A STUDY ON ANTIDIABETIC ACTIVITIES OF SOME INDIAN MEDICINAL PLANTS AND THEIR MODES OF ACTION

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BHAVNA SHARMA

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE - 247 667 (INDIA)

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INDIAN INSTITUTE OF TECHNOLOGY ROORKEE ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **A STUDY ON ANTIDIABETIC ACTIVITIES OF SOME INDIAN MEDICINAL PLANTS AND THEIR MODES OF ACTION** in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during the period from January 2005 to September 2008 under the supervision of Dr. Partha Roy, Assistant Professor, Department of Biotechnology & Dr. C. B. Majumder, Assistant Professor, Chemical Engineering Department, Indian Institute of Technology Roorkee, Roorkee, India.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(BHAVNA SHARMA)

This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

(Dr. C. B. Majumder)
Superviser

(Dr. Partha Roy)
Superviser

Date: 22.10.2008

The PhD Viva-Voce Examination of **Ms, Bhavna Sharma** Research Scholar has been held on .....

Signature of Supervisors

Signature of External Examiner

ABSTRACT

Diabetes mellitus is complex metabolic disorder resulting from either insulin insufficiency (type I diabetes) or resistance of body towards the insulin (type II diabetes) and it leads to an improper regulation of carbohydrate and lipid metabolism. Both these conditions result in increased concentration of glucose in the blood, which in turn damages many of the body's systems. The major chronic complications associated with diabetes include retinopathy, neuropathy, nephropathy and artherosclerotic vascular disease. Diabetes is a multifunctional disease leading to several complications and therefore, demands a multiple therapeutic approach. Although several options for diabetes treatments are available in market, they are associated with various side effects and resistance of body towards their pharmacological effects. Hypoglycemia, weight gain, gastrointestinal disturbances, liver toxicity etc. are some of the associated side effects. Further the complex pathophysiology of this disorder disgraces the use of unidirectional and single drug therapeutic approach in the management of diabetes. In contrast, traditional medicine follows a holistic approach, its prime focus being activation of the body's defense, protective and repair mechanisms.

Although traditional medicines have the immense potential to cure most of the disease, yet this system of medicine had not achieved the respect it deserved in the current scientific community. The prime reason of this situation is the lack of proper standardization and authentication of the herbal extracts. Due to increasing instances of drug associated secondary failures and body's resistance towards the conventional therapy measurements, traditional medicinal system is gaining new thrust. It follows a

holistic approach including medicinal preparations containing herbs and other organic and inorganic materials for the treatment. The ancient texts of India, China, Egypt, Rome and Greek system of medicines are replete with mention of medicinal plants. Currently 80% of the world population depends on plant derived medicines for the first line of primary health care.

The present thesis work is an attempt to validate antidiabetic, antihyperglycemic and hypolipidemic components of some of the Indian medicinal plants using various *in vitro* and *in vivo* approaches. Several medicinal plants were screened for their antidiabetic potential and further fractions rich in particular group of compounds were analyzed for their detailed mechanism of action. Two antidiabetic compounds were purified and their detailed mode of action was studied using *in vivo* and *in vitro* techniques. Flavonoids rich fraction of *Eugenia jambolana* and alkaloids rich fraction of *Capparis decidua* were used for the detailed analysis. Apart from these enriched fractions two potential antidiabetic and antihyperglycemic compounds *guggulsterone* and *pterostilbene* were isolated from *Commiphora mukul* and *Pterocarpus marsupium,* respectively. Detailed analysis of these compounds using *in vitro* and *in vivo* techniques was undertaken in order to prove the efficacy of these two compounds for the treatment of diabetes mellitus.

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PAPERS PUBLISHED/ COMMUNICATED OUT OF THIS THESIS WORK

1. **Sharma Bhavna**, Satapathi Santosh Kumar and Roy Partha. 2007. Hypoglycemic and antihyperglycemic effect of *Aegel marmelos* (L.) leaf extracts on streptozocin induced diabetic mice. *International Journal of Pharmacology*, 3:444-452.

2. **Sharma Bhavna**, Gunda Viswanath, Salunke Rajani and Roy Partha. 2008a. Effects of flavonoid-rich extract from seeds of *Eugenia jambolana* (L) on carbohydrate and lipid metabolism in diabetic mice. *Food Chem.*, 110:697-705.

3. **Sharma Bhavna**, Balomajumder Chanderjit. and Roy Partha. 2008b. Hypoglycemic and hypolipidemic effects of flavonoid rich extract from *Eugenia jambolana* seeds on streptozotocin induced diabetic rats. *Food and Chem. Toxicol.*, 46(7):2376-2383.

4. **Sharma Bhavna**, Salunke Rajani, Kumar Vikas, Balomajumder Chanderjit and Roy Partha. Hypoglycemic and hypolipidemic effects of alkaloids rich fraction of *Capparis decidua* seeds on streptozotocin induced diabetic mice." *Journal of Ethnopharmacology*. **(submitted).**

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6. **Sharma Bhavna,** Balomajumder Chanderjit and Roy Partha. Study on antidiabetic potential of *Guggulsterone* from *Commiphora mukul*. **(submitted).**

7. **Sharma Bhavna,** Balomajumder Chanderjit and Roy Partha. *Pterostilbene*: understanding of antidiabetic nature of *Pterocarpus marsupium*. **(submitted).**

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ABBREVIATIONS USED

µg	Microgram
µm	Micrometer
4-AAP	4-Aminoantipyrine
ACC	Acetyl CoA Carboxylase
Acrp-30	Adipocyte Complement-Related Protein-30
ADA	American Diabetes Association
ADP	Adinosine Diphosphate
AGEs	Advanced Glycation Endproducts
ALT	Alanine Amino Ttransferase
AMPK	Adinosin Monophosphate Activated Protein Kinase
aP2	Adipocyte P2 Enhancer
APC	Antigen-Presenting Cells
aPKC	a Typical Protein Kinase C
ATP	Adinosine Triphosphate
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
bp	Base Pair
BSA	Bovine Serum Albumin
cAMP	Cyclic AMP
CAT	Catalase
cDNA	Complementary DNA
CHOD-PAP	Cholestrol Oxidase - Phenyl Ampyrone

CoA	Co Enzyme A
DEPC	Diethyl Pyrocarbonate
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DNase	Deoxyribonuclease
DPP	Diabetes Prevention Program
DPP-IV	Dipeptidyl Peptidase-IV
DPS	Diabetes Prevention Study
DTT	Di thiothritole
EDTA	Ethylene Diamine Tetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked ImmunoSorbent Assay
eNOS	Nitric Oxide Synthase
ER	Endoplasmic Reticulum
EtOAc	Ethyl Acetate
FATP	Fatty Acid Transport Protein
FBG	Fasting Blood Glucose
FBP	Fructose 1-6 Biphosphatase
FBS	Fetal Bovine Serum
FFA	Free Fatty Acids
Fig.	Figure
FKOγ	Fat PPARγ Knock-Out
FOXO	Forkhead Members Of The Class O

g	Gram
G3P	Glycerol-3-Phosphate
G6Pase	Glucose-6-Phosphatase
GAD	Glutamic Acid Decarboxylase
GC-MS	Gas Chromatography Mass Spectrometry
GIP-1	Gastric Inhibitory Peptide
GK	Glucokinase
GLP-1	Glucagon-Like Peptide-1
Glut	Glucose Transporter
GO	Glucose Oxidase
GOD/POD	Glucose Oxidase Peroxidase
GPCRs	G-Protein-Coupled Receptors
GPO	Glycerophosphate Oxidase
GSK	Glycogen Synthase Kinase
GSK-3	Glycogen Synthase Kinase-3
GTT	Glucose Tolerance Test
GTT	Glucose Tolerance Test
H_2O_2	Hydrogen Peroxide
HBSS	Hank's Balanced Salt Solution
HDL	High Density Lipoprotein
HFD	High Fat Diet
HK	Hexokinase
HPLC	High Performance Liquid Chromatography

h	Hour
HSL	Hormone-Sensitive Lipase
HSP	Heat Shock Protein
I	Insulin
IAPP	Islet Amyloid Polypeptide
IBMX	Isobutyl-3-Methylxanthine
ICA'S	Cytoplasmic Islet Cell Autoantibodies
IDDM	Insulin Dependent Diabetes Mellitus
IDL	Intermediate Density Lipoprotein
IFN-γ	Interferon Gamma
IGF-2	Insulin-Like Growth Factor-2
IKK	IκB Kinase
IL-6	Interleukin-6
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
JM	Juxtamembrane Domain
kD	Kilo Dalton
Kg	Kilogram
KRB	Krebs Ringer Bicarbonate
KRPH	Krebs Ringer Phosphate HEPES
l	Litre
L1	Leucine-Rich Repeat Domain-1
L2	Leucine-Rich Repeat Domain-2

LCMS	Liquid Ghromatography Mass Spectrometry
LDH	Lactate Dehydrogenese
LDL	Low Density Lipoprotein
LDM	Low Density Microsome
LN2	Liquid Nitrogen
LPL	Lipoprotein Lipase
LPO	Lipid Peroxidase
m	Meter
M	Molarity
MAPK	Mitogen Activating Protein Kinase
MC3	Muscarinic-3
MD	Mild Diabetes
MES	2-(N-morpholino) Ethane Sulfonic acid
mg	Milligram
MHC	Major Histocompatibility Complex
Min	Minutes
ml	Millilitre
mM	Millimole
mRNA	Messenger Ribonucleic acid
MT	Muscle Tissue
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFA	Mono-Unsaturated Fatty Acids
MW	Molecular Weight
NAD	Nicotinemide Adinine Dinucleotide

NADH	Nicotinemide Adinine Dinucleotide Hydrogen
NASH	Non-Alcoholic Steato Hepatitis
NBT	Nitroblue Tetrazolium
NCBI	National Center for Biotechnology Information
NF-kβ	Nuclear Factor-kβ
ng	Nanogram
NIDDM	Non Insulin Dependent Diabetes Mellitus
NO	Nitric Oxide
NPH	Neutral Protamine Hagedorn
°C	Degree Centigrade
OGTT	Oral Glucose Tolerance Test
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	phosphate Buffered Saline
PC2	Prohormone Convertase Enzyme-2
PCR	Polymerase Chain Reaction
PDE3	Phosphodiesterase 3
PDK	PI3K-Dependent Serine/threonine Kinases
PEPCK	Phosphoinolpyruvate Carboxikinase
PFK	Phospho-Fructokinase
PGC-1	PPAR Gamma Co-activator 1
PH	Pleckstrin Homology
PI	Phospho-Inositol
PI3K	Phosphoinositol 3 Kinase

PIP3	Phosphoinositol 3 Phosphatase
pNPP	Para- nitrophenyl Phosphate
PO	Peroxidase
PPAR	Peroxisome Proliferator Activated Receptor Gamma
PP-Cell	Pancreatic Peptide- Cell
PPG	Post Prandial Glucose
PPRE	Peroxisome Proliferator–Response Elements
PTB	Phosphotyrosine Binding
PTP-1BE	Protein Tyrosine Phosphatase-1 beta
PUFA	Poly-Unsaturated Fatty Acids
PVDF	Polyvinylidene Difluoride
PYK	Pyruvate Kinase
RNase	Ribonuclease
rpm	Rotation Per Minute
RT-PCR	Reverse Transcriptase –Polymerase Chain Reaction
RXR	Retinoid-X Receptors
SD	Sever Diabetes
SDS	Sodium Dodecyl Sulphate
Sec	Second
S.E.M.	Standard Error of Mean
SOD	Superoxide Dismutase
SREBP	Sterol Regulatory Eelement-Binding Protein
STAT	Signal Transducers and Activators of Transcription

STZ	Streptozotocin
TAE	Tris Acetate
TBH-3	3-Hydroxy-2,4,6-Tribomobenzoic Acid
TC	Total Cholestrol
TCA	Tri Chloroacetic Acid
TE	Tris EDTA
TG	Triglyceride
TH	Tyrosine Hydroxylase
TK	Tyrosine Kinase
T_m	Melting Temperature
TM	Trans-Membrane Domain
TNF-α	Tumor Necrosis Factor-α
TNF-R	Tumor Necrosis Factor- Receptor
TZD's	Thiazolidinediones
U	Units
UV/Vis.	Ultraviolate/visible
VLDL	Very Low Density Lipoprotein
w/w	Weight/weight
α	Alpha
β	Beta
γ	Gamma
δ	Delta
ρ-HBS	ρ-Hydroxybenzene Sulfonate

CHAPTER- I

CHAPTER-I

INTRODUCTION

1.1 GENERAL INTRODUCTION

Physiological system is a complex and nonlinear interplay of different metabolic pathways and neuro-hormonal regulation. An effective balance and homeostasis of both these systems maintains a normal glycemic control along with other physiological conditions. Insulin is the prime hormone for the regulation of carbohydrate, lipid and protein metabolism. Under normal physiological conditions maintenance of normal glucose level depends on three events that need a right coordination: (1) Stimulation of insulin secretion; (2) Insulin mediated suppression of endogenous (glycogenolysis and gluconeogenesis) glucose production and (3) insulin mediated stimulation of glucose uptake by peripheral tissues, primarily muscle.

This normal glycemic control is lost in diabetic condition, either due to pancreatic β-cell destruction; hence a severe lack of insulin, known as type I diabetes; or resistance of body towards the metabolic effects of insulin, designated as type II diabetes.

In type I diabetic condition due to the lack of insulin all the insulin regulated metabolic pathways are affected i.e. production of endogenous glucose does not decrease after glucose ingestion and peripheral glucose uptake does not increase hence the result is hyperglycemia.

In patients with type II diabetes the rate of basal glucose production is excessive because of accelerated gluconeogenesis and also increased glycogenolysis. Muscle tissue in patients with type II diabetes is resistant to insulin (Defronzo, 1981; Bonadonna et al., 1996). Defects in insulin receptor function, insulin receptor signal transduction pathway,

glucose transport and phosphorylation, glycogen synthesis and glucose oxidation contributes towards muscle insulin resistance. Excessive endogenous glucose production and lack of glucose uptake from peripheral tissue contributes almost equally to the excessive postprandial hyperglycemia. Under both the condition the ultimate result is hyperglycemia and body's inability to utilize glucose as the primary energy source, which finally results in severe diabetic complications like, neuropathy, nephropathy, macro and micro vascular complications such as, neuropathy, nephropathy, macro and microvascular complications, like retinopathy, amputation and cardiovascular disorders in association with oxidative stress and hyperlipidemia.

For type I diabetic patient's insulin therapy is the only remedy. The exogenous supply in form of injection or suspension regulates the metabolic machinery for an improved glycemic control. In contrast with type I diabetic condition, patients suffering from type II diabetes have multiple treatment options available depending upon the nature of the problem, for example α-glucosidase inhibitors, delays the carbohydrate digestion and prevents the acute elevation of post prandial glucose level (Dimitriadis et al., 1982; Lebovitz, 1997). Biguanidine and thiazolidienedione enhances peripheral insulin sensitivity by direct and indirect methods and enhances peripheral glucose uptake while sulphonylurea class of members increase insulin secretion (Defronzo, 1999). Other than these available drugs, Protein-tyrosine phosphatase-1B (PTP-1B) inhibitors, Dipeptidyl peptidase-IV (DPP-IV) inhibitors and incretins are also available as therapeutic options.

Although several options for diabetes treatments are available in market as stated above, yet incidences of diabetes are in an increasing trend. Presently this disease affects

5% of the total world population (Dewanjee et al., 2008; Mukherjee et al., 2006) and it is expected that by the year 2010 the total number of people affected worldwide would be close to 293 million (Rao et al., 2004). At present drugs used for the management of diabetes are associated with side effects and resistance of body towards the pharmacological effects of these drugs. Hypoglycemic comma, diarrhea, obesity, abdominal discomfort, excessive flatulence, anorexia, edema, anemia and pulmonary edema etc. are just few side effects of conventional antidiabetic drugs (Cheng and Fantus, 2005; Bailey and Day, 2003; Defronzo, 1999).

Increasing incidence of diabetes mellitus and socio-economical issue associated with it has led to a thrust towards the discovery of new drugs and techniques for the treatment of this disease. The complex pathophysiology of this disorder disgraces the use of unidirectional and single drug therapeutic approach in the management of diabetes (Tiwari and Madhusudana, 2005). Also the available therapeutic measurements of conventional therapies emphasize more on a particular symptom or target, in spite of considering it as an error in a complex and interrelated physiological system.

Due to above mentioned drawbacks, associated secondary failures and body's resistance towards the conventional therapy measurements, traditional medicinal system is gaining new thrust. Traditional medicinal system is the product of thousands years of practices and experience and follows a holistic approach which believes in the activation of body's repair mechanism and defense system (Wagner, 2005). Under traditional therapeutic approach a combination of life style management which includes diet control and adequate exercise and medicinal preparation containing herbs and other organic and inorganic materials is applied for the treatment.

Medicinal plants and their parts are the major components of the medicinal preparations. About 500 plants with medicinal properties are mentioned in ancient texts of Indian, Chinese, Egyptician, Roman and Greek system of medicines. Currently 80% of the world population depends on plant derived medicines for the first line of primary health care; even in United States, 25% of pharmaceutical prescriptions contain at least one plant derived product. During 1950-1970 only, approximately 100 new plants based new drugs were introduced in USA drug market, including desepridine, rescinnamine, reserpine and vinblastine etc (Samy and Gopalakrishnakone, 2007). Several hypoglycemic, hypolipidemic and antihyperglycemic compounds have been isolated and analyzed for their therapeutic potential (Mukherjee et al. 2006; Modak et al., 2007; Gilbert, 2000; Tang et al., 2008). These compounds have not only proven their effectiveness but at the same time in most of the cases they are free of side effects.

Although traditional medicines have proven their efficacy, even using modern scientific approaches in many cases, still the traditional system of medicine could not achieve its deserved importance in scientific community. The reason behind this is the lack of proper systematic regulation for these traditional medicines. In the absence of proper regulation, the qualitative and quantitative standardization and authentication of a particular extract or herbal mixture is questionable. The safety of these herbal preparations pose a major concern as several studies have shown these herbal preparations to contain heavy metal contaminants and certain undesired/hazardous compounds (Itankar et al., 2001; Saper et al., 2004).

Initial purification of phytochemicals to eliminate any kind of detrimental components prior to their use in herbal preparation, is the right approach to handle this

problem. Purification followed by a sequential activity based analysis along with the identification as well as standardization of active principles using certain modern analytical procedures can clarify the doubts of scientific community and prove the efficacy of these herbal drugs.

1.2 AIMS AND OBJECTIVES

The present thesis work is an attempt to validate antidiabetic, antihyperglycemic and hypolipidemic components of some of the Indian medicinal plants using various *in vitro* and *in vivo* approaches. Several medicinal plants were screened for their antidiabetic potential and further fractions enriched for a particular group of compounds were analyzed for their detailed mechanism of action. Also two antidiabetic compounds were purified and their detailed mode of action was studied using *in vivo* and *in vitro* techniques.

Nine medicinal plants: *Tinospora cordifolia*, *Phyllanthus emblica*, *Murraya koenigii*, *Capparis decidua*, *Eugenia jambolana*, *Aegle marmelos*, *Pterocarpus marsupium*, *Eucalyptus globules* and *Commiphora mukul* were screened for their antidiabetic activity using streptozotocin (STZ) induced diabetic mice, and on the basis of preliminary screening five most potential plants *Aegle marmelos, Eugenia jambolana, Capparis decidua, Commiphora mukul* and *Pterocarpus marsupium* were taken further for detailed analysis and fractionation in order to identify the active constituent. Out of these above stated five plants aqueous extract of *Aegle marmelos* leaves, flavonoids rich fraction of *Eugenia jambolana* and alkaloids rich fraction of *Capparis decidua* were used for the detailed analysis. Apart from these enriched fractions two potential antidiabetic and antihyperglycemic compounds *guggulsterone* and *pterostilbene* were isolated from

Commiphora mukul and *Pterocarpus marsupium,* respectively. Detailed analysis of these compounds using *in vitro* and *in vivo* techniques has proven the efficacy of these two compounds for the treatment of diabetes mellitus. Overall, this study attempted to achieve the following objectives –

1. To extract the polar and non-polar antidiabetic compounds from the plant parts.
2. To screen the medicinal plants for their antidiabetic potential.
3. Further extraction of some selected plants to prepare enriched fractions, in order to study the detailed mechanism of action of antidiabetic compounds using *in-vitro* and *in vivo* approaches.
   - 3.1 Glucose tolerance test and fasting blood glucose levels to measure the insulin sensitivity.
   - 3.2 Enzyme assays to study the effects on carbohydrate regulatory enzymes.
   - 3.3 Gene expression and western blot analysis to measure the effects of test extracts and compounds.
   - 3.4 Lipid profile and serum insulin profile analysis to measure hypolipidemic and antidiabetic effects
   - 3.5 Histopathological analysis to examine the effects of plant extracts and compounds on pancreas, liver, and adipose.
4. HPLC analysis of the plant extracts and isolated compounds for the identification of the active components demonstrating antidiabetic effects.

5. Finally isolation of two potent antidiabetic molecules and detailed study of the mechanism of action for enriched fractions and isolated compounds using different *in vitro* assays.

5.1 Glucose uptake assay

5.2 Insulin secretion from isolated pancreatic islets

5.3 PPAR α and γ transactivation assay

5.4 Adipocytes differentiation assay

5.5 PTP-1B inhibition assay

5.6 α-glucosidase inhibition assay

CHAPTER- II

CHAPTER-II

LITERATURE REVIEW

2.1 INTRODUCTION

Diabetes mellitus is a heterogeneous group of metabolic disorders characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both. When fully expressed, diabetes is characterized by fasting hyperglycemia, but the disease can also be recognized during less overt stages, most usually by the presence of glucose intolerance. Diabetes mellitus has become an international healthcare crisis that requires new approaches for prevention and treatment. During last 20 years, the prevalence of diabetes has increased dramatically in many parts of the world. Although genetic factors play a role in the etiology, especially of type II diabetes, the growing problem of obesity that parallels improved economic status in some developing countries is a major environmental factor in this epidemic of diabetes (WHO, 1999). On the other hand, in many parts of the developing world, low birth weight and maternal malnutrition during pregnancy may be a major factor underlying the insulin resistance syndrome and thus in an increased risk of diabetes in later life.

