun et al.<sup>90</sup> reported that SIRT-1 improves insulin sensitivity by repressing PTP-1B transcription at the chromatin level. PTP-1B is the negative regulator of the insulin signal transduction cascade, which dephosphorylates insulin receptors and insulin receptor substrates<sup>91</sup>. 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<sup>45</sup>. 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<sup>92-94</sup>. 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<sup>94</sup>. 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<sup>95, 96</sup>. The transcription factor PPAR gamma directly regulates the expression of genes such as adiponectin and GLUT-4 which can play an important role in insulin sensitivity<sup>97, 98</sup>. 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 *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<sup>99</sup>. Insulin resistance caused by prolonged high fructose feeding is associated with increased accumulation of lipids in liver and muscle tissues of rats<sup>12</sup>. Investigations involving high-fat fed rodent models also detected the intra-muscle triglyceride accumulation as a causative factor in the initiation of insulin resistance<sup>100, 101</sup> and it is confirmed as one of the most consistent markers of whole-body insulin resistance<sup>102</sup>. The insulin sensitivity can be improved by the reduction of elevated intracellular TG content in muscle<sup>103</sup>. 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<sup>104</sup> and obese insulin resistant Zucker diabetic fatty rats<sup>105</sup> and its elevated expression in skeletal muscle contributes to abnormal lipid metabolism and progression of obesity and type 2 diabetes<sup>106</sup>. 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<sup>107</sup>. 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<sup>108</sup>. 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.

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## Summary and Conclusion

The alarming picture of the incidence and prevalence of diabetes worldwide is uncovered by the epidemiological studies of various agencies such as IDF, ADA and WHO. India, especially Kerala is becoming the global diabetic capital in recent years. Elucidating the causes of diabetes remains the key step towards eradicating the disease, but the prevention and amelioration of hyperglycemia and diabetes induced secondary complications is equally important for the millions of people who already living with the disease knowingly or unknowingly. Report from ministry of health, Govt. of India, says that ‘diabetes in India is no longer a disease of the affluent or a rich man’s disease; it is becoming a problem even among the middle income and poorer sections of the society’. It should be noted that poor diabetic subjects are more prone to secondary complications as they have less access to quality health care.

Traditional medicine or indigenous knowledge always serves as a low cost and easily available treatment option for a multifactorial disease like diabetes. Traditional formulations are much attractive due to their increased tolerance and synergistic effects, as they contain diverse kind of bioactives. Moreover, traditional medicines had paved way for development of the most effective modern drug like metformin from *Galega officinalis*. *Ayurveda*, a system of traditional medicine native to Indian subcontinent always plays major role in primary health care of both rural and urban populations of India.

*Symplocos cochinchinensis* (SC) is one of the key ingredients of *Nisakathakadi Kashayam*; a very effective *Ayurvedic* preparation for diabetes mentioned in the ancient script ‘*Sahasrayogam*’. But for wider acceptability of the health benefits of SC, a detailed scientific investigation on its mode of action on various biochemical targets relevant to diabetes is mandatory. But detailed study illustrating the mechanism of action of SC or its biochemical targets relevant to diabetes is not available in literature. Keeping this in mind, the main objective of the study was to see the various biochemical mechanisms responsible for its antidiabetic property by analysing its effects on selected biochemical and molecular targets relevant to diabetes using *in vitro* and *in vivo* models.

For this, bark of the plant was collected, identified and processed for extraction. The



ethanolic extract of the bark of SC (SCE) and its fractions (hexane, dichloromethane, ethyl acetate and 90% ethanol) were evaluated by various *in vitro* cell free and cell based methods against multiple targets relevant to diabetes such as the alpha glucosidase inhibition, glucose uptake, adipogenic potential, oxidative stress, pancreatic beta-cell proliferation, inhibition of protein glycation, protein tyrosine phosphatase-1B (PTP-1B) and dipeptidyl peptidase-IV (DPP-IV). HPLC analysis was also conducted to all the fractions for partial chemical characterization for its chemical composition. AAS analysis of SCE was conducted for mineral composition. The antihyperglycemic activity of SCE was confirmed by OSTT studies in normal, mild diabetic and severely diabetic streptozotocin (STZ) induced Sprague Dawley (SD) rats using acarbose and metformin as positive controls.

The beneficial effects of SCE have also been explored against hyperglycemia associated secondary complications in STZ-induced diabetic SD rat model. The experimental groups under this study consist of normal control (NC), N + SCE 500 mg/kg bwd, diabetic group (DC), D + metformin 100 mg/kg bwd, D + SCE 250 and D + SCE 500. SCE 250/500, metformin or vehicle were administered daily for 21 days and sacrificed on day 22. Oral glucose tolerance test, plasma insulin, % HbA1c, urea, creatinine, aspartate aminotransferase, alanine aminotransferase, albumin and total protein were analysed. Aldose reductase (AR) activity in the eye lens, hepatic, renal oxidative stress and function markers; liver and muscle glycogen and histopathological alterations of pancreas were also checked in this STZ induced diabetic model.