Depending upon the nature of the disease, insulin and certain synthetic drugs like sulphonylureas, biguanidines and acarbose are widely used in its treatment. In recent years, evidence of cases of "insulin resistance" and the occurrence of side effects from prolonged administration of conventional drugs have triggered the search for safe and effective alternatives. The biggest problem with the conventional drug therapy is that it considers only the symptoms or a specific target for that disease or disorders (ex. the

tumor cell or the pathogenic microorganism) but not the root cause of a particular disease. On the other hand, traditional medicines are much safer and follow a holistic approach, its prime focus being activation of the body's defense, protective and repair mechanisms (Wagner, 2005). In spite of having enormous potential to cure many diseases, traditional medicines could not achieve the expected popularity among the scientific community due to some or other reasons. This necessitates a rigorous scientific validation of any drug for the treatment of a particular disease. The probable reasons for lesser use of these medicines are lack of authentication, identification and qualitative as well as quantitative standardization and at the same time the presence of heavy metal contaminants and undesired compounds which impose hazardous effect on the physiological system (Samy and Gopalakrishnakone, 2007; Vaidya and Devasagayam, 2007; Wagner, 2005)

The only way to surmount these problems is to adopt for initial purification of medicinal products so as to eliminate any kind of detrimental components before using in herbal preparation, followed by a sequential activity based analysis along with the identification as well as standardization of active principles using certain modern analytical procedures. Based on the objectives of the present thesis, major emphasis has been given on the understanding the role of medicinal plants in treatment and management of diabetes mellitus.

2.2 ENDOCRINOLOGY OF DIABETES MELLITUS

Hormonally this metabolic disorder is caused by the interplay of several hormones, enzymes and organs. This part of the review will discuss each one of them sequentially.

2.2.1 Pancreas

The human pancreas, a racemose, lobulated gland that weighs about 100 g, and 13 to 25 cm long, is located just caudal to the stomach and opposite the liver along the gastrointestinal tract (Fig. 2.1). Its head (proximal portion) lies in the crook of the duodenum, and its tail (distal portion) contacts the spleen. The pancreas consists primarily of exocrine, endocrine, and ductal cell types that together with a blood supply coordinate to regulate nutritional equilibrium (Bockman, 1993). The exocrine function of the pancreas is carried out by acinar cells, which secretes digestive enzymes and other non-enzymatic components into the duodenum. The endocrine function of the pancreas is carried out by the islets of Langerhans. These are compact, spheroid clusters of cells scattered throughout the more abundant exocrine tissue. Islets consist of four different cell types that secrete hormones into the bloodstream to regulate glucose homeostasis. Islets are therefore penetrated by a network of fenestrated microvasculature and nerve fibers that help administer this regulation (Henderson and Daniel, 1979; Handerson and Moss, 1985; Sundler and Bottcher, 1991).

2.2.1.1 Exocrine Pancreas

The exocrine pancreas made up of acinar cells constitutes the bulk of pancreatic tissue. These cells are pyramidal in shape with a spherical nucleus, basophilic cytoplasm and an extensive secretary apparatus at the apical end, including numerous zymogen granules (Yamamoto and Kataoka, 1985). The base of each pyramidal acinar cell lies on the basement membrane which surrounds each acinus. Beneath this basement membrane there is a rich capillary network. Zymogen granules contain digestive enzymes, including amylases, proteases, nucleases, and lipases, which are secreted into the duodenum.

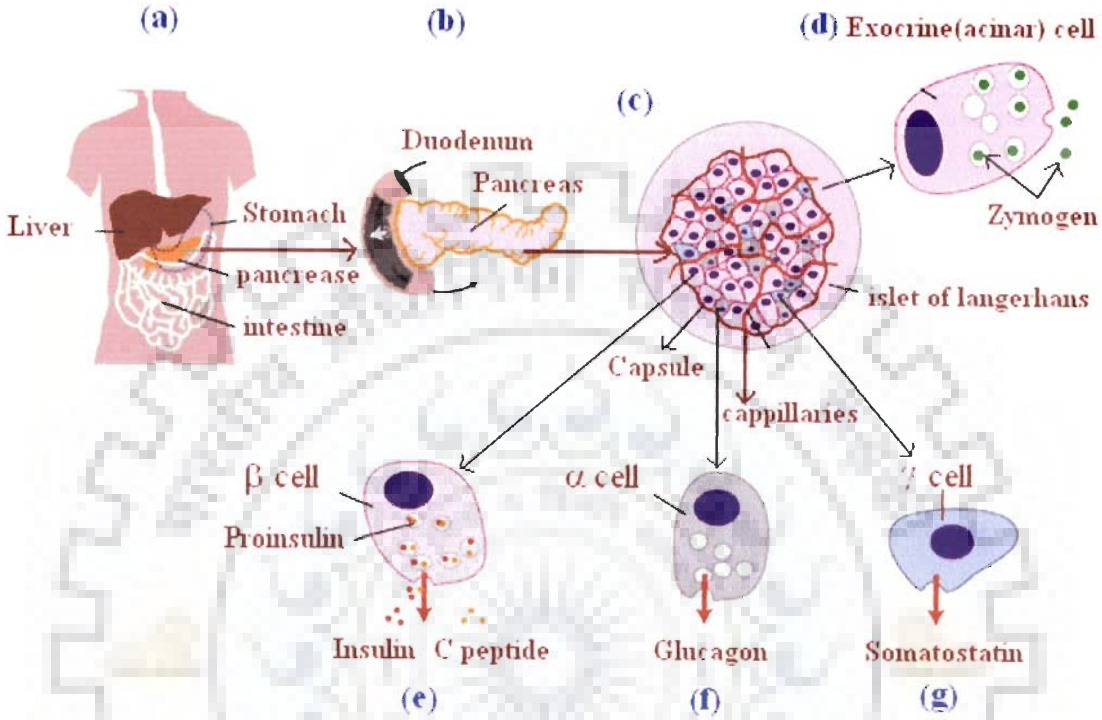


Figure 2.1. (a) The pancreas located in the upper abdomen, close to the liver and behind the stomach. (b) The pancreas secretes digestive enzymes via its duct into the duodenum. (c) An islet is a collection of endocrine cells supplied by capillaries. A thin fibrous capsule separates them from the surrounding (d) Exocrine cells, which produce and secrete zymogen. The endocrine cells include: (e) β cells, which synthesizes proinsulin and is cleaved into insulin (stored in granules) and C peptide (f) α cells, which secrete glucagon and (g) δ cells, which secrete somatostatin.

Initially, these are produced and secreted as inactive proenzymes, which are then activated by limited proteolysis once they enter the digestive tract (Alumets et al., 1977; Bonner and Smith, 1994).

2.2.1.2 Endocrine Pancreas

The endocrine function of the pancreas is performed by a number of cell types in the islets of Langerhans identified by the German physician Paul Langerhans in 1869. There are four cell types found in pancreatic islets: α-cells, β-cells, δ-cells, and PP-cells (pancreatic peptide; also called γ-cells) (Fig. 2.1). β-cells, the major endocrine cell of the pancreas, constitute approximately 70-80% of the organ and secrete insulin, the insulin antagonist amylin, and other peptides (Guest et al., 1991). The α, δ, and PP-cells secrete glucagon, somatostatin, and pancreatic polypeptide, respectively.

2.2.1.2.1 β-cells

The β-cells are polyhedral and are usually well granulated having approximately 10,000 granules per cell with 250 to 300 nm diameter (Bonner and Smith, 1994). These cells constitute around 70 to 80% of the total islet content. There are two forms of insulin granules: (i) mature granules, that have an electron-dense core and a loosely fitting granule-limiting membrane with the appearance of a spacious halo and (ii) immature granules, with little or no halo, moderately electron-dense contents, and a clathrin coat. Immature granules are the major site of conversion from proinsulin to insulin (Dean, 1973). Other changes, such as the shedding of the clathrin coat, acidification of the granule contents, and crystallization of insulin, occur as the granule matures from a proinsulin-rich granule to an insulin-rich granule (Kuliawat and Arvan, 1994; Amherdt et

al., 1996; Mutkoski et al., 1987). In some species, the electron-dense core of the mature granule is visibly crystalline.

2.2.1.2.2 α-cells

The α-cells secretes counter regulatory hormone, glucagons. These cells are usually smaller and more columnar than the β-cells and well granulated, with granules 200 to 250 nm in diameter. Almost 15-20% of the total islets cells are glucagons producing α-cells. The granules are electron-dense with a narrow halo of less-dense material and a tightly fitting granule-limiting membrane.

2.2.1.2.3 δ-cells

The δ-cells are smaller than either α- or β-cells, well granulated, and are often dendritic in shape. They form 5 to 10% of total islets cells. The electron density of granules within a δ-cell varies greatly. Each granule is 200 to 250 nm in diameter, contains material of homogeneous and moderate density that fills the granule-limiting membrane. δ-cells secretes somatostatin hormone.

2.2.1.2.4 PP-cells

PP cells are pancreatic polypeptide producing cells in the islets of Langerhans. About 15%-20% of total islets cells are PP-cells. They contain secretary granules with variable shapes and electron density in different species. In humans the granules are elongated, extremely electron dense, and 120 to 160 nm in diameter whereas in dogs and cats, the granules are spherical, variable in electron density, and approximately 300 nm in diameter. Granules exhibit a distinct double membrane which is either slightly scalloped or smooth, conforming to a round, oval or evenly indented shape. The material is mostly

homogeneous in some granules, but also others may have more condensed parts of the matrix.

2.2.2 Insulin

Humans have a single insulin gene, located on chromosome 11, at 2p21 between the tyrosine hydroxylase (*THI*) gene and the insulin-like growth factor-2 (*IGF-2*) gene. The gene contains two introns, one interrupting the 5′ untranslated sequence and the second interrupting the sequence encoding the C-peptide. This general structure is conserved, with few exceptions, among the other mammalian insulin genes, as well as among the related IGF genes. The sequences upstream of the transcription start site comprise the insulin-gene promoter (Docherty et al., 1995 and Steiner and Chan, 1988) (Fig. 2.2). The promoter directs RNA polymerase II to the correct transcription start site, restricts insulin-gene transcription to the β-cell, and responds to metabolic, hormonal, and neural signals by modulating the rate at which insulin-gene transcription is initiated. Pancreatic β-cells synthesize insulin from a single-chain precursor of 110 amino acids termed *preproinsulin*. After translocation through the membrane of the rough endoplasmic reticulum (ER), the 24-amino-acid N-terminal signal peptide of *preproinsulin*, which is rich in hydrophobic residue and facilitates the penetration through rough endoplasmic reticulum, is cleaved rapidly to form *proinsulin* (Fig. 2.3). Thereafter, *proinsulin* undergoes appropriate folding so that the disulfide linkage between the A- and B-chains of insulin is correctly aligned. During conversion of human *proinsulin* to insulin, four basic amino acids and the remaining connector or C-peptide are removed by proteolysis.

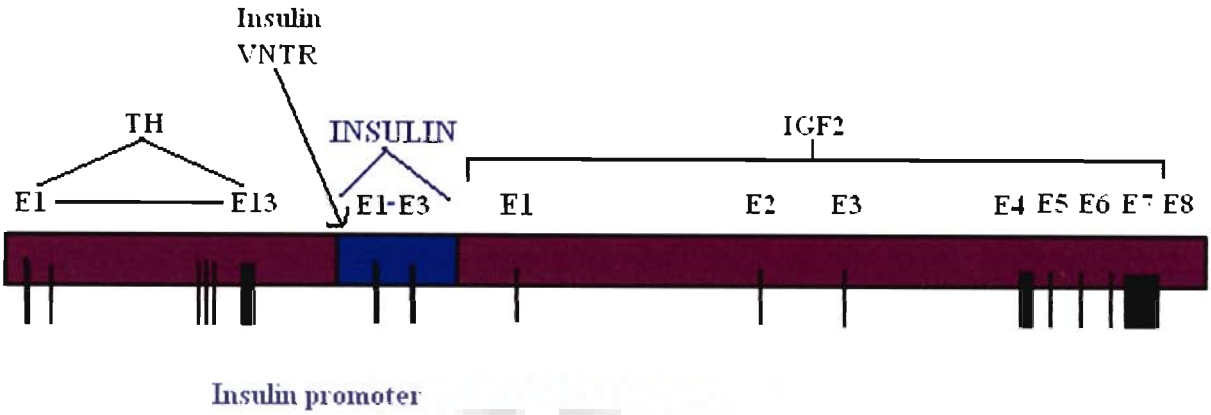


Figure 2.2. The human insulin gene (INS) and surrounding loci on chromosome number 11. IGF2, insulin like growth factor-2; TH, tyrosine hydroxylase.

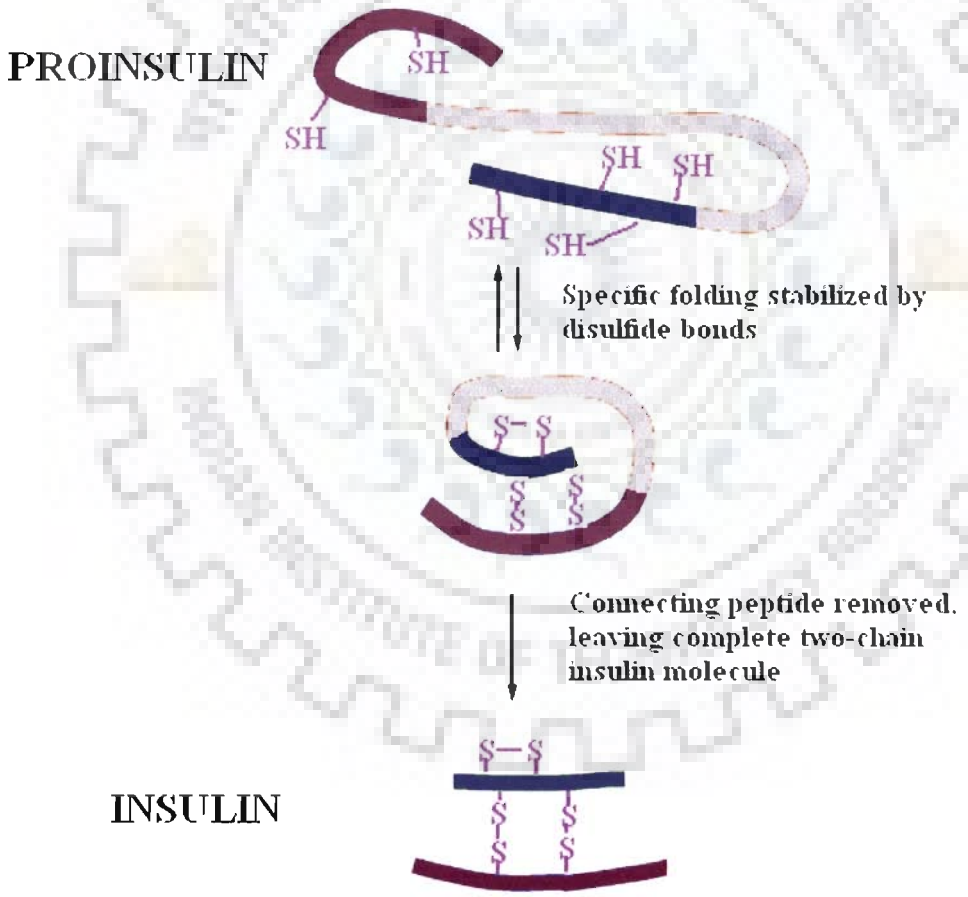


Figure 2.3. Conversion of proinsulin to insulin. Conversion occurs within acidifying secretory granules.

This gives rise to the A (21 amino acids) and B (30 amino acids) peptide chains of the insulin molecule (MW~ 57 D), containing one intra-subunit and two inter-subunit disulfide bonds (Campbell and Hellqvist, 1982; Rhode et al., 1994).

The combination of granule acidification and conversion of *proinsulin* to insulin provides the scenario for insulin crystallization within the mature granule. Insulin is known to be able to associate into dimers, and in the presence of Zn^{2+}, the dimers associates to form hexamers by the histidine residue at position 10 of the insulin B-chain (Amherdt et al., 1986). Crystallization depends on a high local concentration of insulin and Zn^{2+}, as well as an acidic milieu which are satisfied within the granule (Cutfield et al., 1980). The dense core of the secretary granule observed by electron microscopy is thus considered to be an insulin crystal (Hodgkin et al, 1972). The C-peptide does not co-crystallize with insulin to any significant extent and is therefore excluded from the crystal which is then found in the clear halo surrounding the dense core of granules (Orci, 1982; Swift et al., 1987) with other soluble granule constituents such as Prohormone Convertase Enzyme-2 and 3 (PC2 and PC3), chromogranin A, and Islet amyloid polypeptide (IAPP) (Beckers et al., 1987).

Two distinct Ca^{2+}-dependent endopeptidases, present in the islet cell granules and in other neuroendocrine cells are responsible for the conversion of *proinsulin* to insulin. These endoproteases, PC2 and PC3, have catalytic domains related to that of subtilisin (serine endopeptidase) and cleave at Lysine-Arginine or Arginine-Arginine sequences (Seal et al., 1992, Bennett et al., 1992). PC2 selectively cleaves at the C peptide-A chain junction. PC3 preferentially cleaves at the C-peptide-B chain junction but has some action at the A chain junction as well. Although there are at least two other members of

the family of endoproteases (PC1 and furin), PC2 and PC3 appear to be the enzymes responsible for processing *proinsulin* to insulin (Thomas et al., 1992; Fricker, 1988).

2.2.3 Regulation of insulin secretion and degradation

Insulin secretion is a tightly regulated process designed to provide stable concentrations of glucose in blood during both fasting and post feeding conditions. This regulation is achieved by the coordinated interplay of various nutrients, gastrointestinal hormones, pancreatic hormones, and autonomic neurotransmitters. Glucose, amino acids, fatty acids, and ketone bodies promote the secretion of insulin. Glucose is the principal stimulus for insulin secretion in human beings and is an essential permissive factor for the actions of many other secretagogues (Grapengiesser et al., 2004).

Insulin secretion occurs in a biphasic condition under the stimulation of glucose. The first phase reaches a peak after 1 to 2 min and is short-lived; the second phase has a delayed onset but lives for a longer duration. A rising plasma glucose concentration initiates a series of events that leads to the depolarization of pancreatic cell membrane. Glucose enters the β-cell by facilitated transport or diffusion, which is mediated by glucose transporter-2 (Glut-2), a specific subtype of glucose transporter, whereupon the sugar is phosphorylated to glucose-6-phosphate (G-6-P) by glucokinase (Grapengiesser et al., 2005). The increase in oxidizable substrate (glucose and G-6-P) enhances adenosine triphosphate (ATP) production, thereby increasing the ATP-adenosine diphosphate (ADP) ratio and inhibiting an ATP-sensitive K^+ channel. This decrease in K^+ conductance causes E_m to rise, opening a voltage-sensitive Ca^{2+} channel.

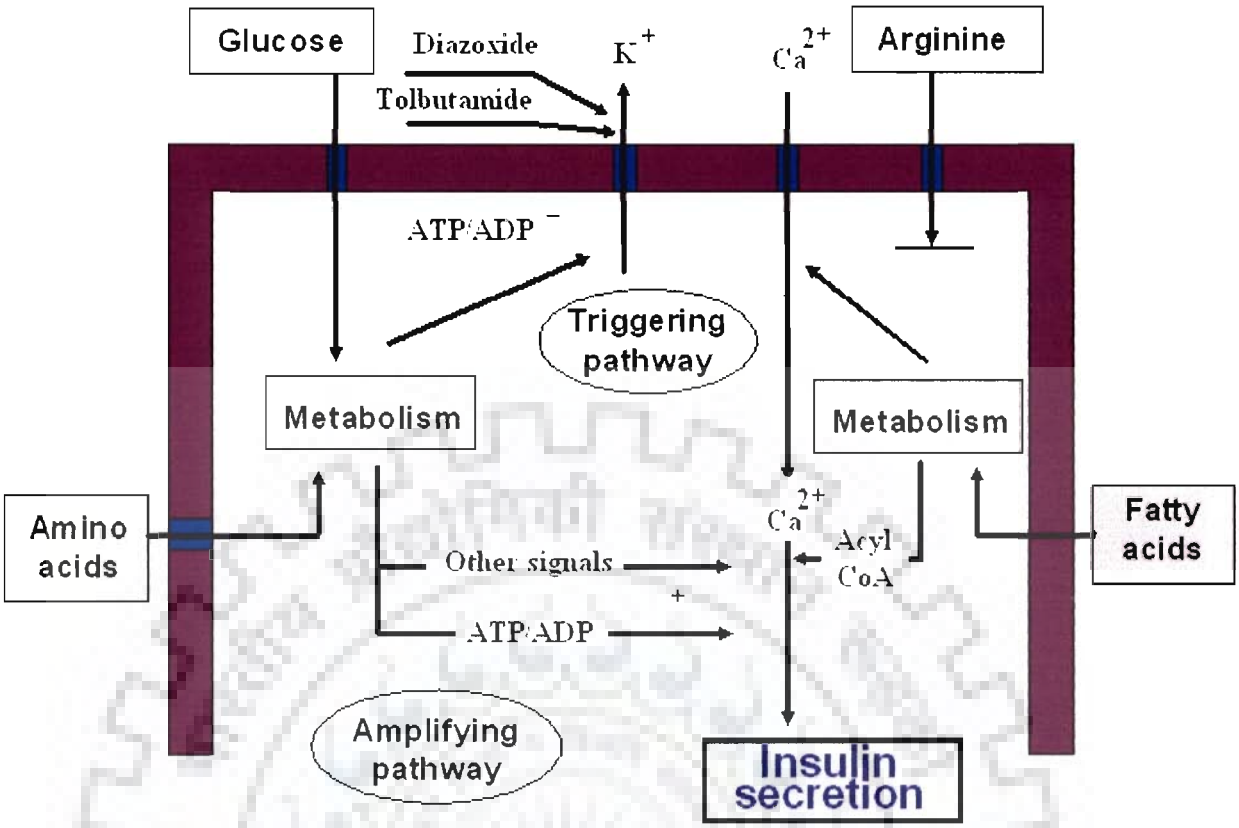


Figure 2.4. Schematic representation of the triggering and amplifying pathways of the stimulation of insulin secretion by glucose, and of the mode of action of other nutrients: +, stimulation; - inhibition; ATP, adenosine triphosphate; ADP, adenosine diphosphate; CoA, coenzyme A.

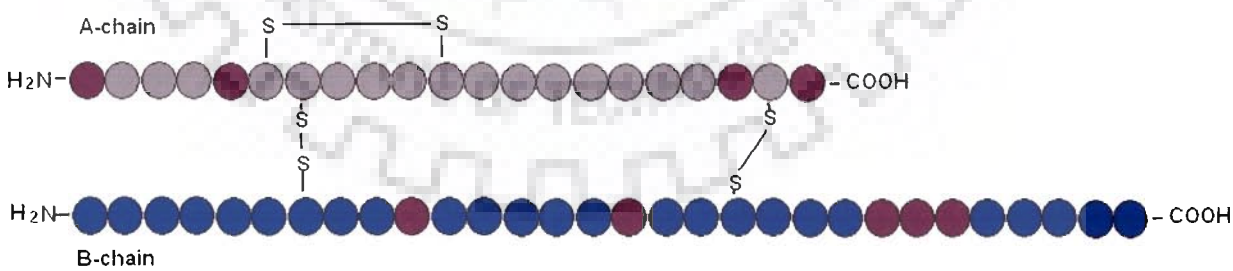


Figure 2.5. Structure of human insulin, insulin consists of two peptide chains which are connected by disulfide bonds.

In this situation intracellular Ca^{2+} acts as the insulin secretagogues (Fig. 2.4) (Grill and Cerasi, 1973; Tengholm and Gylfe, 2008). The half-life of insulin in plasma is about 5 to 6 min in normal subjects and patients with uncomplicated diabetes. Degradation of insulin occurs primarily in liver, kidney, and muscle (Emmanouel et al., 1983; Paugh et al., 1983). About 50% of the insulin that reaches the liver *via* portal vein is destroyed and never reaches the general circulation. Insulin is filtered by the renal glomeruli and is reabsorbed by the tubules, which also degrades it.

2.2.4 Insulin structure

Insulin is composed of two polypeptide chains that are linked to one another by disulfide bonds. The A- and B-chains of human, porcine, and bovine insulin like those of most other vertebrate insulin are composed of 21 and 30 amino acids, respectively. The two peptide chains are covalently linked to one another by two cystine disulfides, one between CysA7 and CysB7 and the other between CysA20 and CysB19. An additional intra chain disulfide connects cysteine A6 and A11 (Fig. 2.5).

Insulin structure is highly conserved in vertebrate evolution (Sanger, 1959). Out of the entire structures of insulin the most homologues regions include the positions of cysteine that form the disulfide bridges, the amino- and carboxyl-terminal regions of the A-chain, and certain hydrophobic residues at the carboxyl-terminus of the B-chain (Welsh et al., 1985). Mutations and chemical modifications at invariant or highly conserved positions generally diminish or abolish receptor binding potency and biological activity, emphasizing the importance of these positions in either creating or maintaining a three-dimensional structure suitable for receptor recognition (Brandenburg and Wollmer, 1980; Gammeltoft, 1984).

2.2.5. Insulin Receptor

The insulin receptor (IR) belongs to a subfamily of receptor tyrosine kinases and is a heterotetrameric membrane protein, consisting of two identical extracellular α-subunits (MW 135 kD) that bind insulin, and two identical transmembrane β-subunits (MW 95 kD) that have intracellular tyrosine kinase activity (Gautier et al., 1992; Ablooglu and Kohanski, 2001). The IR gene product is synthesized as a prepro-receptor, from which a 30 amino acid signal peptide is cleaved. The pro-receptor is then processed further, undergoing glycosylation, folding, and dimerization. In the Golgi apparatus, the dimerized amino acid chains are then cleaved into α- and β-subunits. In the mature receptor a subunit consist of 719-731 amino acids and the β-subunit is composed of 620 residue including 194 amino acids for the extracellular portion, a 23 amino acid trans-membrane domain and a 403 residue cytoplasmic extension (Fig. 2.6) (Kahn and White, 1988; Youngren, 2007).

The tertiary structure of α-subunit has not yet been fully determined. According to proposed model of Bajaj et al. (1987) two similar, independently folded domains, L1 and L2, each one of which form a β-pleated sheet flanked by α-helical structure. These extend from residues 1-119 and 311-428, respectively with an intervening cysteine rich domain from 115 -312. Cysteine rich regions of IR and epidermal growth factor receptor (EGFR) (approximately 150 amino acids residues with 24-26 cysteine) consisted of three repeats of eight cysteine residues (Bajaj et al., 1987). The disulfide bond within the cysteine rich residue provides the scaffolding to support the tertiary structure of the L1 and L2 domains which confer high affinity for insulin. The high affinity binding require only one insulin molecule to bind both α-subunit, contacting the L1 domain of one and L2 domain of the other.

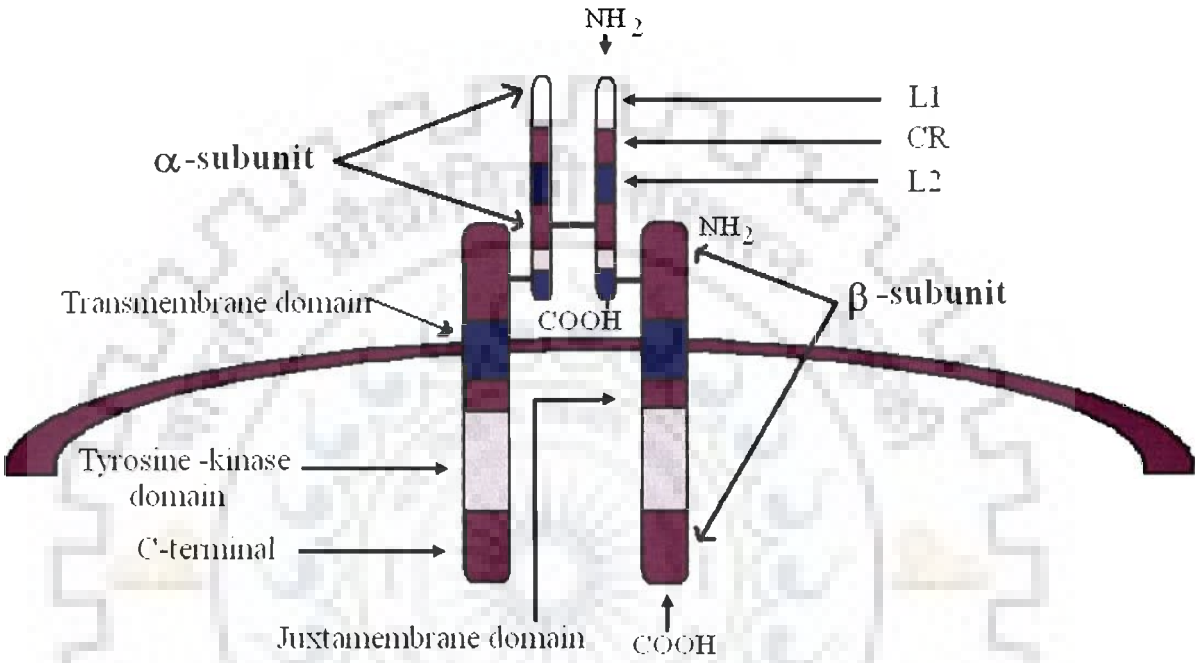


Figure 2.6. Structure of human insulin receptor; Leucine-rich repeat domains are denoted as L1 and L2, the cysteine-rich region as CR domain.

The β-subunit of insulin receptor contains several functionally distinct domains. The IR is anchored to the plasma membrane by the trans-membrane domain (TM). There are seven tyrosine residues in the cytoplasmic domain of the β-subunit that have been identified as autophosphorylation sites. The juxtamembrane domain (JM) contains two tyrosine residues that are autophosphorylated in response to insulin binding. Of these, tyrosine 972 is instrumental in binding the phosphotyrosine binding (PTB) domains of insulin receptor substrate-1 (IRS-1) and Shc (SHC-adaptor protein). The tyrosine kinase domain (TK) contains the enzymatic active site of the molecule, as well as the ATP-binding site and three key tyrosines (1158, 1162, and 1163) which must be phosphorylated to produce full kinase activity of the IR (Langlois and Olefsky, 1994). Phosphorylation of tyrosines in different regions regulates separate aspects of IR function (Fig. 2.6).

2.2.6 Insulin Signaling

The binding of insulin to the extracellular agonist binding domain of IR results in a conformational change in juxta-positioned cytosolic β-subunit. This conformational change leads to a depression i.e. activation of the kinase activity in the β-subunit. This is followed by initial autophosphorylation, then a trans-phosphorylation and finally leading to the increase in total kinase activity (Avruch, 1998) (Fig. 2.7).

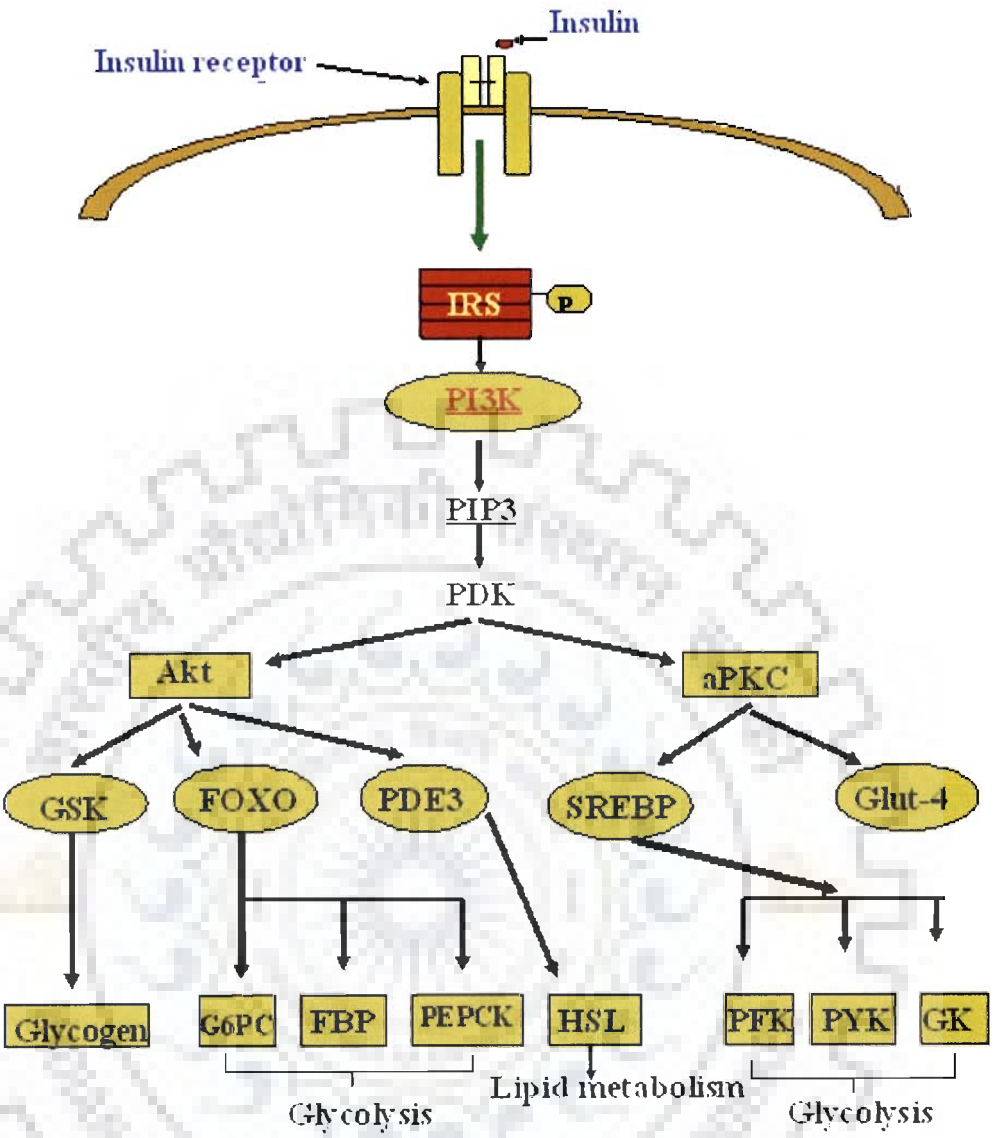


Figure 2.7. Mechanism of insulin signaling and regulation of metabolic pathways; IRS, Insulin receptor substrate; PI3K, Phosphoinositol 3 kinase; PIP3, Phosphoinositol 3phosphatase; PDK, PI3K-dependent serine/threonine kinases; aPKC, a typical protein kinase C; GSK, Glycogen synthase kinase; FOXO, forkhead box O, PDE3, phosphodiesterase 3; SREBP, Sterol regulatory element-binding protein; Glut-4, Glucose transporter protein-4; G6PC; Glucose 6 phosphatase, FBP, Fructose 1-6 biphosphatase, PEPCK, Phosphoinolpyruvate carboxikinase; HSL, Hormone-Sensitive Lipase; PFK, phospho-fructokinase; PYK, Pyruvate kinase; GK; Glucokinase.

This phosphorylation reveals the binding sites for recruitment of Src homology-2(SH2) or PTB domain containing protein, including IRS 1-6 and Shc. IRS protein following recruitment and phosphorylation amplify signaling through recruitment of numerous other adaptor, activator proteins and kinases via their phosphorylated tyrosine motif for example phosphoinositol-3-kinase (PI3K), Growth factor receptor-bound protein-2 (Grb-2), adaptor protein CT-10 related kinase (Crk), Nck, Fyn. IRS activates PI 3-kinase, which is essential for stimulation of glucose transport and phosphorylation by activating glucose transporter Glut-4 and hexokinase II (HKII) (Taniguchi et al., 2006; Avruch, 1998). PI3K also mediates insulin-induced increases in nitric oxide (NO) in endothelial cells (Seino et al., 1989). PI3K pathway is mainly involved in mediating the metabolic effects of insulin, such as glucose transport, glycogen and protein synthesis, ion and amino acid transport, and lipid metabolism (Kim and Novak, 2007). Insulin receptor substrate-1 (IRS-1) associated PI3K pathway also mediates insulin-induced lipolysis in adipocytes via inhibition of hormone sensitive lipase (HSL). Mitogen-activated protein (MAP) kinase pathway is another insulin signalling pathway that is necessary in regulating cell proliferation, differentiation and apoptosis (Youngren, 2007; Saltiel and Kahn, 2001).

2.2.7 Cellular actions of insulin

Insulin elicits a remarkable array of biological responses. The important target tissues for regulation of glucose homeostasis by insulin are liver, muscle and fat, in addition it also exerts potent regulatory effects on other cell types as well. Insulin is the primary hormone responsible for controlling the uptake, use and storage of cellular nutrients. Insulin's anabolic actions include the stimulation of intracellular use and

storage of glucose, amino acids, and fatty acids, whereas it inhibits catabolic processes such as the breakdown of glycogen, fat, and protein. It accomplishes these general purposes by stimulating the transport of substrates and ions into cells, promoting the translocation of proteins between cellular compartments, activating and inactivating specific enzymes, and changing the amounts of proteins by altering the rates of gene transcription and specific mRNA translation.

2.2.7.1 Glucose metabolism

Glucose provides a major energy supply for cells. The blood glucose concentration is maintained within narrow limits (4-7 mM) by the regulated production of glucose by liver from glycogenolysis and gluconeogenesis, which is counterbalanced by the glucose absorption from the intestine and regulated peripheral clearance of glucose by tissues such as skeletal muscle, adipose tissue, and the splanchnic bed (intestine and liver tissues are collectively referred as splanchnic bed), including the liver (DeFronzo, 1981; Yki-Jarvinen, 1993).

The metabolic effect of insulin on cellular level is via PI3K. The PI3K is a heterodimeric enzyme consisting of p85 regulatory subunit as well as p110 catalytic subunit. Activated PI3K specifically phosphorylates phospho-inositol (PI) substrates to produce PI(3)P, PI(3,4)P_2, and PI(3,4,5)P_3. These phospholipids acting as second messanger recruit the PI3K-dependent serine/threonine kinases (PDK1) and Akt from cytoplasm to the plasma membrane by binding to the "pleckstrin homology domain" (PH domain) of kinases. Lipid binding and membrane translocation leads to conformational changes in Akt that is subsequently phosphorylated on Thr 308 and Ser 473 by PDK1. Phosphorylation by PDK1 leads to full activation of Akt (Saltiel and Kahn, 2001; Kim

and Novak, 2007; Pessin and Salteil, 2000). Activated Akt phosphorylates and regulates the activity of many downstream proteins involved in multiple aspects of cellular physiology (Fig. 2.7).

The liver produces glucose by glycogenolysis or via *de novo* gluconeogenesis mainly from lactate, alanine, pyruvate and glycerol (Wahren et al., 1972; Joseph et al., 1996; Patel, 1989). Insulin decreases endogenous glucose production primarily by suppressing glycogenolysis and by decreasing the glucose synthesis via gluconeogenesis. Insulin dramatically inhibits the transcription of the gene encoding phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting step in gluconeogenesis. Insulin also decreases transcription of the genes encoding fructose 1, 6-bisphosphatase (FBP1) and glucose 6-phosphatase (G6Pase) and increases transcription of those encoding glycolytic enzymes such as glucokinase (GK) and pyruvate kinase and lipogenic enzymes such as fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC). Several transcription factors like Hepatic nuclear factor-3-4 (HNF3-4), Sterol regulatory element-binding protein-1c (SREBP-1c) and the forkhead transcription factor (FKHR; also known as FOXO1) play significant role in this insulin-mediated regulation (Sugiyama et al., 1999; Chakravarty et al., 2001; Kruger, 2001).

Activated Akt phosphorylates and regulates glycogen synthase kinase-3 (GSK-3). This inactivates GSK-3, resulting in a decrease in the phosphorylation of glycogen synthase (GS) and an increase in its active state which leads to increased glycogen synthesis. Insulin can also influence glucose metabolism indirectly via its anti-lipolytic effect. Acute elevations of plasma free fatty acids (FFA) increase gluconeogenesis and

lower glycogenolysis, while acute lowering of FFA acts reciprocally where endogenous glucose production remains unchanged. This phenomenon is known as auto regulation of endogenous glucose production (Consoli, 1992).

Glucose is a polar molecule and thus is unable to cross the cell membrane through simple diffusion. For this reason the final step in insulin action leading to cellular glucose uptake, involves translocation of glucose transporter proteins (Glut) from intracellular compartments to the cellular membrane where they facilitate glucose uptake. There are currently 13 known members of the Glut family with a distinct tissue distributions and biochemical properties and contribute to the precise disposal of glucose under varying physiological conditions, out of which Glut-4 is associated with insulin-stimulated glucose transport in adipocytes and muscle (Bryant et al., 2002).

Under normal physiological conditions different glucose transporter isoforms has different Km values i.e. differential affinity for glucose in different isoforms. K_m for Glut-1 is 16.9-26.2 mM and under the same conditions Glut-4 has a K_m of 1.8-4.8 mM and Glut -3 has a K_m of 10.6 mM. This means that Glut-3 and Glut-4 have a higher affinity for glucose than Glut-1, ensuring that glucose transport will be maximal in tissues containing these isoforms even when glucose concentrations are low especially for the brain, which expresses Glut -3, and relies on glucose as its only source of energy. Expression of Glut-1 and 3 has been shown to be regulated by insulin in L6 muscle cells and 3T3-F442A adipocytes.

Glut-2 has a very low affinity for glucose with a K_m of 40 mM. Since normal circulating glucose concentration is 3.9-5.6 mM, therefore, in the postprandial state, when

circulating glucose levels are high, there is a net in flux of glucose into hepatocytes and pancreatic β-cells. Increasing glucose transport inside the β-cell by Glut-2 increases glucose 6 phosphate concentrations, which increases glucose metabolism, and stimulates the β-cell to secrete insulin.

Glut-4 transporter proteins are sequestered into specialized storage vesicles that remain within the cell's interior under basal conditions. As post-prandial glucose levels rise, the subsequent increase in circulating insulin activates intracellular signaling cascades that ultimately result in the translocation of Glut-4 storage compartments to the plasma membrane. Regulation of glucose uptake into muscle and fat cells via Glut-4 is a fundamental action of insulin, and this process is impaired in type 2 diabetes.

Hexokinase (HK) catalyzes glucose phosphorylation, which is the first step in glucose uptake in skeletal muscle. Two HK isoforms, HK-I and HK-II, are expressed in human skeletal muscle, but only HK-II is regulated by insulin (Vogt et al., 2000), which is a physiological regulator of HK-II mRNA expression in skeletal muscle *in-vivo* (Shulman et al., 1995). The balance between GS and glycogen phosphorylase activities determines *in vivo* net glycogen synthesis (Fig. 2.7).

2.2.7.2 Lipid metabolism

The liver synthesizes triglycerides from FFA or via *de novo* lipogenesis from carbohydrate. Triglycerides are either stored in the hepatocytes or released to the circulation as very low density lipoprotein (VLDL) particles. Post-prandially insulin stimulates intravascular lipolysis via lipoprotein lipase (LPL) action on chylomicrons and VLDL. Fatty acids are taken-up by muscle and adipose tissue where they are oxidized or esterified into triglycerides.

In adipocytes, glucose is stored primarily as lipid. This is the result of increased uptake of glucose and activation of lipid synthetic enzymes, including pyruvate dehydrogenase, FAS and ACC (Saltiel and Kahn, 2001). Insulin also profoundly inhibits lipolysis in adipocytes, primarily through inhibition of the enzyme hormone-sensitive lipase (HSL). This enzyme is acutely regulated by control of its phosphorylation state, activated by PKA-dependent phosphorylation, and inhibited owing to a combination of kinase inhibition and phosphatase activation. Insulin inhibits the activity of the lipase primarily via reductions in cAMP levels due to the activation of a cAMP-specific phosphodiesterase in fat cells (Stralfros et al., 1984).

2.2.7.3 Protein metabolism

Amino acids are an important source of energy and precursors of nitrogenous compounds. The balance between protein synthesis and breakdown determines the protein content of the whole body and individual tissues, as well as the concentrations of specific proteins. Insulin has been shown to stimulate protein synthesis both *in-vitro* and *in-vivo* (Jefferson et al., 1977; Kimball et al., 1998). Several studies in rodent models has shown that at low insulin levels, as occurs with fasting or β-cell destruction, the protein synthesis is down regulated which returns back to normalcy on insulin replacement (Pain et al., 1974). Studies on perfused or incubated rat diaphragm, skeletal muscle, and heart (Pain et al., 1983; Rannels et al., 1975; Munger et al., 1974) have shown that insulin also decreases the breakdown of muscle protein. Insulin may influence protein synthesis through numerous sites of regulation including effects on gene transcription (mRNA production), mRNA stability, ribosome biogenesis, initiation and elongation steps of mRNA translation and the regulation of pre-existing enzymes (Munger et al., 1974).