Then, we analysed the therapeutic potential of SCE against diet induced insulin resistance model and the molecular mechanisms of its activity in SD rats. The experimental groups under this study consist of normal diet animal (ND), ND + SCE 500 mg/kg bwd, high fructose saturated fat diet (HFS), HFS + metformin 100 mg/kg bwd, HFS + SCE 250 and HFS + SCE 500. Initially the animals were kept under HFS diet for 8 weeks and at the end of 8 week period, animals of HFS group were found to be developed insulin resistance and dyslipidemia which was evident from oral glucose tolerance test, plasma insulin and lipid profiles. SCE 250/500, metformin or vehicle were administered daily for next 21 days and sacrificed on day 22. OGTT, ITT, plasma analysis of insulin, lipid profile, % HbA1c and uric acid were conducted. Liver glycolytic and gluconeogenic

enzyme activities, triglyceride and glycogen content in liver and muscle, plasma level of proinflammatory cytokines like MCP-1, IL-6, TNF alpha were also checked in HFS model. The mRNA expression of genes involved in the lipid metabolism like SCD-1, SREBP-1c, FAS and PPAR alpha were analysed using qRT-PCR. The hepatic expression levels of genes involved in carbohydrate metabolism and insulin signalling such as G6Pase, PEPCK, GLUT-2, Akt-2, GLUT-4, PTP-1B and SIRT-1 were also evaluated. The expression pattern of various proteins like IRS-1, AKT-2 and GLUT-2 in the liver; IRS-2, AKT-2 and GLUT-4 in the muscle and PPAR gamma, IRS-1, AKT-2, and GLUT-4 in adipose tissue were analysed by western blotting.

Overall results from *in vitro* cell free and cell line based studies revealed that among the five extracts analysed, SCE exhibited comparatively better activity via alpha glucosidase inhibition, insulin dependent glucose uptake in L6 myotubes, pancreatic beta cell regeneration in RIN-m5F and reduced triglyceride accumulation in 3T3-L1 cells, protection from hyperglycemia induced generation of reactive oxygen species in HepG2 cells with moderate antiglycation, DPP-IV and PTP-1B inhibition. The *in vivo* studies involving OSTT after the oral administration of single dose of SCE confirmed the marked antihyperglycemic activity of SC in both STZ treated mild diabetic (SLM) and severely diabetic (STZ-S) SD rats. In SLM, treatment with 500 mg/kg bw of SCE reduced the whole glycemic response by 12.88% while acarbose and metformin caused 15.73% and 17.12% reduction respectively. SCE (500 mg/kg bw) treatment in STZ-S caused 23.48% improvement in blood glucose profile after 5 h of treatment and acarbose and metformin showed 30.27% and 33.18% respectively. These potential effects of SC contribute significantly to its antidiabetic property. SCE has been taken forward for the *in vivo* study as it exhibited better activity under *in vitro* assays, increased yield of more bioactive molecules and less toxicity of the solvent.

The long term (21 days) administration of SCE in STZ-induced diabetic SD rats showed that SCE can exert beneficial effects on liver, kidney, pancreas, eye lens and muscle against hyperglycemia induced secondary complications. Treatment with SCE protected from the deleterious alterations of biochemical parameters in a dose dependent manner including histopathological alterations in pancreas. SCE also exhibited of glucose lowering effect and decreased HOMA-IR, % HbA1c, lens aldose reductase activity, and

hepatic, renal oxidative stress and function markers compared to the diabetic control group. Considerable amount of liver and muscle glycogen was replenished by SCE treatment in STZ-induced diabetic SD rats.

The SCE was also found to be an effective agent against high fructose saturated fat diet model of insulin resistance and dyslipidemia in high fructose saturated fat fed SD rats. The study of multiple aspects of diabetes and insulin resistance displayed the antidiabetic potential of SCE via improving hepatic glucose homeostasis and lipid metabolism in the liver of rats. Increased insulin sensitivity was noticed in peripheral tissues like muscle and adipose by SCE administration which was the result of improved expression level and phosphorylation state of proteins involved in the insulin signalling pathway. SCE treatment reduced the level of serum proinflammatory cytokines and glycated hemoglobin which protected from further inflammatory response and tissue damage. SIRT-1 mediated down regulation or inhibition of the activity of PTP-1B enzyme may be one of the mechanisms behind the insulin sensitizing and improved hepatic glucose homeostatic property of SCE. The improvement of insulin resistance and hepatic steatosis by SCE may be due to the down regulation of SCD-1 gene expression which modulate SREBP-1c dependent and independent hepatic lipid accumulation.