2.2.7.4 Vascular function

Insulin slowly increases blood flow in skeletal muscle under intravenously maintained normoglycaemic-hyperinsulinaemic conditions (Chen and Messina, 1996). Insulin also acutely decreases wave reflection in the aorta in healthy subjects (Westerbacka, 1999), and treatment with insulin decreases central aortic pressure in subjects with type II diabetes (Tamminen et al., 2003). The ability of insulin to increase peripheral blood flow is mediated by NO (Chen and Messina, 1996). Insulin also increases endothelial nitric oxide synthase (eNOS) activity in endothelial cells and induces vasodilatation in human forearm vessels by activating of endothelial $Na^{+}K^{+}$ - ATPase (Tack et al., 1996; Yki-Järvinen and Utriainen, 1998).

2.2.7.5 Other actions

Physiological concentrations of insulin increase the activity of the sympathetic nervous system (Anderson et al., 1991) and inhibit platelet aggregation in healthy subjects (Trovati et al., 1988; Westerbacka et al., 2002). Insulin also acutely lowers serum potassium concentrations by stimulating potassium uptake by skeletal muscles and the splanchnic bed (DeFronzo et al., 1980). In addition, insulin increases intracellular calcium concentrations in vascular smooth muscle cells (Ceolotto et al., 2001) and inhibits uric acid (Ter Maaten et al., 1997), sodium, and potassium and phosphate excretion by the kidney (DeFronzo et al., 1975).

2.2.8 Diabetes Mellitus

Diabetes mellitus (*DM*) consists of a group of syndromes characterized by hyperglycemia, altered metabolism of lipids, carbohydrates, and proteins, and an increased risk of complications from vascular diseases. Most patients can be classified

clinically as having either type I or type II diabetes. Diabetes or carbohydrate intolerance is also associated with certain other conditions or syndromes. Criteria for the diagnosis of diabetes have been proposed by several medical organizations. According to the American Diabetes Association (ADA), a person would be diagnosed as diabetic if he shows symptoms like polyuria, polydipsia, unexplained weight loss, a random plasma glucose concentration of greater than 200 mg/dl (11.1 mM), a fasting plasma glucose concentration of greater than 126 mg/dl (7 mM), or a plasma glucose concentration of greater than 200 mg/dl (11 mM) 2 h after the ingestion of an oral glucose load (NDDG, 1979; WHO, 1999).

There are more than 125 million people with diabetes in the world today, and by 2010, this number is expected to approach 293 million (Rao et al., 2004). The increase in the number of affected people with type I and type II diabetes can be attributed to increasing age, obesity, sedentary lifestyle, and low birth weight.

2.2.8.1 Type I diabetes

Type I diabetes is a chronic disease characterized by deficient insulin secretion due to the loss of insulin-producing β-cells in the endocrine pancreatic islets. The disease remains sub clinical until the number of β-cells is too low to produce the amount of insulin needed to maintain glucose homeostasis, resulting in symptoms of hyperglycaemia, such as polyuria and polydipsia with associated weight loss.

Type I diabetes is preceded by an asymptomatic preclinical period characterized by the presence of markers of β-cell autoimmunity which are closely associated with progressive destruction of the insulin-producing β-cells. The immune cell mediated destruction of β-cells is thought to be dependent on three factors: firstly, a feedback

circuits of antigen transport / presentation/recognition between antigen-presenting cells (APC's) and T-lymphocytes (T-cells), secondly the amount and type of cytokines produced by these cells, and thirdly, the β-cell defense capacity. All these functions in a combined fashion thus results in type I diabetic condition.

2.2.8.1.1 Mechanism

Type I diabetes is a chronic disease characterized by deficient insulin secretion due to the loss of insulin-producing β-cells in the endocrine pancreatic islets. The disease remains sub clinical until the number of β-cells is too low to produce the amount of insulin needed to maintain glucose homeostasis, resulting in symptoms of hyperglycaemia, such as polyuria and polydipsia with associated weight loss. Overt type I diabetes is preceded by an asymptomatic preclinical period characterized by the presence of markers of β-cell autoimmunity which are closely associated with progressive destruction of the insulin-producing β-cell. Although the subjects most likely to develop the disease can be identified, prediction on an individual level is still uncertain and no specific characteristics that could completely discriminate the subjects en route to clinical type I diabetes have been identified so far.

2.2.8.1.2 Causes

2.2.8.1.2.1 Genetic predisposition

Genetic predisposition has traditionally been considered to originate from the class II major histocompatibility complex (MHC) genes on chromosome 6p21, and is associated with HLA–DR and DQ. These genes are expressed on the surface of antigen-presenting cells, and present antigenic peptides to CD4 T-lymphocytes (Reijonen and Concannon, 2006). Approximately 30% of type I diabetic patients are heterozygous for

the high risk HLA-DR3/4 or HLA-DQ2/DQ8. Some also lack the protective HLA-DR2 or HLA-DQ6 which increases type I diabetes susceptibility (Resic-Lindehammer et al., 2008; Morran et al., 2008).

2.2.8.1.2.2 Autoimmunity

Type I diabetes caused due to autoimmunity, is a type of self-allergy that induces the T-lymphocytes of the immune system to attack the pancreatic islets, as if they are a foreign invader. Research on the autoimmune response towards islet cell antigens has shown that it can be demonstrated in pre-diabetic individuals often several years before clinical presentation of diabetes (Morran et al., 2008). Cytoplasmic islet cell autoantibodies (ICA's) are measured by immunohistochemistry on sections of pancreas. In addition to these auto-antibodies, many investigators are using antibodies against insulin, Glutamic acid decarboxylase (GAD) 65, and ICA512 (IA-2)/IA-2β as markers of the autoimmune process (Huurman et al., 2008). Additional auto-antigens have been identified, however, they are less well characterized, less specific, and/or less prevalent, e.g. heat shock protein (HSP), GAD67, Glima 38 and ICA69 (Karavanaki et al., 2008; Richer and Horwitz; 2008 and Abdi et al., 2008)

2.2.8.1.2.3 Immune cells

Immune cells such as macrophages and T-cells have been identified as the mediators of the pancreatic islet β-cell destruction and have been shown to act by releasing cytotoxic molecules including cytokines, oxygen free radicals and NO (Reijonen and Concannon, 2006). Perforin and granzymes released from the granules of cytotoxic T-cells are toxic to the β–cells resulting in their destructions. T-cell induced β-cell death can also be mediated via the cell death receptors, such as Fas (CD95/APO-1)

and tumor necrosis factor receptor-1 (TNF-R1) or by secretion of the pro-inflammatory cytokines, e.g. interleukin-1 (IL-1), interferon gamma (IFN-γ), and tumor necrosis factor-α (TNF-α) (Suk et al. 2001 and Mckenzie et al., 2006), inducing β-cell apoptosis via the activation of β-cell gene networks under the control of the transcription factors NF-kB and STAT-1. NF-κB activation leads to the production of NO and chemokines and depletion of endoplasmic reticulum calcium. The execution of β-cell death occurs through activation of MAPK, via triggering of endoplasmic reticulum stress and by the release of mitochondrial death signals.

2.2.8.1.2.4 Environmental factors

Environmental factors have also been suggested as inducers of autoimmunity since several studies showed its involvement in the pathogenesis of type I diabetes. First, the concordance rate for type I diabetes in monozygotic twins is less than 50%. Second, the epidemiological data and seasonality of the disease strongly indicate the contribution of environmental factors. The environmental factors can be classified into three major groups: viral infections (e.g., Enterovirus, Cytomegalovirus and Rubella virus), early infant diet (eg, breast feeding versus early introduction of cow's milk components), and toxins (eg, N-nitroso derivatives). In addition to these, several other factors like antenatal and perinatal factors, stressful life events and a combination of several environmental risk factors are also associated with type I diabetes.

2.2.8.2 Type II diabetes

Type II diabetes is characterized by insulin resistance and, at least initially, a relative deficiency of insulin secretion. In absolute terms, the plasma insulin concentration (both fasting and meal-stimulated) usually is increased, although "relative"

to the severity of insulin resistance, the plasma insulin concentration is insufficient to maintain normal glucose homeostasis (Defronzo, 1997; Bonadonna et al., 1996). With time, however, there is progressive β-cell failure and absolute insulin deficiency ensues. In a minority of type II diabetic individuals, severe insulinopenia is present at the time of diagnosis and insulin sensitivity is normal or near normal (Christopher et al., 1995; Pass et al., 2002). Most individuals with type II diabetes exhibit intra abdominal and visceral obesity, which is closely related to the presence of insulin resistance (Kozec et al., 2004). In addition, hypertension, dyslipidemia (high triglyceride and low HDL-cholesterol levels, postprandial hyperlipemia) and elevated plasminogen activator inhibitor-1 (PAI-1) levels often are present in these individuals. This clustering of abnormalities is referred as the "insulin resistance syndrome" or the "metabolic syndrome". Because of these abnormalities, patients with type II diabetes are at increased risk of developing macrovascular complications (myocardial infarction and stroke).

2.2.8.2.1 Causes

2.2.8.2.1.1 Obesity

Obesity is a disease, with excess body fat accumulated to such an extent that health may be adversely affected. Obesity is commonly defined as a body mass index (BMI; weight divided by height squared) of 30 kg/m^2 or higher. The consequences of obesity are serious. Obese individuals are predisposed to a cluster of metabolic disturbances known as 'syndrome X' or the metabolic syndrome, which comprises glucose intolerance (the inability to metabolize glucose adequately), type II diabetes mellitus, hypertension, dyslipidaemia (high triglyceride levels accompanied by a raised concentration of low-density lipoproteins (LDL) and diminished high-density

lipoproteins (HDL), leading to an increased risk of stroke and cardiovascular disease (Eckel et al., 20004; Klein et al., 2004; Arias al., 2007; Burke et al., 2008). Epidemiological studies have shown that both overall and upper-body obesity are closely correlated with insulin resistance. Upper body obesity is characterized by an increase in visceral fat (Kahn et al., 2006; Havel et al., 1970), which has been suggested to have greater ability to mobilize FFA than subcutaneous fat and links visceral adiposity to insulin resistance in the liver. This "Portal Theory" which implicates that, increased lipolytic activity in the visceral fat and therefore increased delivery of FFA to the liver, ultimately leading to liver insulin resistance however, has been criticized, because catheterization studies have suggested that FFA released by the splanchnic bed account for maximally 10% of total FFA delivery to the liver (Fried et al., 1998; Crips and Ozanne, 2005). Although intra-abdominal adipocytes have been shown to be more insulin resistant than subcutaneous adipocytes and generate FFA more easily, according to catheterization studies, in upper body obesity increased delivery of FFA to the liver is due to excessive release by subcutaneous rather than visceral adipose tissue (Bonora, 2000; Park, et al., 2007; Frayn 2001). Visceral fat could also induce insulin resistance via release of adipokines such as interleukin-6 (IL-6). Omental fat secretes 3-fold more IL-6 than subcutaneous fat, although IL-6 secreted from isolated adipocytes accounts only for ~10% of total adipose tissue release (Fried et al., 1998).

2.2.8.2.1.2 High fat diet

Diets high in total fat are energy dense and may have low satiety properties. Thus they may be associated with an increased energy intake and consequently an increased risk of obesity and the metabolic syndrome. High intake of saturated fat may be

associated with impairment insulin sensitivity, although human data are sparse on this aspect. Animal studies have suggested that both the type and the amount of dietary fat modulate insulin sensitivity. Moderate increase of fat in the diet (45% of saturated fat) did not impair insulin sensitivity in healthy subjects, although a diet with high monounsaturated fat content may improve insulin sensitivity (Dessein et al., 2000). Addition of n-3 fatty acids (ω-3 fatty acids) to the diet did alter insulin sensitivity or insulin secretion (Tsitouras et al., 2008; Sneddon et al., 2008). LDL cholesterol also increased significantly on the saturated fatty acids (SAFA) diet and decreased on the mono unsaturated fatty acids (MUFA) diet. It is not known in humans, which tissue(s) contribute to improved insulin sensitivity during dietary interventions. An inverse correlation between insulin sensitivity and dietary intake of total fat, oleic acid and ω-6 poly-unsaturated fatty acids (PUFA) was found in insulin resistance and arthrosclerosis study but it was no longer significant after adjusting for BMI. Although higher consumption of fish and long-chain ω-3 fatty acids is associated with a lower risk for cardiovascular diseases and total mortality (Hu et al., 2003), long chain ω-3 fatty acid supplementation doesn't seem to enhance insulin sensitivity in humans (Luo et al., 1998; Dey et al., 2005 and Dey et al., 2006).

2.2.8.2.1.3 Physical inactivity

Physical inactivity is associated with insulin resistance and hyperinsulinemia (Rivellese and Lilli, 2003) and an increase in the risk of developing type II diabetes independent of other factors (Manson et al., 1992; Helmrich et al. 1991). Low physical fitness within men increases the risk of type II diabetes by 2.6-fold even after adjusting for age, smoking, alcohol consumption and family history of diabetes (Wei et al., 1999).

Physical training has been shown to increase muscle insulin sensitivity in normal subjects and type II diabetic patients, when measured directly using the insulin clamp technique combined with positron emission tomography or catheterization techniques. AMPK is an energy-sensing enzyme, which responds to decreased ATP/AMP ratio in conditions such as muscle contraction, hypoxia and ischaemia. AMPK activation enhances peripheral insulin sensitivity and increases fat oxidation in skeletal muscles. AMPK activation increases fatty acid oxidation and inhibits glucose production in liver and also decreases fatty acid, triglyceride and sterol synthesis. In rodents high-intensity treadmill exercise activates liver AMPK and increases plasma glucagon concentrations whereas prolonged low-intensity running has no effect on liver AMPK activity (Musi and Goodyear, 2003).

2.2.8.2.1.4 Counterregulatory hormone excess

Counterregulatory hormones like glucagon, catecholamines, growth hormone, and cortisol are released during hypoglycaemia and other conditions associated with mental or physical stress. All these hormones have insulin-antagonistic effects both in the liver and peripheral tissues. Glucagon and catecholamines, such as adrenalin, act rapidly, whereas the action of cortisol and growth hormone occurs over a period of several hours (Lager, 1991). Glucagon has an important role in regulating glucose counter regulatory factors in hypoglycemia. Glucagon stimulates hepatic gluconeogenesis and glycogenolysis during hypoglycaemia (Stevenson et al., 1987). In pheochromocytoma (a state of catecholamine overproduction) and agromegaly (a state of excessive amount of growth hormone production) a marked effect on insulin resistance has been observed. Growth hormone infusion has been shown to increase plasma insulin concentrations without altering suppression of glucose production and cause insulin resistance in

humans due to impairment in effect of insulin in both liver and skeletal muscle (Rizza et al., 1982). In Cushing's syndrome the overproduction of cortisol causes insulin resistance in humans. Cortisol overproduction stimulates hepatic gluconeogenesis and increases hepatic secretion of VLDL and decreases the uptake of LDL by the liver, and cortisol-infusion has also been shown to induce both hepatic and peripheral insulin resistance (Chrousos, 2000). Plasma glucagon, cortisol and growth hormone responses to insulin-induced hypoglycemia are impaired in patients with type II diabetes (Bolli et al., 1984).

2.2.8.3 Gestational diabetes

Gestational diabetes is a temporary condition that occurs during pregnancy. Gestational diabetes affects 2 to 4% of all pregnancies and involves an increased risk of developing diabetes for both mother and child (Ben-Haroush et al., 2008; Radesky et al., 2008). Several factors are associated with risks for developing gestational diabetes i.e. a family history of diabetes in parents or brothers and sisters, presence of a birth defect in a previous pregnancy, obesity in the woman, BMI greater than 29, older maternal age (over the age of 30), a history of pregnancy induced high blood pressure, urinary tract infections, hydramnios (extra amniotic fluid) and so on (Lee et al., 2008; Galtier-Dereure et al., 1995).

2.2.9 Pathophysiological effects

Diabetes mellitus is associated with decrease in the circulating concentrations of insulin (insulin deficiency) and/or a decrease in the response of peripheral tissues to insulin (insulin resistance). These abnormalities lead to alterations in the metabolism of carbohydrates, lipids, ketones, and amino acids, the central feature of which is hyperglycemia.

Insulin lowers the concentration of glucose in blood by inhibiting hepatic glucose production and by stimulating the uptake and metabolism of glucose by muscle and adipose tissues. These two important effects occur at different concentrations of insulin. In case of human, the production of glucose is inhibited half maximally by an insulin concentration of about 20 μ units/ml, whereas glucose utilization is stimulated half maximally at about 50 μ units/ml.

In both types of diabetes, glucagon (the levels of which are elevated in untreated patients) opposes the hepatic effects of insulin by stimulating glycogenolysis and gluconeogenesis, but it has relatively little effect on peripheral utilization of glucose. Thus, in the diabetic patient with insulin deficiency or insulin resistance and hyperglucagonemia, there is an increase in hepatic glucose production, a decrease in peripheral glucose uptake, and a decrease in the conversion of glucose to glycogen in the liver (Hoffman, 2007).

Alterations in secretion of insulin and glucagon also profoundly affect lipid, ketone, and protein metabolism. At concentrations below those required to stimulate glucose uptake, insulin inhibits the hormone-sensitive lipase in adipose tissue and thus inhibits the hydrolysis of triglyceride stores (Carmen and Víctor, 2006). This counteracts the lipolytic action of catecholamines, cortisol, and growth hormone and reduces the concentrations of glycerol (a substrate for gluconeogenesis) and free fatty acids (a substrate for production of ketone bodies and a necessary fuel for gluconeogenesis) (Watt and Spriet, 2004). These actions of insulin are deficient in the diabetic patients, leading to increased gluconeogenesis and ketogenesis.

The liver produces ketone bodies by oxidation of free fatty acids to acetyl CoA; this involves the inter conversion of the coenzyme A (CoA) and carnitine esters of fatty acids by the enzyme acylcarnitine transferase (Haley-Andrews and Mackenzie, 2005, Wolfsdorf et al., 2007). The activity of CoA is inhibited by intra mitochondrial malonyl CoA, one of the products of fatty acid synthesis. Under normal conditions, insulin inhibits lipolysis, stimulates fatty acid synthesis (thereby increasing the concentration of malonyl CoA), and decreases the hepatic concentration of carnitine all of which finally decreases the production of ketone bodies (Cardella, 2005). In the diabetic patient, particularly the patient with type I diabetes mellitus, the consequences of insulin deficiency and glucagon excess provide a hormonal milieu that favours ketogenesis and in the absence of appropriate treatment, may lead to ketonemia and acidosis.

Insulin also enhances the transcription of lipoprotein lipase in the capillary endothelium, which hydrolyzes triglycerides present in VLDL and chylomicrons, resulting in release of intermediate-density lipoprotein (IDL) particles (Bellomo et al., 2007). In absence of insulin IDL particles are converted by the liver to more cholesterol-rich LDL, resulting in hypertriglyceridemia and hypercholesterolemia. In addition, deficiency of insulin may be associated with increased production of VLDL (Hartge et al., 2007).

Insulin stimulates amino acid uptake and protein synthesis and inhibits protein degradation in muscle and other tissues. It thus causes a decrease in the circulating concentrations of most amino acids for example glutamine and alanine, the major amino acid precursors for gluconeogenesis (Kruszynska, 2003; Tremblay et al., 2007). In poorly controlled diabetics, excess alanine converts into glucose, contributing to the enhanced

rate of gluconeogenesis. The increased conversion of amino acids to glucose also results in increased production and excretion of urea and ammonia.

Another pathognomonic feature of diabetes mellitus is thickening of the capillary basement membrane and other vascular effects resulting in progressive narrowing of the vessel lumina, causing inadequate perfusion of critical regions of certain organs (Clark, 2008). The matrix expansion and cellular proliferation in many large vessels further contributes to luminal narrowing. These pathological changes contribute to some of the major complications of diabetes, including premature atherosclerosis, intercapillary glomerulosclerosis, retinopathy, neuropathy and ulceration and gangrene of the extremities (Rosolova et al., 2008).

Prolonged exposure of tissues to elevated concentrations of glucose results in the formation of glycosylated products and eventually may form cross-linked proteins termed advanced glycation end products (AGEs) (Hyogo and Yamagishi, 2008; Sugimoto et al., 2008). Such nonenzymatic glycosylation may be directly responsible for expansion of the vascular matrix and the vascular complications of diabetes. The macrophages possess receptors for advanced glycosylation end products Binding of such proteins to macrophages stimulate the production of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), which in turn, induces degradative and proliferative cascades in mesenchymal and endothelial cells, respectively (Yan et al., 2008; Makinen et al., 2008; Kero et al., 2003; Faulkner et al., 2007; Nogueira-Machado and Chaves, 2008).

Intracellular glucose is reduced to its corresponding sugar alcohol, sorbitol, by the enzyme aldose reductase, and the rate of production of sorbitol is determined by the ambient glucose concentration. The increased intracellular concentration of sorbitol

contributes to an increased osmotic effect and ultimately causing tissue damage (Oyama et al., 2006; Oates, 2008).

In neural tissues and perhaps in other tissues, glucose competes with myoinositol for transport into cells. Reduction of cellular concentrations of myoinositol may contribute to altered nerve function and neuropathy. Hyperglycemia also may enhance the *de novo* synthesis of diacylglycerol, which could facilitate persistent activation of protein kinase C (Rogers et al., 2008; Edwards et al., 2008).

2.2.10. Treatment

Diabetes mellitus has become an international healthcare crisis that requires new approaches for its prevention and treatment. Current scientific advances have increased our understanding on the mechanism of insulin action and insulin resistance, and modes of controlling insulin secretion. Pharmaceutical products, surgical measurements, diet control, life style management or herbal formulation are the vast variety of options, available for the treatment of diabetes. These available therapeutic options can be divided in 2 basic categories (1) conventional therapy and (ii) traditional therapy

2.2.10.1 Conventional therapy

Conventional therapy is currently accepted and widely used treatment for a certain type of disease, based on the results of past scientific research. It includes life style management, surgical measurement and drug therapy.

2.2.10.1.1 Weight loss

2.2.10.1.1.1 Diet

The beneficial effects of weight reduction on insulin resistance by caloric restriction are well documented (Abete et al, 2008; Astrup, 2008). Two large intervention

studies - diabetes prevention study (DPS) and diabetes prevention program (DPP) showed clearly that with the changes of lifestyle, especially with weight reduction and increase of exercise, the development of diabetes could be prevented by 58% (Tuomilehto et al., 2001; Knowler et al., 2002). Several studies have documented the beneficial effect of weight loss on peripheral (van der Crabben, et al., 2008; de Mello et al., 2008) and hepatic insulin sensitivity (De Taeye et al., 2007; Rosa et al., 2008). The improvement of insulin sensitivity and glucose metabolism could be mediated by weight loss induced reduction of intramuscular and liver fat content; however, the data on this aspect are sparse (Schrauwen, 2007).

2.2.10.1.1.2 Surgery

Gastric bypass, gastric partioning, gastroplasty, and recently gastric banding are surgical procedures, which can be used to treat massively obese patients. The two most common types of procedures currently used in the United States are adjustable gastric bands and Roux-en-Y gastric bypass (Meneghini, 2007). Morbidly obese (BMI 52 kg/m2) subjects, who underwent gastric bypass operation, lost 47 Kg of body weight, intramuscular lipid deposits decreased by ~30%, and insulin sensitivity improved by 92% (Luyckx et al., 1998; Galtier et al., 2006). Weight loss induced by gastroplasty has also been shown to decrease significantly liver fat content in obese subjects with steatohepatitis (Bult et al., 2008; Furuya et al., 2007; Jaskiewicz et al., 2006). Improvements in insulin resistance and β-cell function are related to surgically induced weight loss, the rapid post-operative improvement in glycemia is possibly due to a combination of decreased nutrient intake and changes in gut hormones as a result of the bypassed intestine (Meneghini, 2007).

2.2.10.1.1.3 Exercise

Both acute exercise and physical training have been shown to increase insulin stimulated glucose uptake in skeletal muscle. The ability of exercise to increase insulin sensitivity has been suggested to be mediated through enhanced glucose transport, increased glycogen synthesis, increased muscle mass and augmented muscle blood flow (Bikman et al., 2008; Rossner et al., 2008). Physical training has also been shown to enhance insulin-induced glucose uptake in skeletal muscles by improving insulin action on oxidative enzymes, increasing activity of glycogen synthase and by increasing the proportion of oxidative red fibers in skeletal muscles. In addition, improved mitochondrial oxidative enzyme capacity and activation of the glucose transport system have been suggested to contribute in the improvement of insulin sensitivity (Balkau et al., 2008; Kriska et al., 2008). Skeletal muscle adapts to exercise, such as prolonged running or swimming with an increase in mitochondrial density (Spassiani et al., 2008; Orozco et al., 2008). Physical exercise has also been found to increase mitochondrial gene expression and oxidative capacity, possibly through an increase in the expression of the peroxisome proliferator-activated receptor gamma (PPARγ) co-activator 1 (PGC-1), the over expression of which in muscles markedly increases insulin sensitivity, GLUT-4 expression and the proportion of oxidative red fibers (Ojuka et al., 2003; Lumini et al., 2008; Narkar et al., 2008).

2.2.10.1.2 Antidiabetic Drugs

2.2.10.1.2.1 Insulin or Insulin mimetic

The insulin therapy comes as a last option when oral agents are unable to achieve or sustain adequate glycemic control or symptoms become severe. Insulin replacement

can either be in the form of long acting basal insulin, which is given once daily or as meal time (bolus) insulin, which is rapid acting and given pre-prandially to cover meal time glucose fluctuations. The mealtime insulin is the rapid-acting analogues or short-acting regular human insulin. They simulate the basal level of insulin occurring between meals, through the night and with fasting. Insulin is commercially available in concentrations of 100 or 500 units per ml.

2.2.10.1.2.1.1 Mealtime insulin

Insulin in a solution self-associates and forms larger aggregates called hexamers. These large aggregates need to dissociate after subcutaneous injection before diffusion of insulin into the circulation (Heinemann and Richter, 1993). The first rapidly acting insulin analogue approved for human administration was insulin lispro (Humalog), in which the terminal proline and lysine residues of the B-chain of insulin are inverted, resulting in decreased self-association properties of the insulin (Burge and Schade, 1997; Howey et al., 1994). Lispro insulin is absorbed much more rapidly than regular insulin. Lispro begins acting within 15 min, reaches peak biologic effects in 60 to 90 min and continues to act for 4 to 5 h. Insulin aspart (NovoLog/NovoRapid) is another rapid-acting insulin analogue similar to lispro. Due to lower risk of nocturnal hypoglycemia, insulin lispro and aspart are much safer than regular human insulin.

2.2.10.1.2.1.2 Basal Insulin

Basal insulin is the suspension of insulin to delay their absorption from subcutaneous sites for a prolonged action. On the basis of onset of their action, peak activity and duration, different forms of basal insulins are available in the market for example, intermediate-acting human insulin, Neutral Protamine Hagedorn (NPH) and

Lente insulin, with a onset of action within 2.5 to 3 h of injection, and a peak action in 5 to 7 h, long acting human insulin, Ultra lente, with an onset of action in 4 h after injection, peak action is at 8 to 10 h and duration of action is up to 20 h. Detemir, another long acting insulin analogue, is characterized by a peak activity at 6 to 8 h after injection and prolonged 24 h duration of action.

2.2.10.1.2.1.3 Premixed Insulin

Premixed insulin is a combination (90-10 to 50-50 ratio) of short and intermediate acting insulin. Use of premixed insulin avoids the potential problems of self-mixing and reduces the number of steps before injection, thereby reducing the chances of possible errors. The premixed insulin`s are preferred by elderly patients and those with visual or fine-motor impairment (Coscelli et al., 1992). However, premixed insulin's do not permit easy adjustment of mealtime and basal insulin doses and are inappropriate for patients with type I diabetes. They are valuable and frequently used in the treatment of type II diabetes. The improper regulations of doses of various forms of insulin may result in following disorders:

Hypoglycemia: The most common adverse reaction to insulin is hypoglycemia. This may result from an inappropriately large dose, from a mismatch between the time of peak delivery of insulin and food intake, or from superimposition of additional factors that increase sensitivity to insulin (*e.g.*, adrenal or pituitary insufficiency) or that increase insulin-independent glucose uptake (*e.g.*, exercise). The more vigorous the attempt to achieve euglycemia, the more frequent is the episodes of hypoglycemia.

Insulin allergy and resistance: Although there has been a dramatic decrease in the incidence of resistance and allergic reactions to insulin with the use of recombinant

human insulin or highly purified preparations of the hormone, these reactions still occur as a result of reactions to the small amounts of aggregated or denatured insulin in all preparations, to minor contaminants, or because of sensitivity to one of the components added to insulin in its formulation (protamine, Zn^{2+}, phenol, *etc.*). The most frequent allergic manifestations are IgE-mediated local cutaneous reactions, although on rare occasions patients may develop life-threatening systemic responses or insulin resistance.

Lipoatrophy and lipohypertrophy: Atrophy of subcutaneous fat at the site of insulin injection (lipoatrophy) is probably a variant of an immune response to insulin, whereas lipohypertrophy (enlargement of subcutaneous fat depots) has been ascribed to the lipogenic action of high local concentrations of insulin. Both problems may be related to some contaminant in insulin and are rare with more purified preparations.

Insulin edema: Some degree of edema, abdominal bloating, and blurred vision develops in many diabetic patients with severe hyperglycemia or ketoacidosis that is brought under control with insulin. This is associated with a weight gain of 0.5 to 2.5 kg.

2.2.10.1.2.2 Enhancers of insulin release

Secretagogues stimulate the pancreas to secrete insulin, which then reduces hepatic glucose production and improves glucose uptake by muscles. There are two classes of secretagogues: sulfonylureas, nonsulfonylureas and incretin mimetics.

2.2.10.1.2.2.1 Sulfonylureas

The sulfonylureas are substituted arylsulfonylureas and were developed in during 1950's. They differ by substitutions at the *para* position on the benzene ring and at one nitrogen residue of the urea moiety. They are potent insulin secretagogues and directly stimulate first phase insulin secretion. They initiate their action by binding to a specific

sulphoylurea receptor on pancreatic β-cells. This closes a potasium dependent adenosine triphosphate channel, leading to a decrease in K^+ influx and depolarization of β-cell membrane. This result in a increased Ca^{2+} influx into the β-cell, activating a cytoskeleton system that cause a translocation of secretory granules to the cell surface and extrusion of insulin through exocytosis. Sulfonylureas are used to control hyperglycemia in diabetic patients who cannot achieve appropriate control with changes in diet alone. In all patients, continued dietary restrictions are essential to maximize the efficacy of the sulfonylureas. The clinical efficiency of sulphonylurea is directly related to the pretreatment level of fasting plasma glucose and glycosylated hemoglobin (HbA1c) i.e the higher the fasting plasma glucose level, the greater its decrease from base line (Fonseca et al., 2008). On the basis of their relative potency, doses and duration of activity, sulphonylurea group of compounds have been categorized in 3 generations of drugs as shown in Table 2.1 (Webb et al., 2000).The usual initial daily dose of tolbutamide is 500 mg, and 3000 mg is the maximally effective total dose. Tolazamide and chlorpropamide usually are initiated in a daily dose of 100 to 250 mg, with maximal doses of 1000 (tolazamide) or 750 mg (chlorpropamide). Tolbutamide and tolazamide often are taken twice daily 30 min before breakfast and dinner. The initial daily dose of glyburide is 2.5 to 5 mg and daily doses of more than 20 mg are not recommended. Therapy with glipizide usually is initiated with 5 mg given once daily. Although the maximal recommended daily dose is 40 mg, but daily doses of more than 15 mg should be avoided. In summary, the treatment with the sulfonylureas must be guided by the patient's response, which needs to be monitored frequently (Bailey and Day, 2003, Cheng, 2005; Goldfine, 2005).

Table 2.1. Relative Potency, doses and duration of activity of 3 generation drugs of sulphonylurea class

Generations	Drug	Act Over	Dose range	Relative potency	Dose/day
Ist generation	Orinase (Tolbutamide)	6-10 h	500-3000 mg	1	2-3
	Diabenese (Chlorpropamide)	24-72 h	100-500 mg	6	1-2
IInd generation	Glucotrol (Glipizide)	12 h	2.5-40 mg	75	1-2
	Glucotrol XL (Ex. Rel. glipizide)	24 h	2.5-20 mg	150	1
	Micronase diabeta (Glyburid)	18-24 h	1.25-2.0 mg	150	1-2
	Glynase (Micronized gly)	24 h	3-12 mg	250	1-2
IIIrd generation	Amaryl (glympiride)	24 h	1-8 mg	350	1

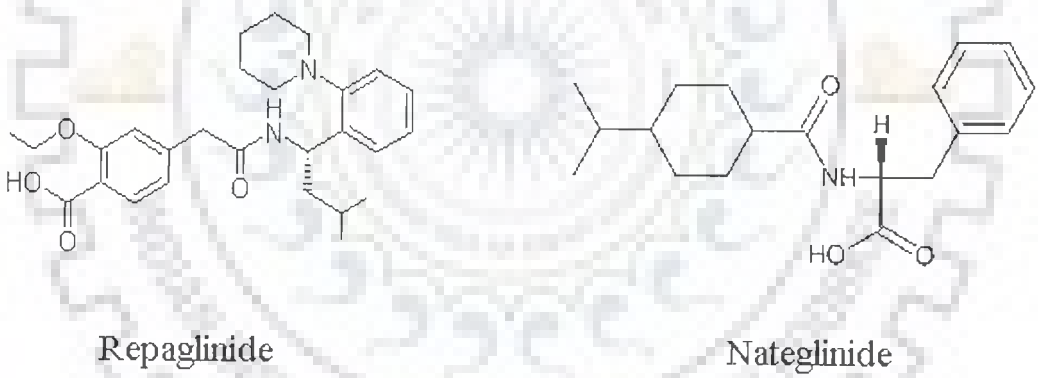
The major side effect associated with sulphonylurea class of drugs is hypoglycemia as a result of intermittent excess in insulin production and release. Excessive weight gain due to edema and reduction of the osmotic diuresis, abdominal

upset, headache and hypersensitivity reactions are some of the other side effects related to **sulphonylurea class of drugs (Fonseca et al., 2008; Cheng 2005).**

2.2.10.1.2.2.2 Nonsulfonylurea secretagogues

Non sulphonylurea or meglinitides are benzoic acid and amino acid derivatives, which bind to the ATP-dependent K^+ channel on the cell membrane of pancreatic β-cell in a manner similar to sulphonylurea but at a different binding site. Meglinitides are unique in their function as they stimulate the first phase of insulin secretion in a glucose sensitive manner and hence reducing the risk of hypoglycemia (DeFronzo, 1999).

Compared to sulphonylurea they have a quick onset and a short duration of action. Repaglinide (16mg/day) and nateglinide (36mg/day) are 2 members of this class (Fig. 2.8).



Repaglinide Nateglinide

Figure 2.8. Structure of miglinitides class compound

Repaglinide is metabolized primarily by the liver to inactive derivatives. Repaglinide should be used cautiously in patients with hepatic insufficiency because a small proportion (about 10%) of repaglinide is metabolized by the kidney. As with

sulfonylureas, the major side effects of repaglinide is hypoglycemia (Fonseca et al. 2008; Bailey and Day, 2003).

The major therapeutic effect of nateglinide is reducing postprandial glycemic elevations in type II mellitus patients. Nateglinide is approved by the FDA for use in type II mellitus and is most effective if administered in a dose of 120 mg about 10 min before a meal. Nateglinide is metabolized primarily by the liver and thus should be used cautiously in patients with hepatic insufficiency (Austin et al., 2003; ACP, 2007).

Major side effects associated with this class of compound are hypoglycemia and weight gain but the magnitude of these side effects is less than sulphonylurea group of members.

2.2.10.1.2.2.3 Incretin mimetics

Incretins are a type of gastrointestinal hormone that causes an increase in the amount of insulin released from the β-cells of the islets of Langerhans after diets, even before blood glucose level gets elevated. They also slow the rate of absorption of nutrients into the blood stream by reducing gastric emptying and may directly reduce food intake. As expected, they also inhibit glucagon release from the α-cells of the islets of Langerhans. The two main candidate molecules that fulfill the criteria for an incretin are glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (glucose-dependent insulinotropic peptide or GIP).

Both GIP and GLP-1 exert their actions by the engagement of structurally distinct G protein-coupled receptors (GPCRs). Activation of both incretin receptors on β-cells leads to rapid increase in the levels of cAMP and intracellular calcium, followed by insulin exocytosis, in a glucose-dependent manner. More sustained incretin receptor

signaling is associated with the activation of protein kinase A, induction of gene transcription, enhanced levels of insulin biosynthesis, and stimulation of β-cell proliferation. Both GLP-1 and GIP receptor activation also promote resistance to apoptosis and enhanced β-cell survival, in both rodent and human islets (Ranganath, 2008). Further, GLP-1 also inhibits glucagon secretion, gastric emptying, and food ingestion, and promotes enhanced glucose disposal through neural mechanism which contributes substantially for the control of glucoregulation (Drucker, 2003; Aronoff et al., 2004). Since, GLP-1 is rapidly metabolized in the circulation by dipeptidyl peptidase-IV (DPP-IV); its use in therapy is limited. Therefore, research is in progress for creating GLP-1 mimetic that are resistant to DPP-IV, as well as compounds that block DPP-IV activity. Strategies designed to circumvent degradation of GLP-1 by DPP-IV have resulted in GLP-1 mimetics (GLP-1-R agonists) with longer half-lives than natural GLP-1. Two synthetic GLP-1 analogs have entered clinical trials. One approach to make longer-acting forms has been to modify the GLP-1 molecule to promote albumin binding, which results in slow absorption from subcutaneous injection sites and a longer plasma half-life with persistent activity (Stonehouse et al., 2008). Liraglutide (NN2211, Novo Nordisk, Copenhagen, Denmark), the most studied compound in this group, includes a C-16 fatty acyl derivative, that binds to albumin. It has an elimination half-life of 12 hours following a subcutaneous dose. Exendin-4 is derived from the salivary gland of the Gila monster and has 53% homology with human GLP-1. Exendin-4 is resistant to DPP-IV and has full agonist activity for GLP-1 receptors. The most common adverse effect was mild to moderate transient nausea, reported by 30 to 50% of treated patients compared with 7 to 23% of controls (Hunziker et al., 2005; Pei, 2008; Demuth et al., 2005).

2.2.10.1.2.2.4 Dipeptidyl Peptidase IV (DPP-IV) Inhibition

The rationale behind using DPP-IV inhibitors as antidiabetic agents is to enhance plasma concentrations of intact, biologically active, endogenous GLP-1. Since DPP-IV inactivates GIP and other peptides, DPP-IV inhibitors might also improve glycemic control by increasing the levels of these hormones (Campbell and White, 2008; Pei, 2008). The experience so far with DPP-IV inhibitors is that they are well tolerated with few adverse effects. They do not seem to have any major effect on gastric function or body weight.

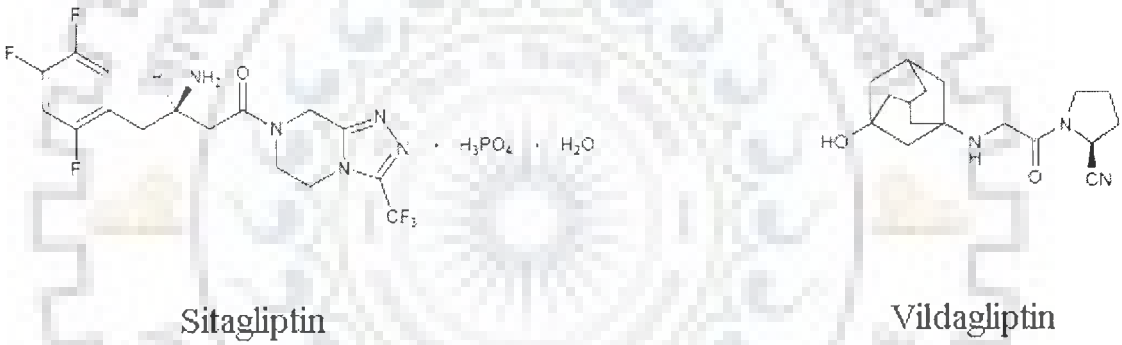


Figure 2.9. Structure of some DPP-IV inhibitors

Till date, sitagliptin (Januvia) and vildagliptin (Galvus) are two known DPP-IV inhibitors (Matsuyama-Yokona et al., 2008) (Fig. 2.9). Some other DPP-IV inhibitors are also being studied for their potential and safety (Matsuyama-Yokona et al., 2008; Sattigeri et al., 2008). Since the main mechanism of action of the DPP-IV inhibitors is mediated by GLP-1, and because GLP-1 potentiates insulin release only during conditions of hyperglycemia, the glucose-lowering effect of the DPP-IV inhibitors is self

limiting. In theory, therefore, hypoglycemia should not be a problem with the DPP-IV inhibitors, and this has been proved in clinical studies. A theoretical risk associated with DPP-IV inhibition is the prolongation of action of a multitude of small peptide hormones, neuropeptides, growth factors, cytokines, and chemokines that normally are cleaved by this protease (Campbell and White, 2008). Despite these theoretical concerns, clinical studies have shown a particularly good safety profile.

2.2.10.1.2.3 Enhancers of insulin action

The agents, which enhance insulin actions function through a variety of mechanisms, are called as enhancers. They can inhibit gluconeogenesis and glycogenolysis, inhibit hepatic glucose absorption, or increase glucose uptake in fat and muscles. Biguanides (metformin), thiazolidinediones, PTP-1B inhibitors, GSK-3 inhibitors are the member of this category.

2.2.10.1.2.3.1 Metformin

Metformin (Fig. 2.10) is a biguanide derivate, which lowers blood glucose concentrations and inhibits hepatic glucose production both *in vitro* and *in vivo*. It also increase glucose uptake by skeletal muscle to some extent (Kirpichnikov et al., 2002). Metformin has also been found to be effective for greater reductions of haemoglobin A(1c) and fasting plasma glucose values when used combination therapy with insulin (Raskin, 2008). In addition, metformin has beneficial effects on plasma lipid concentrations and significantly reduces the risk of cardiovascular diseases (Levetan, 2007; Eriksson et al., 2007).

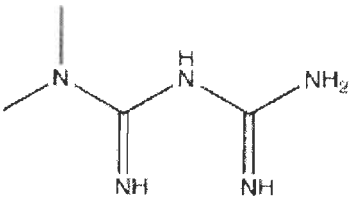


Figure 2.10. Structure of Metformin

Although metformin has been used in the management of type II diabetes for more than 40 years, its molecular mechanism of action has not yet been well understood. Zhou et al. (2001) has recently demonstrated that in isolated rat hepatocytes metformin decreases hepatic glucose production by activating adenosin monophosphate activated protein kinase (AMPK), an enzyme normally activated by adenosine monophosphatase, the breakdown product of adenosine triphosphate and a cellular signal for increased energy requirement. Activation of AMPK suppresses acetyl-CoA carboxylase (ACC) activity, stimulates fatty acid oxidation, muscle glucose uptake and expression of cAMP-stimulated gluconeogenic genes such as PEPCK and G6Pase (Misra and Chakrabarti, 2007). Metformin stimulates chronic activation of AMPK and induces the expression of muscle hexokinase and Glut-4 accompanied by enhanced peripheral glucose disposal (McCarty, 2004). High concentrations of metformin in isolated hepatocytes have also been found to reduce mitochondrial NADH:NAD+ ratio, thus lowering cellular ATP levels, which lead to increased flux through pyruvate kinase and reduced gluconeogenesis. Moreover, metformin has been shown to inhibit oxidative phosphorylation and lower cellular ATP levels (Hawley et al., 2002) and increase the active forms of glycogen synthase and glycogen phosphorylase in the liver and skeletal muscles of diabetic mice indicating enhanced glycogen turnover. AMPK also decrease

the SREBP-1 expression, a factor responsible for pathogenesis of insulin resistance, dyslipidemia and diabetes, and contributes to decreased triglyceride synthesis and hepatic steatosis (Cheng and Fantus, 2005). Synthetic derivatives of Metformin (cysteinyl metformin) have also shown effect on pancreatic cell performance and oxidative status of treated rats (Liu et al., 2008). In addition, metformin also showed anti-inflammatory effects on endothelial cells and inhibited TNF-α induced IKKα/β phosphorylation, IκB-α degradation and IL-6 production in human umbilical vein endothelial cells. This effect was related to PI3K-dependent AMPK phosphorylation (Huang et al., 2008).