The presence of known insulin sensitizers and AG inhibitors like phloretin 2'-glucoside, oleanolic acid and beta-sitosterol in SC may be playing an important role in these multifaceted activities of SC with respect to diabetes. Furthermore, SCE does not affect these cited parameters in normal control rats. These findings indicate that SCE only affects the markers under diseased conditions and suggests that this herb is safe for consumption by healthy subjects.

The detailed investigation related to the antidiabetic effect of SC revealed its therapeutic potential against key biochemical targets like alpha glucosidase inhibition, oxidative stress induced diabetic secondary complications, diet induced insulin resistance and dyslipidemia. The present study also revealed presence of bioactives and minerals which are reported to have the therapeutic benefit to diabetes and other related complications. From overall results, it can be concluded that SC is the potential medicinal plant from Indian system of medicine to be exploited for the development of lead molecule or standardised extract for the control and management of diabetes. On the basis of these

preliminary investigations, SC is recommended for detailed future study on various models like *in vitro* and *in vivo* models for SGLT-2 inhibitors, db/db mice etc. using standardised extract or isolated molecules from this plant for the generation of new chemical entities for future drug.

## LIST OF PUBLICATIONS

1. **Antu KA**, Riya MP, Mishra A, Anilkumar KS, Chandrakanth CK, Tamrakar AK, Srivastava AK, Raghu KG (2014) Antidiabetic property of *Symplocos cochinchinensis* is mediated by inhibition of alpha glucosidase and enhanced insulin sensitivity. *PLoS ONE* 3:9(9):e105829. doi:10.1371/journal.pone.0105829.
2. **Antu KA**, Riya MP, Mishra A, Sharma S, Srivastava AK, Raghu KG (2014) *Symplocos cochinchinensis* attenuates streptozotocin-diabetes induced pathophysiological alterations of liver, kidney, pancreas and eye lens in rats. *Exp Toxicol Pathol* 66(7):281-291.
3. Riya MP<sup>1</sup>, **Antu KA**<sup>1</sup>, Pal S, Srivastava AK, Sharma S, Raghu KG (2014) Nutraceutical potential of *Aerva lanata* (L.) Juss. ex Schult ameliorates secondary complications in streptozotocin-induced diabetic rats. *Food Funct* 5(9):2086-2095. (<sup>1</sup>Co-first authors)
4. Riya MP, **Antu KA**, S. Pal, Anilkumar KS, Chandrakanth CK, Tamrakar AK, Srivastava AK, Raghu KG (2014) Antidiabetic property of *Aerva lanata* (L) Juss. ex Schult. mediated by inhibition of alpha glucosidase, protein glycation and stimulation of adipogenesis. *J Diabetes* doi: 10.1111/1753-0407.12216.
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9. **Antu KA**, Riya MP, Sharma S, Srivastava AK, Raghu KG. *Symplocos cochinchinensis* enhances insulin sensitivity via the down regulation of hepatic lipogenesis and insulin resistance in high fructose saturated fat diet rodent model. (Manuscript under preparation).
10. **Antu KA**, Riya MP, Sharma S, Srivastava AK, Raghu KG. Protective property of *Symplocos cochinchinensis* against muscle and adipose insulin resistance and renal proinflammatory markers in diet induced insulin resistant *in vivo* rat model. (Manuscript under preparation).
11. Riya MP, **Antu KA**, Sharma S, Srivastava AK, Raghu KG. *Aerva lanata* improves insulin resistance in skeletal muscle and liver of rats fed a high fructose saturated fat diet. (Manuscript under preparation).
12. Riya MP, **Antu KA**, Sharma S, Srivastava AK, Raghu KG. Administration of *Aerva lanata* ameliorates inflammation due to high fructose saturated fat diet. (Manuscript under preparation).

## CONFERENCE PRESENTATIONS

### Oral Presentation

1. “Elucidation of molecular mechanisms responsible for the antidiabetic property of *Symplocos cochinchinensis* (Lour.) S Moore: an *in vitro* approach” **Antu K A** and Raghu K G (paper presented in 12<sup>th</sup> International conference of Ethnopharmacology, ISE-2012) organized by School of Natural Product Studies, Jadavpur University, Kolkata, India from 17<sup>th</sup> -19<sup>th</sup> February, 2012.

### Poster Presentations

1. “*Symplocos cochinchinensis* exhibit antihyperglycemic activity via its antioxidant potential and inhibition of PTP-1B and alpha-glucosidase” **Antu K A** and Raghu K G (poster presented in 5<sup>th</sup> World Congress of Diabetes India-2013 organized by Diabetes India, Ahmedabad) conducted at Kochi, Kerala from 18<sup>th</sup> -21<sup>st</sup> April, 2013.
2. “Bioactives from pineapple residue-a potential source of nutraceutical for diabetes” Riya M P, **Antu K A**, Raghu K G (poster presented in International conference on multidisciplinary approaches to diabetes research and health (ICMADRH-2010)) conducted by Department of Zoology, University of Rajasthan, Jiapur, India from 14<sup>th</sup> -16<sup>th</sup> November, 2010.