2.2.10.1.2.3.2 Thiazolidinediones (TZDs)

TZDs, troglitazone, pioglitazone and rosiglitazone, are a relatively new class of oral antidiabetic drugs (Fig. 2.11). The first agent in this group, troglitazone, was withdrawn due to hepatotoxicity (Gale, 2001). *TZDs* are peroxisome proliferators activated receptor- gamma (PPARγ) agonists. The PPARγ is a nuclear receptor activated by fatty acids and fatty acid–derived eicosanoids. PPARγ forms heterodimers with retinoid-X receptors (RXRs) upon activation. These PPAR-RXR heterodimers bind to DNA-specific sequences called peroxisome proliferator–response elements (PPREs), thus stimulating or dampening the transcription of target genes (Ahmed et al., 2007; Guo and Tabrizchi, 2006). Synthetic ligands for PPARγ are of particular interest for treating patients with type II diabetes because they restore sensitivity to insulin. It has been suggested that *TZDs* act mainly in adipose tissue and the effects on liver and muscle are either indirect via changes of circulating concentrations of fatty acids and adipo-cytokines, or that *TZDs* improve insulin sensitivity by direct interaction with muscle and liver (Hammarstedt et al., 2005; Gross and Staels, 2007). Activation of PPARγ by *TZDs*

induces expression of numerous genes that regulate fatty acid metabolism and lipid storage (Savkur and Miller, 2006; Gelman et al., 2007). *TZDs* increase adipocyte P2 enhancer (aP2) gene expression both in cultured cells and in transgenic mice (Tontonoz et al., 1994), PEPCK gene expression in cultured 3T3-F442A preadipocytes and adipocytes (Tontonoz et al., 1995), and long-chain-acyl-CoA synthetase (acylCoA) activity in liver and adipose tissue in rodents (Schoonjans et al., 1993).

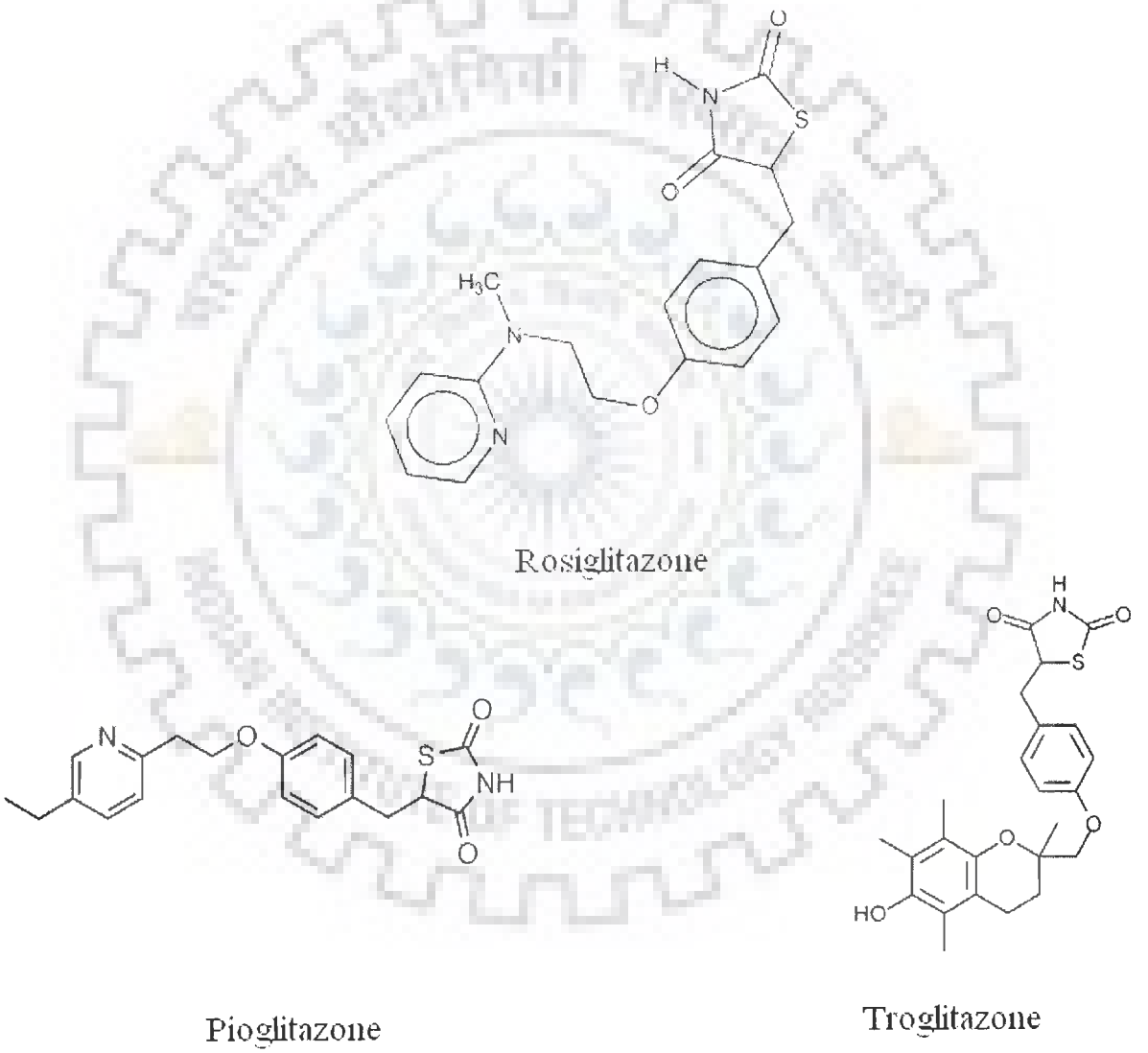


Figure 2.11. Structure of Thiazolidinediones class members

PPARγ agonists also increases fatty acid transport protein 1 (FATP-1) mRNA expression in 3T3-L1 cells, preadipocytes and adipocytes in rat adipose tissue and to a lesser extent in muscle, but not in liver (Martin et al., 1997). Further, *TZDs* have also been reported to activate LPL gene expression in rat adipose tissue and 3T3-L1 preadipocytes (Schoonjans et al., 1996), increase GLUT-4 mRNA expression in cultured fibroblasts, and glucokinase expression in the liver of diabetic ZDF rats. Activation of PPARγ by *TZDs* also improve insulin signaling by increasing the expressions of IRS-1, IRS-2 and p85 subunit of PI3K in cultured 3T3-L1 and human adipocytes. PPARγ agonists also increase the expression of several genes in β-cells like Glut-2 and β-glucokinase leading to the restoration of the glucose-sensing ability of these cells (Kim et al., 2002). Tissue specific knockout mice have been developed to clarify the action of PPARγ on glucose homeostasis in muscle, liver and adipose tissue. Muscle specific PPARγ knockout mice are obese and have hepatic insulin resistance, but insulin-stimulated glucose uptake in muscle is not impaired (Norris et al., 2003). The hepatic insulin resistance might be secondary to altered adipokine release associated with increased adiposity. The expression of 30 kDa adipocyte complement-related protein (Acrp30) in adipose tissue is reduced which also may induce hepatic insulin resistance. The fat tissue PPARγ deficient (FKOγ) mice develop general lipodystrophy, fatty liver with hepatic insulin resistance, and exhibit increased plasma FFA and triglyceride concentrations, decreased leptin and Acrp30 concentrations. Treatment with *TZDs* increases insulin stimulated muscle glucose disposal and normalizes hepatic insulin sensitivity (He et al., 2003). Deletion of PPARγ2 selectively from white adipose tissue (PPARγhyp/hyp) leads to severe lipodystrophy, hyperlipidemia and growth retardation.

Interestingly, PPARγhyp/hyp mice have hepatomegaly with a fatty liver at birth, but the livers of adults did not show any signs of fatty liver, and the adults have only mild glucose intolerance with low serum adiponectin and leptin concentrations. In PPARγ hyp/hyp mice genes controlling FFA catabolism are activated, whereas expression of genes involving in FA synthesis remains unchanged. Treatment with PPARγ agonist alleviated the glucose intolerance, but not the insulin resistance, suggesting that white adipose tissue, not the liver or muscle, is crucial for insulin sensitivity by PPARγ agonists (Koutnikova et al., 2003). Liver-specific ablation of the PPARγ gene in wild type (WT LKO) and AZIP PPARγfl/fl (AZIP LKO) mice were used to study the function of liver PPARγ. Inactivation of liver PPARγ reduced hepatic steatosis in both lipoatrophic AZIP mice and diet-induced obesity, and increased adipose tissue mass and insulin resistance in WT LKO mice. Inactivation of liver PPARγ in the AZIP and WT LKO abolished the hypoglycemic and hypolipidemic effects of rosiglitazone, implying that in the absence of adipose tissue the liver is a primary and major site of thiazolidine action. PPARγ agonists have been demonstrated to decrease liver fat content and serum alanine aminotransferase (ALT) levels and to improve peripheral insulin sensitivity also in patients with Nonalcoholic steatohepatitis (NASH) (Caldwell et al., 2001; Promrat et al., 2004).

The major side effects associated with this class of drugs is weight gain and fluid retention which leads to edema and dilutional anemia.

2.2.10.1.2.3.3 PTP-1B inhibitors

Tyrosine phosphorylation is the fundamental step in insulin mediated physiological effects. It is reversible and governed by the opposing activities of PTP-1B; a cytosolic phosphatase consisting of a single catalytic domain (Charbonneau et al., 1989;

Picha et al., 2007). *In vitro*, it is non-specific protein tyrosine phosphatases (PTP) and dephosphorylates a wide variety of substrates. *In vivo*, it is involved in down regulation of insulin signaling by dephosphorylation of specific phosphotyrosine residues in the insulin receptor. Defective or inappropriate operation of PTP-1B leads to aberrant tyrosine phosphorylation, contributing to the development of diabetes (Kahn, 1994; Vaque and Racch, 1992). PTP-1B is a key negative regulator of insulin signal transduction and a potential therapeutic target in the treatment of type II diabetes and obesity. Thus several pharmaceutical companies are attempting to develop some potential drug molecules that could specifically inhibit PTP-1B activities. Several group of compounds i.e. vanadium, naphthoquinones, pyridazine analoges, difluoromethylene phosphonic acids etc. have been screened for their PTP-1B inhibitor activity and their specificity and potency is being studied extensively (Kasibhatla, 2007)

2.2.10.1.2.3.4 Glycogen synthase kinase 3 inhibitors

Glycogen synthase kinase-3 (GSK-3) is a key enzyme involved in glycogen metabolism and maintenance of cell structure, function and survival (Patel et al., 2008). Phosphorylation of IRS-1 on multiple serine residues by GSK-3 impaires insulin receptor kinase activity and insulin action. GSK-3 acts as a regulatory molecule to limit activation of insulin signaling and inhibitors of GSK-3 enhances response to insulin i.e. lowers blood glucose level and stimulates glucose transport and glycogen synthesis in skeletal muscles (Forde and Dale, 2007; Hooper, 2007). Although GSK-3 posses marked potential as therapeutic target yet effective drugs are not available.

2.2.10.1.2.4 Inhibitors of glucose uptake

2.2.10.1.2.4.1 α-Glucosidase inhibitors

The α-glucosidase is a group of enzymes responsible for the breakdown of oligosaccharides. Oligosaccharides bind on specific site of glucosidase enzymes followed by a hydrolytic cleavage. α-glucosidase inhibitors (acarbose, voglibose, miglitol etc.) are classical competitive inhibitors that compete with natural ligand i.e., oligosaccharides and are resistant to hydrolysis (Fig. 2.12) (Dimitriadis et al., 1982; Lebovitz, 1997). Acarbose, a pseudotetra-saccharide is a natural microbial product derived from culture broths of *Actinoplanes* strain SE 50. Acarbose binds reversibly, competitively and in a dose-dependent manner to the oligosaccharide binding site of α-glucosidase enzymes in the brush border of the small intestinal mucosa. As a consequence, hydrolysis is prevented. It binds to intestinal sucrase with a binding affinity 10^4 to 10^5 greater than that of sucrose. Similarly, voglibose (a potent α-glucosidase inhibitor) inhibits most α-glucosidase enzymes but it is weaker than acarbose at inhibiting sucrase and has little effect on pancreatic amylase. Neither acarbose nor voglibose interferes with glucose absorption through the intestinal sodium-dependent glucose transporter (Mizuno et al., 2008; Hanefeld and Schaper, 2008; Godbout and Chiasson, 2007). Miglitol is another effective α-glucosidase inhibitor and has greater activity than acarbose on isomaltase. Miglitol has no effect on pancreatic amylase but it does mildly interfere with glucose absorption by interacting with the intestinal sodium-dependent glucose transporter (Aoki et al., 2008; Goda et al., 2007; Yokoyama et al., 2007; Singh et al., 2007).

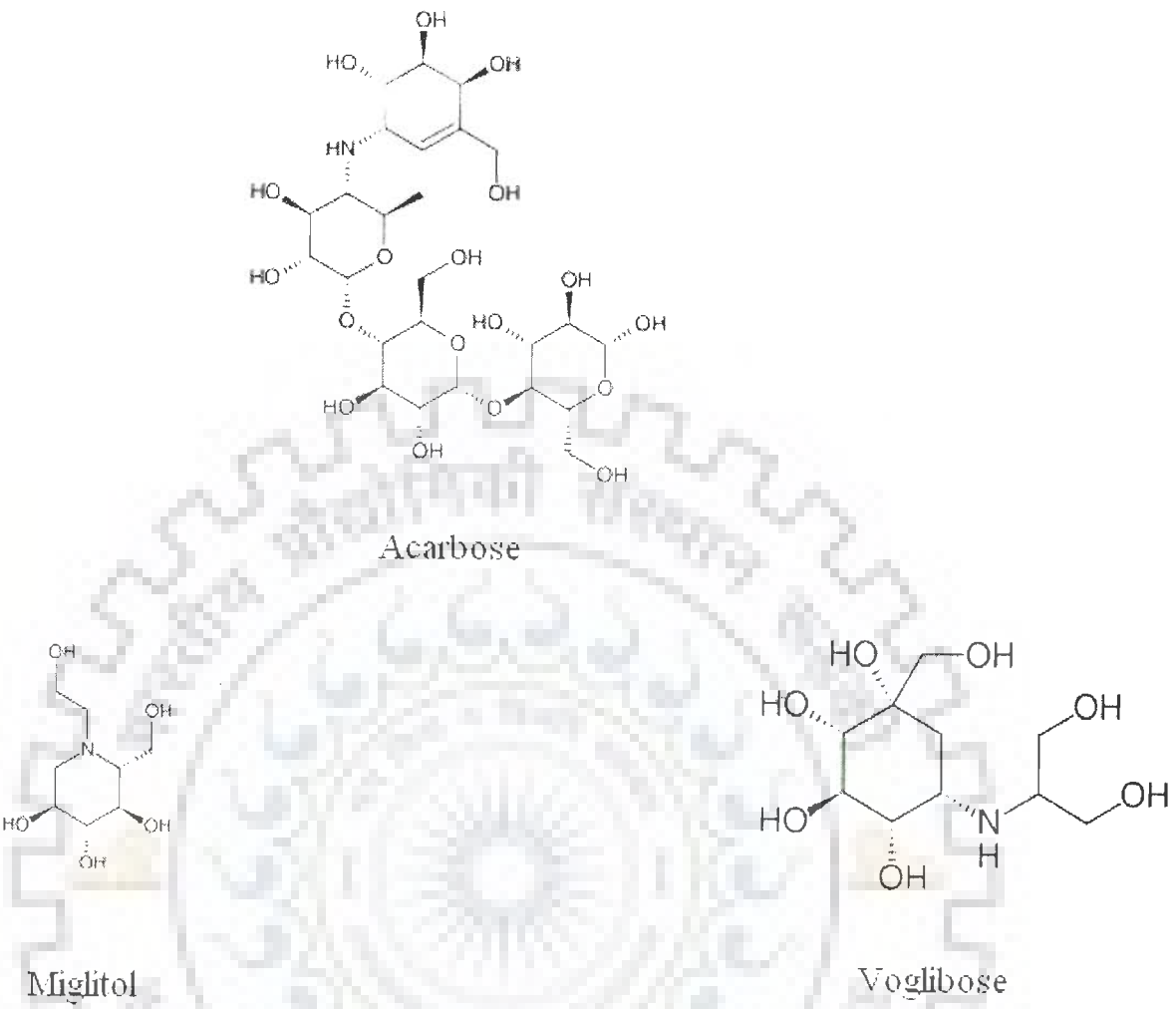


Figure 2.12. Structure of different α glucosidase inhibitors

One of the biggest problem with these inhibitors is that they results in the accumulation of glucose in the intestine. This is because, the α-glucosidase inhibitors prevents the degradation of complex carbohydrates into glucose which finally makes them non-absorbable from the intestine and thus allowing their accumulation in the intestine. In the colon, bacteria will digest the complex carbohydrates, thereby causing gastrointestinal side effects such as flatulence and diarrhea (Defronzo, 1999; Cheng and Fantus, 2005).

2.2.10.2 Traditional therapy

Advances in biomedical research have unraveled complex and nonlinear physiological, biochemical and pathological processes involved in causing/fostering diabetes, along with the multiple cross talk of signaling pathways. Therefore this warrants the use of multiple drugs for different therapeutic targets or a single drug with multiple target potency for the reversal of all or majority of aspects of the diabetes. At the same time conventional therapeutic measurement available for the treatment are associated with severe side effects along with the development of resistance towards a particular treatment across the prolonged administration of the drugs. Also the present conventional therapeutic approach deals with a symptoms or a specific target for a particular disease or disorders not the root cause (Wagner, 2005).

All these drawbacks associated with conventional therapy have triggered the search for a safe and effective alternative source of cure for this disorder. Traditional therapy with a holistic approach and thousand years of experience has come up as the right answer. Traditional therapy comprises, lifestyle management and medicinal preparations, which includes medicinal plants, minerals and organic matters (Tiwari, 2005; Tiwari and Madhusudana, 2005). Medicinal plants are the most important component of these medicinal preparations used by Indian, Chinese, Egyptian, Greek and Roman system of traditional medicines. The plants provide an effective and potential source of hypoglycemic drugs and many plant derived compounds and extracts have been used in the treatment of diabetes. Medicinal plants in the form of extract or herbal formulation have been investigated for their therapeutic potential using modern scientific approach and several active molecules have been isolated/identified and studied for their

role in diabetes treatment (Touny and Banerjee, 2007; Ojewole and Adewunmi, 2003, 2004; Chau et al., 2007). There are a number of reports on compounds from all classes of phytochemicals including flavonoids, alkaloids, terpenes, polysaccharides, peptides and proteins has been isolated and studied for their antidiabetic potential (Mukherjee et al. 2006; Modak et al., 2007; Gilbert, 2008; Bae et al., 2003;Young et al., 2005; Tang et al., 2008). Chemical structure, source and exact molecular mechanism of action of these phytochemicals are explained in the following section.

2.2.10.2.1 Alkaloids

Alkaloids are heterocyclic nitrogen compounds of plant origin with alkali nature and physiological activity. In the plant they exist in a free form or unite to an acid to make a salt. They usually contain one nitrogen atom in the form of primary, secondary or tertiary amine (Fig. 2.13). Basically alkaloids are of three types (i) true, (ii) proto and (iii) pseudo alkaloids, where true and proto alkaloids, both derived from amino acids, differs on the basis of presence or absence of heterocyclic ring, whereas pseudo alkaloids are derived of terpenoids and purines. The pharmacological action of alkaloids varies widely: They are analgesics and narcotic (atropine, homatropine), nervous system stimulator (strychnine, brucine) mydriatics (atropine, homatropine) and myotics (physosigmine, pilocarpin) etc. Alkaloids have restricted distribution among plant kingdom, *Leguminosae*, *Papaveraceae*, *Ranunculaceae*, *Rubiaceae*, *Solanaceae* and *Berbridaceae* etc. are some of the families, containing biologically important alkaloids.

Alkaloids are known for their varied physiological activities since long and hence alkaloid rich fractions and purified alkaloids from numerous medicinal plants have been investigated for their possible antidiabetic activities in *in vivo* and *in vitro* models.

Alkaloid rich fractions isolated from different medicinal plants like *Trigonella foenum graecum, Percea Americana, Commelina communis* have been shown to be effective in management of diabetic condition in several ways (Wang et al., 2007; Helmy et al., 2007; Antia et al., 2005). Apart from crude fractions, purified alkaloids have also been studied for their antidiabetic and hypolipidemic properties. Allicin, berberine and boldine isolated from different plant species are some of the typical examples of such alkaloids which have showed marked potential as hypoglycemic and hypolipidemic agents (Yin et al. 2008; Huang et al., 2006; Ashraf et al., 2005; Konrath et al., 2008; Brien et al., 2005; Dikshit et al., 2006). Specifically, increased AMPK activity, Glut-4 translocation, glycolysis, glucose metabolism, increased and additional antioxidant activities has been reported in berberine treated mice (Huang et al., 2006).

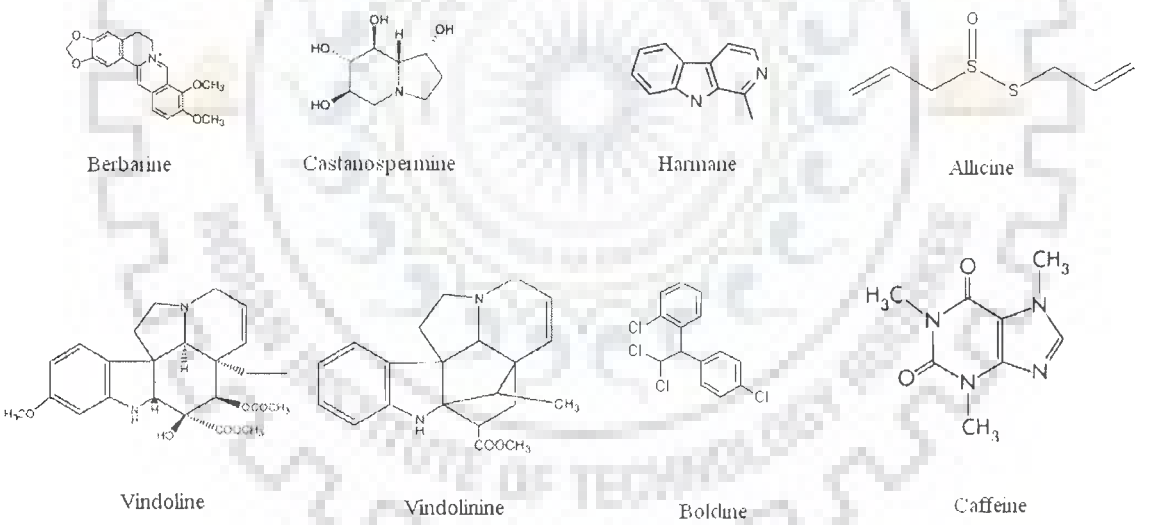


Figure 2.13. Structure of different antidiabetic alkaloids

Caffeine (which is found in coffee), castanospermine obtained from *Castanospermus australe* and chlorogenic acids are other alkaloids having the potential to work on different targets of lipid and carbohydrate metabolism, like TNF-α, α-

glucosidase and G6pase respectively (Stegelmeier et al., 2008; Kang et al., 1995; Kagami et al., 2008; Arion et al., 1998). Several alkaloids have been isolated from the active fractions of different plant extracts with insulinotropic effects for example sparteine and β-carboline. Treatment with sparteine results in increased insulin secretion and lower blood glucose level in a healthy man. Whereas different β-carboline like harmane, norharmane and pinoline stimulates insulin secretion from isolated human islets of Langerhans (Sgambato et al., 1987; Bobkiewicz-Kozłowska et al., 2007; Kirtikar and Basu, 1993). PTP-1B is a new target for antidiabetic treatment which has been shown to be inhibited by an alkaloid called urosolic acid; isolated from a wide variety of plant species (Zhang et al., 2006). Apart from these stated examples some other alkaloids have also been isolated from different plants with antidiabetic properties like thiospartine of *Trigonella foeneum*, tecomine from *Tecoma stans*, vindoline and vinolinine from catharanthus species, harmane and normane from *Tribulus terrestris* and others (Chattopadhyay, 1999).

2.2.10.2.2 Carbohydrates

Carbohydrates are a group of organic compounds consisting of carbon, hydrogen and oxygen usually in the ratio of 1:2:1. They are aldehyde or ketone derivatives of polyhydric alcohols containing carbon, hydrogen and oxygen. Depending upon their complexity and behavior on hydrolysis the carbohydrates are classified into two categories: sugars and non sugars. Polysaccharides are the non sugar carbohydrates with a general formula of $C_nH_{2n}O_n$ where 'n' is usually a large number between 200 and 2500.

Polysaccharides from different plants have the potential to affect the glycemic index of the ingested food, which gives them enormous potential to control the post

prandial hyperglycemia. Dietary carbohydrates and fibers improves glycemic control by reducing or delaying the absorption of carbohydrates and also decreases blood cholesterol level by reducing cholesterol absorption and increasing bile-acid excretion. Polysaccharides from *Lyceum barbarum, Artemisia sphaerocephala*, Fucan from *Himanthalia elongate* and a protein bound polysaccharide from pumpkin reduces blood glucose level , improves insulin resistance and lipid profile in high fat diet and STZ induced diabetic rats (Li, 2007, Zhang et al., 2006; Quanhong et al., 2005). Apart from regulation of post-prandial glucose, polysaccharides from *Pleurotis citrinopiliatus* and polysaccharides CS-F30, CS-F10 of *Cordyceps sinensis* have a marked effect on carbohydrate regulatory enzyme level i.e. G6Pase, hexokinase and glucose 6 phosphate dehydrogenase (Kiho et al., 1996; Hu et al., 2006).

Another study involving insoluble fiber-rich fractions including insoluble dietary fiber, alcohol insoluble solid, and water-insoluble solid, obtained from the peel of *Citrus sinensis* showed that the fiber rich fraction effectively check glucose, retard glucose diffusion and inhibit the activity of alpha-amylase to different extents and may be responsible for decreasing the rate of glucose absorption and concentration of postprandial serum glucose (Chau et al., 2003).

2.2.10.2.3 Phenolic compounds

Phenolic compounds are composed of one or more aromatic benzene rings and attached with hydroxyl groups (C-OH). This enormous class includes numerous plant compounds with structural diversity. On the basis of basic skeleton and number of carbon atom phenolic compounds are divided into 11 classes (Table 2.2).

Table 2.2. Different classes of phenolic compounds

Number of C-atoms	Basic skeleton	Class
6	C_6	simple phenols, benzoquinones
7	$C_6 - C_1$	phenolic acids
8	$C_6 - C_2$	acetophenone, phenylacetic acid
9	$C_6 - C_3$	hydroxycinnamic acid, polypropene, coumarin, isocoumarin
10	$C_6 - C_4$	Naphtoquinone
13	$C_6 - C_1 - C_6$	Xanthone
14	$C_6 - C_2 - C_6$	stilbene, anthrachinone
15	$C_6 - C_3 - C_6$	flavonoids, isoflavonoids
18	$(C_6 - C_3)_2$	lignans, neolignans
30	$(C_6 - C_3 - C_6)_2$	Biflavonoids
n	$(C_6 - C_3)_n$ $(C_6)_n$ $(C_6 - C_3 - C_6)_n$	lignins catecholmelanine (condensed tannins)

Of these various polypenolic compounds, flavonoids constitute of the major class of compounds within this group. Flavonoids are derived from the C_{15} body of flavone. They differ from other phenolic substances in the degree of oxidation of their central pyran ring and, also in their biological properties. The variability of the flavonoids are largely based on the hydroxylation and/ or methylation pattern of the three ring systems (Fig. 2.14). They are widely distributed in plant kingdom and exhibit distinctive pharmacological properties (Murali et al., 2007; Makela et al., 1998). The flavonoids can be widely classified into different categories like flavanols, flavones, catechins, flavanones, anthocyanins etc. Flavonoid shows hypoglycemic effect by altering glucose and oxidative metabolism. Flavonoid rich fractions from *Loandra lacunose* and *Eugenia jambolana* (as described in subsequent chapters) shows significant hypolipidemic and hypoglycemic effect in STZ induced diabetic models (Cunha et al., 2008; Sharma et al., 2008 a,b).

Quercetin is an important flavonoid known to possess a vast array of pharmacological activities. Intraperitoneal administration of quercetin to normal as well as STZ induced diabetic rats resulted in marked reduction in plasma glucose level of diabetic animals while the glucose level of the normoglycemic rats remained unaltered. Quercetin also suppressed the glucose level in diabetic rats in a glucose tolerance tests, reduced plasma cholesterol and triglycerides significantly and increased their hepatic glucokinase activity probably by enhancing the insulin release from pancreatic islets of the diabetic rats (Vessal et al., 2003). Quercetin, naringenin and chrysin significantly enhanced the insulin release from isolated rat islets of Langerhans in the presence of 20 mmol glucose/l (Hii and Howell, 1985). Citrus bioflavonoids, hesperidin and naringin shows multiple effects on blood glucose level, hepatic glucose-regulating enzymes hexokinase, fructose-1,6-bisphosphatase, G6Pase and PEPCK etc., hepatic glycogen concentration, and plasma insulin levels in diabetic animals (Jung et al., 2004).

Narginin treatment in combination with vitamin C also caused decreased glycated hemoglobin and significant elevation in total hemoglobin and body weight. Further the soy isoflavones, genistein or daidzein, also possess hypoglycemic activity as studied in male and female obese Zucker rats, a model of type II diabetes. PPARs are promiscuous nuclear receptors that regulate the transcription of genes involved in lipid and glucose homeostasis and lipid metabolism within the cell. The isoflavones genistein and daidzein significantly improved lipid and glucose metabolism by acting as a hypoglycemic PPARs agonist and increase glucose tolerance and lowered glucagon content (Mezei et al., 2003).

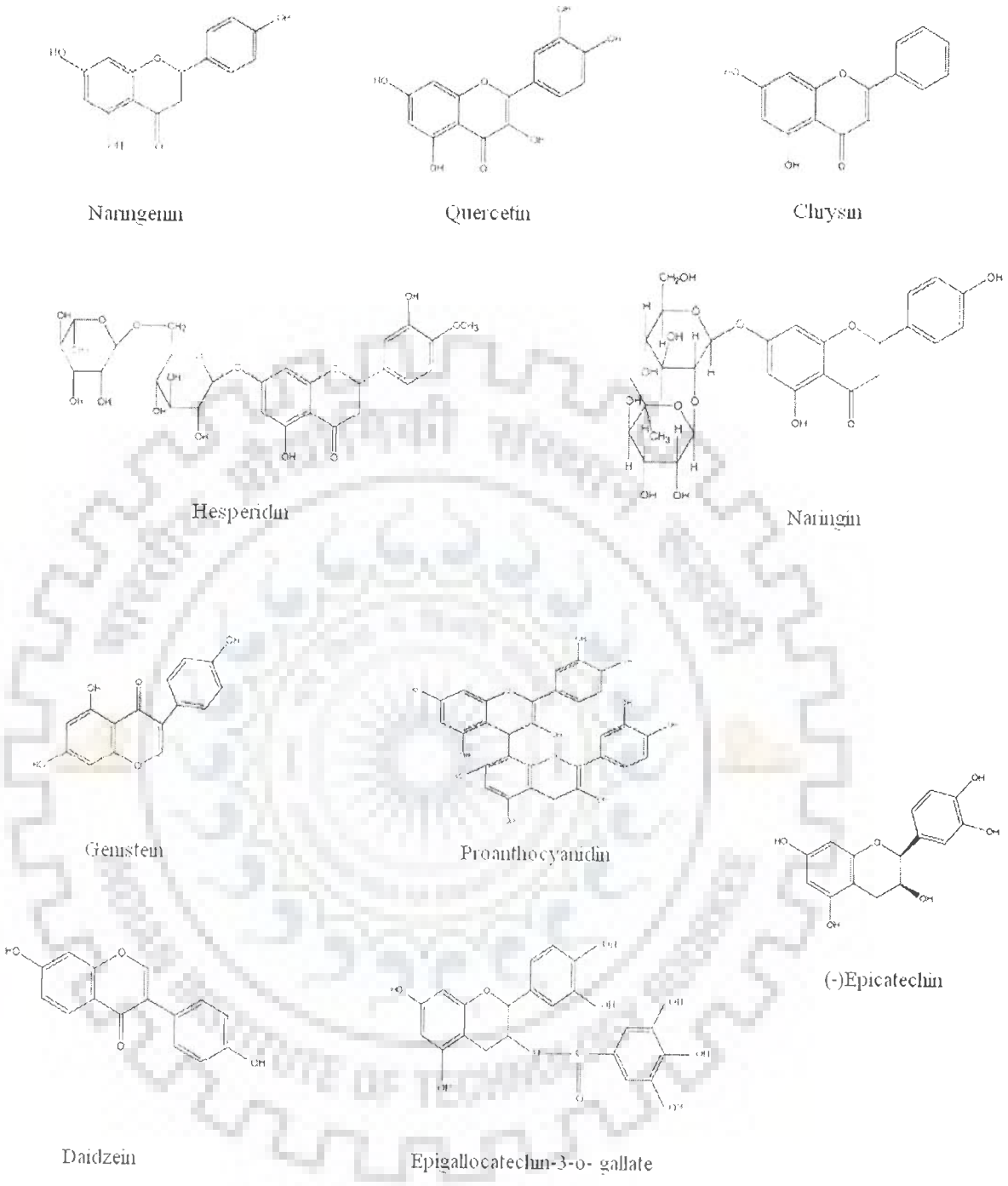


Figure 2.14. Structure of some of the antidiabetic flavonoids

Proanthocyanidins, the flavonoids with an oligomeric structure, improves the pathological oxidative state of diabetic situation. Whereas procyanidins of grape seed produces significant antihyperglycemic activity possibly by it's insulinomimetic activity. Further it also stimulates glucose uptake in insulin sensitive cells *in vitro* (Pinent et al., 2004). Glycoside of flavonoid, Kaempferol 3, neohesperidoside and Kempferitrin (Kaempferol-3,7-O-(alpha)-l-dirhamnoside) stimulates glucose uptake in isolated rat soleus muscle and inhibits preadipocytes differentiation, suggesting that blood glucose lowering activity of the compound attributed to altered intrinsic activity of the glucose transporter (Jorge et al., 2004).

Epigallocatechin gallate, a green tea flavonoid, decreases hepatic glucose production, increases tyrosine phosphorylation of the insulin receptor and inhibits insulin receptor substrate-1 (IRS-1) Ser307 phosphorylation through AMPK activation. It inhibits hepatic gluconeogenesis via activation of 5'-AMP activated protein kinase (AMPK) and also effectively inhibits lipid accumulation in 3T3-L1 cell line. It also reduces PEPCK gene expression in a PI3K dependent manner and mimics insulin by increasing PI3K, MAPK, and p70 (s6k) activity. These findings suggest that epigallocatechin gallate is an important hypoglycemic agent (Waltner-Law et al., 2002). Another flavonoid molecule, (-)-epicatechin, has been reported to possess insulin-like activity and protects the experimental albino rats against the diabetogenic actions of alloxan (Rizvi and Zaid, 2001).

Anthocyanins are a type of water-soluble vacuolar pigment in plants. They belong to a parent class of molecules called flavonoids. Treatment with anthocyanins from *Cabernet sauvignen* resulted in a decreased urine, blood glucose and fatty acid

metabolites concentration in treated animals in comparison to untreated animals (Zhang et al., 2004). Cyanidin 3-O-β-D-glucoside-rich purple corn colour prevented high fat diet induced obesity and insulin resistance along with the suppression of mRNA level of enzymes involved in lipid metabolism (triglycerole synthesis and sterol regulatory elements binding protein-1) in adipose tissue and facilitates triacylglycerol accumulation in white adipose tissue (Tsuda et al., 2003). Pelargonidine, another anthocynidine (sugar-free counterparts of anthocyanins) improves blood glucose, serum lipid, super oxide dismutase (SOD), catalase and fructoseamine profile in STZ diabetic animals (Roy et al., 2008). *Prunus cerasus* anthocyanins show multiple effects for antidiabetic treatment, it reduces fasting blood glucose and improves serum lipids and insulin profile. Further it cures fatty liver and enhances the expression of peroxisome perolifirator activating receptor α (PPARα) level (Seymour et al., 2008).

Stilbenes are organic compounds that contain 1, 2-diphenylethylene as a functional group. There hydroxylated derivatives are called stilbenoids. They are a new group of compound recently explored for their antidiabetic potential. Pterostilbene and resveratrol are the representative members of this family. Pterostilbene have been reported to possess antidiabetic effect by changing the activity of different carbohydrate metabolism regulatory enzymes (Pari and Satheesh, 2006; Pan et al., 2008). Further antidiabetic properties and multi target potency of pterostilbene for the treatment of diabetes have been described in detail in subsequent chapters, where pterostilbene has shown to significantly reduce hyperglycemia and improves liver glycogen content. Different therapeutic target of antidiabetic treatment were studied in detail and pterosrilbene has proved its efficacy for the management of diabetes.

2.2.10.2.4 Terpenes/Terpenoids

Terpenes are the largest group of naturally occurring plant metabolites with over 20,000 known structures. Terpenes biosynthetically derived from isoprene units (C_5H_8) can exist as hydrocarbon chain or have oxygen containing compound such as hydroxyl or carbonyl groups called terpenoids. Terpenes are classified on the basis of isoprene units present in a particular molecule i.e. monoterpenoids (n=2), sesquiterpenoids (n=3), diterpenoids (n=4) etc. (Fig. 2.15). This group is further subdivided into different sub-groups on the basis of rings present in their structure. The skeletal structure of terpenoids may differ from strict addition of isoprene units by the loss or shift of a fragment, generally a methyl group.

Terpenoids are most widespread, chemically interesting and structurally diversified group of compounds with various physiological affects. Several terpenoids have been isolated from different plant families with antidiabetic potentials. Stevioside is a diterpene stervial glycoside extracted from leaves of *Stevia rebudiana*, which possesses insulinotropic, glucagonostatic and antihyperglycemic effect (Jeppesen et al., 2000; Chang et al., 2005). Aviloside a triterpenoid isolated from bitter melon showed antidiabetic effects both in L6 myotubuls and 3T3-L1 adipocytes. It stimulates Glut-4 translocation and fatty acid oxidation (Tan et al., 2008). Ginsenoside RH2 and intestinal metabolites of ginsenosides isolated from Panax ginseng root increases insulin secretion by the stimulation of muscarinic (MC3) receptor in pancreatic cells and also shows antiobesity effect via AMPK signaling pathway (Lee et al., 2007; Hwang et al., 2007).

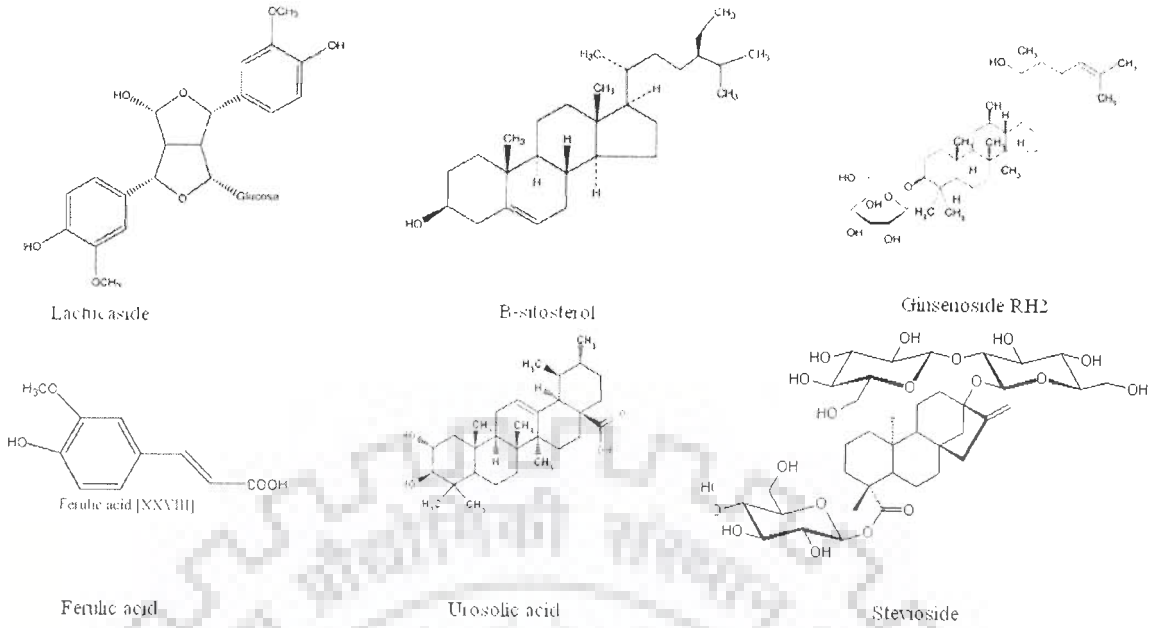


Figure 2.15. Structure of some antidiabetic terpenoids

Corosolic and urosolic acid, from banana leaves and Chinese medicinal plants respectively have significant effect on Glut-4, PTP-1B and gluconeogenesis (Klein et al., 2007; Miura et al., 2004; Zhang et al., 2006). A steroid (triterpenoid) obtained from *Azaractica indica*, has also been shown to have a potential hypoglycemic property. Fucoxanthone a marine carotenoid (tetraterpenoid) treatment resulted in the down regulation of leptin and TNF-α in white adipose tissue. It also improves blood glucose and insulin profile (Shiratori et al., 2005). Carotenoids like lutein and zea xanthia from plants chromoplasts are efficient free-radical scavenger and prevent oxidative damage in retina (Kowluru et al., 2008). Other terpenes like β-sitosterol, lactucain A and lactcaside have also been reported recently to show potent antidiabetic ability in STZ induced diabetic rats (Tan et al., 2008).

2.2.10.2.5 Peptide /protein

Protein and peptide molecules are another class of complex nitrogenous substances. They are biopolymers containing large number of amino acids joined to each other by peptide bonds. Peptide and proteins from different medicinal plants and prokaryotes have been isolated and studied for their hypoglycemic effects and this insulin like molecules are called as glucokinin (Fidalgo et al., 1991 and Xavier-Filho et al., 2003). However, the presence or absence of glucoregulatory glucokinins in plants species is yet a matter of controversy.

Charantin is insulin like molecule isolated from an well known anti-diabetic plant bitter guard. A number of studies are available on the isolation and activity analysis of charantin from bitter guard (Krawinkel and Keding, 2006). Other than bitter guard, hypoglycemic peptide molecules with sequence similarity with insulin have been reported in different plant species i.e. bryophytes (mosses), psilophyta (whisk fern), Lycopodophytea (selaginela), gymnosperms (conifers, cycades) and angiosperms (Xavier-Filho et al., 2003). Insulin like molecules were also isolated and characterized from spinach and *Lemna gibba* G3 and others (Collier et al., 1987). Protein with amino acid sequence homologous to bovine insulin has also been isolated from two leguminous plants, *Vigna unguiculata* (cowpea) and *Canavalia ensiformis* (jack bean) (Venâncio et al., 2003; Silva et al., 2002).

2.3 CONCLUSION

Medicinal plants with the enormous therapeutic potential have come out as a new resource for the treatment of different diseases. In last few years several phytochemicals have been isolated and studied in detail for their physiological effects and has proven to

be an effective candidate for commercial uses. Apart from being safe in comparison to synthetic available rivals these phytochemicals are effective in those cases also where conventional therapeutic measurements are not available or fails to act. The only draw back associated with traditional medicines is the lack of standardization and authentication. In order to address this drawback in a better way various strategies along with modern analytical technique like GCMS, LCMS, HPLC etc. has been developed. By implementing these techniques several active phytochemicals have been isolated from different medicinal plants known for their therapeutic potential and in some cases herbal formulations comprising of various plant extracts has been standardized on the basis of qualitative and quantitative analysis of their active ingredients. All these techniques in addition to isolation of phytochemicals also helped further for proper validation of these chemicals making them further safe for human consumption. Taken all together, current scientific developments and increasing interest in the area of phytomedicins has given a new thrust to the pharmaceutical industries for the management of diabetes mellitus and other diseases.

CHAPTER- III

CHAPTER III

MATERIAL AND METHODS

3.1 MATERIALS

The routine chemicals used in this thesis were purchased from the SRL (Mumbai, India), SD Fine Chemicals (Mumbai, India), Himedia (Mumbai, India) and Rankem (Mumbai, India). Some of the specialized chemicals like streptzotocin, quercitine, kaempferol, myristine and genestien, sodium vanadate, acarbose and collagenase etc. were from Sigma (St. Louis, USA). The chemicals and enzymes used in the molecular biology were purchased from Bangalore Genei (Bangalore, India), Himedia (Mumbai, India), Sigma (St. Louis, USA) and Genescript (Bangalore, India). The media and other reagents used in the cell culture were obtained from GIBCO-BRL (Rockville, USA). Kits used for Glucose estimation was obtained from Excel Diagnostics Pvt. Ltd. (Hyderabad, India). The kits used for the estimation of total cholesterol (TC), high density lipoprotein (HDL) cholesterol and triglycerides (TG) were from Transasia Bio Medical Ltd. (Mumbai, India). The insulin estimation kit was obtained from Cayman Chemicals (MI, USA). Radioisotopes were obtained with the kind assistance of Dr. Subeer S. Majumder, National Institute of Immunology, Delhi, India. The antibodies used in this study were from Santacruz Biotech (Santacruz, USA), kindly provided by Dr. Arun Bandyopadhyay, Indian Institute of Chemical Biology, Kolkata, India.

3.2 METHODOLOGY

3.2.1 Samples preparation

In the present thesis, the experiments were carried out in two phases:

Phase 1: Initial screening was carried out for following 9 medicinal plants as an aqueous or ethanolic extracts as shown in Table 3.1.

Phase 2: Out of the 9 plants screened above, 4 most promising plants were selected i.e. *Eugenia jambolana* (*EJ*), *Capparis decidua* (*CD*), *Commiphora mukul* (*CM*) and *Pterocarpus marsupium* (*PM*) for further analysis.

Table 3.1. Medicinal plants and solvent system used for extract preparation

Plant Name (Indian names)	**Plant parts used**	**Solvent system**
Tinospora cordifolia (Guduchi)	Stem	Ethyl alcohol
Phyllanthus emblica (Amla)	Dried fruits	Ethyl alcohol
Murraya koenigii (Curry leaves)	Leaves	Ethyl alcohol
Capparis decidua (Kair)	Dried fruits	Ethyl alcohol
Eugenia jambolana (Jamun)	Seeds	Ethyl alcohol
Aegle marmelos (Bael)	Leaves	Water
Pterocarpus marsupium (Beejak)	Stem	Ethyl alcohol
Eucalyptus globulus (Safeda)	Leaves	Ethyl alcohol
Commiphora mukul (Guggul)	Resin	Ethyl alcohol

Out of these plants, first two plants were analyzed for a rich fraction of a group of phytochemicals like flavonoids and alkaloids for their antidiabetic activities. For the rest i.e., *Commiphora mukul* and *Pterocarpus marsupium*, active principle was further purified to almost homogeneity (*guggulsterone* and *pterostilbene*, respectively) and studied in detail for their modes of action.

3.2.1.1 Plant materials

Various parts of the selected plants were used for the purpose of screening and identification of the antidiabetic molecules (Table-1). All the plants used in this study were identified and authenticated as per the literature of Ayurveda and by a local expert of herbal gardens and also confirmed by Dr. H. S. Dhaliwal, Professor of Plant Biotechnology, Indian Institute of Technology Roorkee, Uttarakhand, India.

3.2.1.2 Preparation of aqueous extract

A routine procedure was followed for the preparation of aqueous extract (Saenphet et al. 2006). Briefly, various parts of the respective plants were collected and were thoroughly washed with water, and dried in shade. Hundred grams of the air dried parts (for example, leaves) were ground into fine powder and soaked in water for 7-8 h and stirred occasionally. After soaking, the mixture was filtered using Whatmann No. 1 filter paper. The filtrate was then centrifuged at 10,000 rpm at room temperature (25°C) and the pellet was discarded. The supernatant was concentrated up to 100 ml under reduced pressure on a Bucchi rotatory evaporator. The concentrated crude extract was lyophilized into powder. Prepared doses were stored at -20°C until gavaged to animals. For final doses the extracts were dissolved in 0.01% ethanol.

3.2.1.3 Preparation of ethanolic Extract

Ethyl alcohol was used for general organic solvent extraction and the procedure followed was according to the methods described earlier (Kokate et al., 1994). Briefly the plant materials used for the extraction was thoroughly washed and dried in shade. Dried plant material was then ground to fine powder in presence of liquid nitrogen (LN_2) and soaked in 75% ethanol for 24 h with continuous stirring. The procedure was repeated

thrice to ensure the maximum extraction. Extracts were then pooled together and centrifuged at 10,000 rpm at room temperature. The pellet was then discarded and the supernatant was concentrated under reduced pressure on a Bucchi rotatory evaporator. Finally, the concentrated crude extract was lyophilized into powder. For final doses extracts were dissolved in 0.01% ethanol prepared doses were stored at -20 °C until used.

3.2.1.4 Preparation of flavonoid rich fraction

Flavonoid rich fraction was prepared using a general flavonoid extraction method as reported earlier (Harborne, 1998). Briefly, the plant materials (for example, *EJ* seeds) were thoroughly washed with water, and dried in shade. One hundred grams of air dried material were then ground in fine powder and soaked in 75% ethanol for 24 h with continuous stirring. This procedure was repeated thrice to ensure maximum extraction. After soaking, the extracts were then pooled together and the mixture was filtered with Whatmann No.1 filter paper. The filtrate was then centrifuged at 10,000 rpm at room temperature (25°C) and the pellet was discarded. The supernatant part of the extract was then concentrated under reduced pressure on a Bucchi rotatory evaporator. The concentrated extract was then dissolved in as little water as possible and washed three times with chloroform to remove lipids. Residual layer was extracted three times with ethyl acetate (EtOAc) and all the extracts were then pooled together and concentrated using Bucchi rotatory evaporator. Ethyl acetate extract was re-dissolved in 20 ml. of water.

For further purification of desired flavonoid rich fraction, polyamide column chromatography was used. The peptide groups of polyamide absorbs polar compounds

containing hydroxyl and carboxyl groups like phenolic compounds from methanolic and aqueous solutions under reverse phase mode.

The polyamide resin (particle size 50-160 μm) was packed into a glass column (0.5 cm x 30 cm). In order to obtain flavonoids rich fraction the column was first washed with water to remove any unbound impurity, followed by increasing concentrations of methanol in water to elute the bound flavonoid rich fraction. The enrichment of the flavonoids in the eluted fractions was estimated by the method as described earlier by Mace (1993). Finally the prepared doses were stored at -20°C until gavaged to animals.

3.2.1.5 Preparation of alkaloid rich fraction

Alkaloid rich fraction was prepared by a general acid-base extraction method for alkaloids, according to the methods described earlier (Houghton and Raman, 1998). Briefly, the dried plant material (for example, *CD* fruits) was ground to a fine powder and soaked in methanol for 24 h and stirred occasionally. After soaking the mixture was filtered using Whatmann No.1 filter paper. The filtrate obtained was concentrated under reduced pressure on a Bucchi rotatory evaporator. The concentrated crude extract was then mixed with 1M hydrogen chloride and filtered again to remove any precipitate. To this filtrate 2N sodium hydroxide was added slowly to get an off white precipitate. For the extraction of precipitated alkaloids dichloromethane was added at the ratio of 100 ml/l of extract and mixed thoroughly for 10 min and then left for another 15 min for settling down of dichloromethane layer. Dichloromethane layer was then separated and this procedure was repeated thrice. All the extracted dichloromethane layers were pooled together and concentrated on Bucchi rotatory evaporator. Extract was freed of solvent and quantification of alkaloids was done by the Dragondorff's reagent (5% bismuth

nitrate, 4% acetic acid and 2% of KI). Total yield was 0.97% w/w of the starting material. Extract was stored at -20°C until used.

3.2.1.6 Purification of guggulsterone

Guggulsterone was purified following the method of Bajaj and Dev (1982) with slight modifications as per our laboratory conditions. Briefly, the resin was soaked in EtOAc at room temperature for 24 h with continuous stirring. Extract was then filtered and procedure was repeated four times to ensure complete extraction and all the extracts were then combined together. Combined extract was then concentrated on Bucchi rotatory evaporator. Concentrated EtOAc extract was then further washed with 3N hydrogen chloride and 10% sodium bicarbonate to get a neutral fraction. Neutral fraction was washed three times with brine and again concentrated on rotatory vacuum evaporator and a dark brown gummy neutral fraction was obtained. Mixture of the earlier obtained neutral fraction along with 10% semicarbazide on silica and toluene were stirred and heated at 60-65°C for 14 h. The mixture was then cooled at room temperature and filtered. Silica was then washed with toluene three times and refluxed with 10% oxalic acid and toluene for 2.5 h and then filtered. Silica gel was then extracted several times with EtOAc and combined extracts were then washed with water and brine and finally freed of solvent to get the required ketonic fraction. Ketonic fraction was further purified using silica gel column chromatography as described below and schematically shown in fig 3.1.

For silica gel column chromatography, silica gel (mesh size 150-200) was packed in a glass column and equilibrated with n-butanol. Concentrated ketonic fraction was loaded on the column. The column was then initially washed with n-butanol, followed by

elution with increasing amount of EtOAc in benzene (C_6H_6). Fractions rich in guggulsterone E and Z were then eluted with 15-25% EtOAc in C_6H_6. Extract was then further concentrated, freed of solvent and stored at -20°C prior to use.

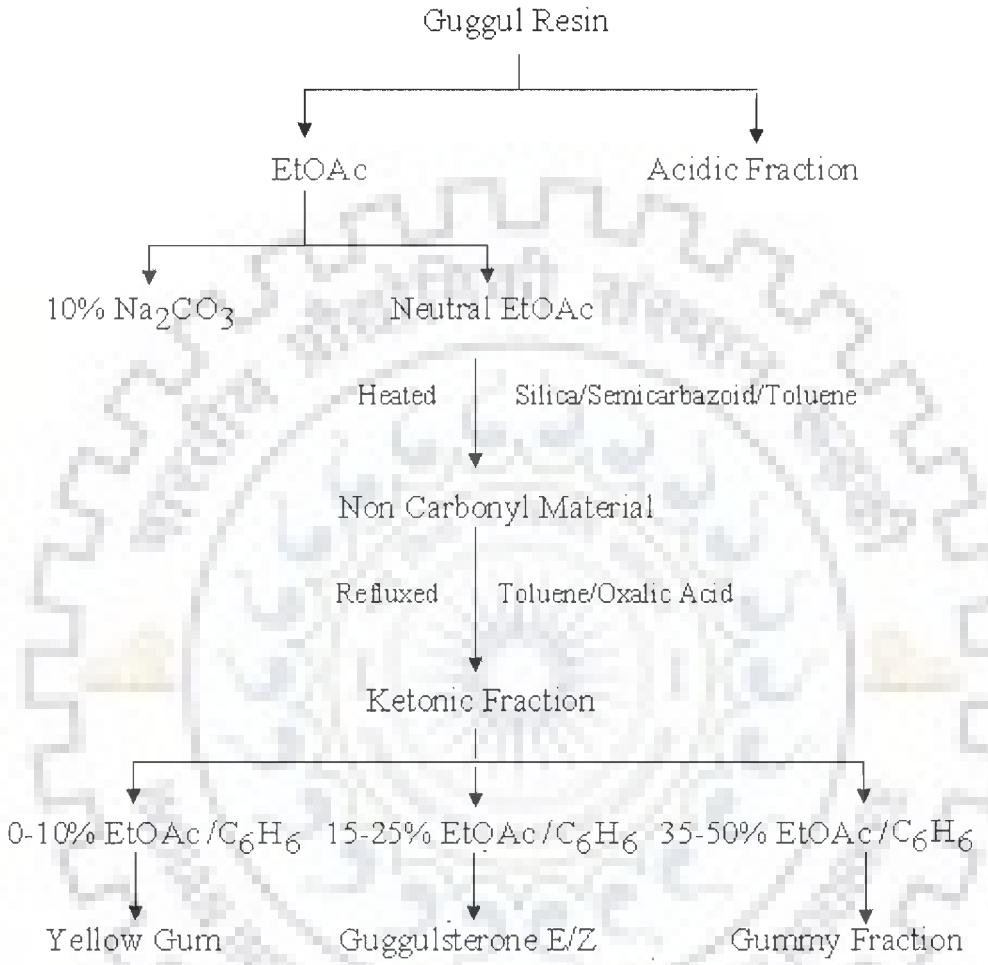


Figure 3.1. Schematic presentation of purification of Guggulsterones from *Commiphora mukul*

3.2.1.7 Purification of pterostilbene

Stem pieces of *Pterocarpus marsupium* (*PM*) were thoroughly washed with water, and dried in shade. Dried stem pieces were ground into fine powder and overnight socked in warm water with continuous stirring. After soaking, the mixture was filtered with Whatmann No.1 filter paper. Procedures were repeated until water stopped changing

its color after soaking. Combined filtrate was lyophilized to obtain a concentrated solution.

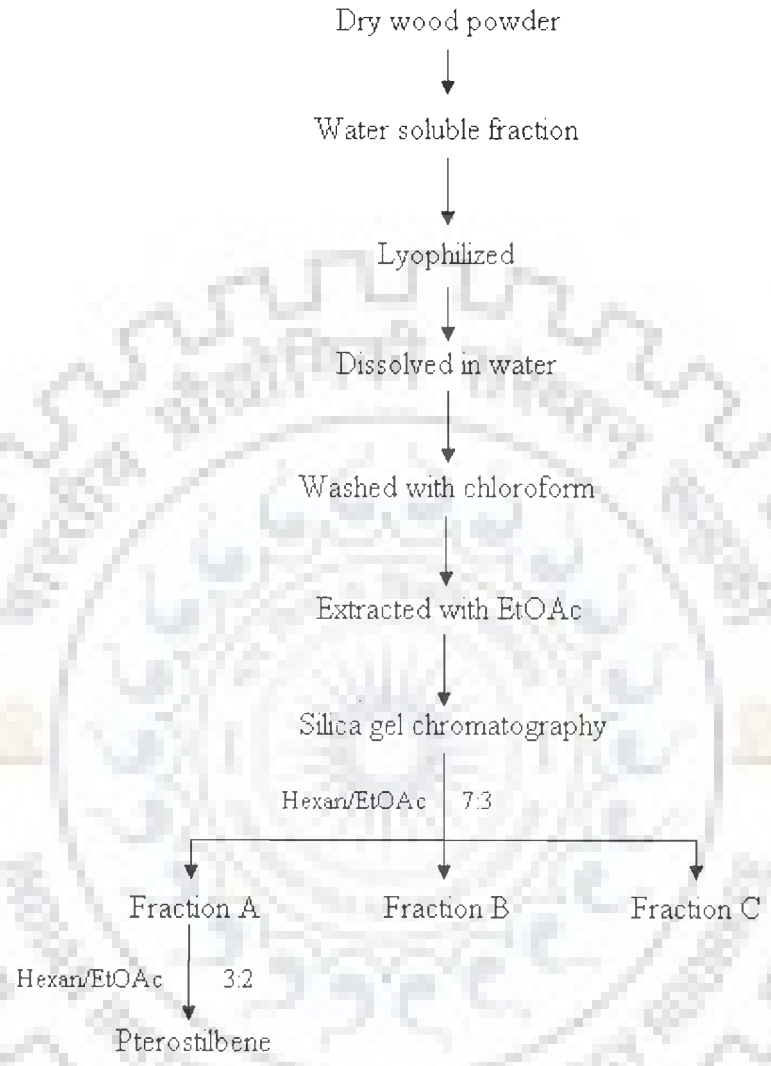


Figure 3.2 Schematic presentation of *Pterostilbene* purification from *Pterocarpus marsupium*

The powder was then re-dissolved in as little water as possible and washed three times with chloroform to remove lipids. Residual layer was extracted three times with EtOAc. All the extracts were then pooled together and concentrated using Bucchi rotatory evaporator under reduced pressure. Obtained viscous material was chromatographed over silica gel column using *n*-hexane, EtOAc and MeOH as elutents.

Elution with hexane and EtOAc (3:1 ratio) gave an active fraction A; showing potent antihyperglycemic effect. Active compound was identified as *pterostilbene* using commercial standard with the help of HPLC. The whole procedure was performed in dark to avoid any photo conversion of active components.

3.2.2 Experimental Models (Animals)

The *in vivo* study was performed using adult male albino rats, *Rattus norvegicus* and mice, *Mus musculus* of age group almost 8-10 weeks. Animals were purchased from the animal house facility of All India Institute of Medical Sciences (New Delhi, India), National Institute of Pharmaceutical Education & Research, (Chandigarh, India) and Zamia Hamdard University (New Delhi, India) and were in healthy condition at the time of purchasing. They were housed in a well-ventilated animal house at a temperature of 22–23°C, humidity 50–55% and lighting cycle of 14 h light: 10 h dark. The animals were fed with a balanced animal feed (Ashirwad Animal Feed Industries; Punjab, India) other than specified and had access to hygienic drinking water *ad libitum*. The animals were acclimatized to the animal house condition for 10 days prior to beginning of each of the experiments. All the procedures used in this thesis with animals were approved by the Institutional Animal Ethics Committee and confirmed to the UFAW Handbook on the Care and Management of Laboratory Animals.

3.2.2.1 Streptozotocin

Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is a commonly used substance in development of diabetic animal models for the study of diabetes. Originally isolated from *Streptomyces achromogenes* as an effective broad-spectrum antibiotic drug, STZ also possesses anti-tumour, diabetogenic and oncogenic

properties. Rakieten and his associates first demonstrated the diabetogenic property of STZ in dogs and rats in 1963.

The STZ molecule consists of a glucose residue with a methylated nitrosurea linked to carbon-2 (Fig. 3.3). Its nitrosourea moiety especially at the O_6 position of guanine is responsible for β-cell toxicity, while deoxyglucose moiety facilitates transport across the cell membrane via Glut-2 glucose transporter. Inside the cell, STZ is decomposed and reactive methylcarbonium ions are produced, which may alkylate the DNA and cause cross-links between the DNA strands. This leads to the activation of DNA repair mechanisms and increases in the activity of poly-ADP-ribose synthetase, which in turn depletes NAD in β-cells. This causes a deficiency in cofactors for oxidative phosphorylation with subsequent lack of ATP, and causes diminished protein synthesis, insulin release and reduced activity of the ion pumps, which ultimately lead to β-cell death (Fig. 3.3).

For the induction of diabetes over night starved rats/mice were injected with STZ at a dose of 60 mg/kg bw. The chemical was injected intra-peritoneally (i.p.) within 10 min after dissolving in 0.025 M sodium citrate at pH 4.0. The animals from control groups were injected with sodium citrate buffer as vehicle control. Fasting blood glucose (FBG) was estimated at the time of induction of diabetes and post prandial glucose (PPG) was checked regularly until stable hyperglycemia was achieved. The rats/mice exhibiting blood glucose level ~250 mg/dl and above, were included in the study as stable hyperglycemic animal.

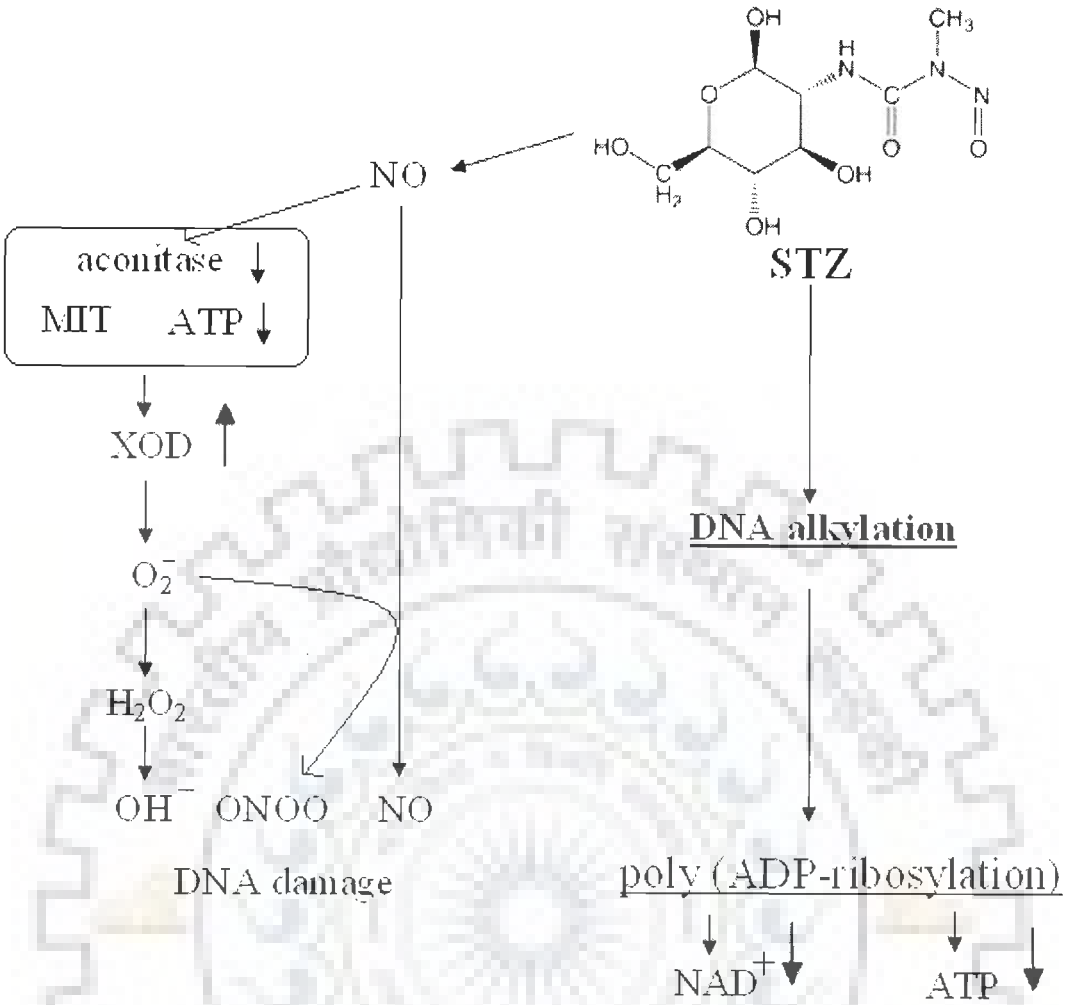


Figure 3.3 Mechanism of streptozotocin induced toxic events in β-cells of pancreas

3.2.2.2 High fat diet

High fat diet induced diabetic rat models were used to study the effect of test compound in type II diabetic condition because diet-induced obese animals are considered to be a better comparable model for human obesity than single mutation genetically obese one (Winzell and Ahren, 2004).

The formulation of the high fat (HF) diet was according to the D#12451 (D12451; Research Diets, New Brunswick, NJ), containing 45% fat (w/w). Lard with high

cholesterol content was the key ingredient at the expense of sucrose and corn starch to achieve the desirable energy content derived from fat source.

All powder ingredients (as shown in Table 3.2) were added into the mixer and mixed well for 5 min. Lard and corn oil was then added and the mixture was finally mixed for another 10 min. Prepared HF diet was stored at -20°C until use. The diet in storage could last for 14 days.

Table 3.2. Composition of Experimental High Fat Diet.

Ingredients	**gm%**	**kcal%**
Casein	200	800
Corn starch	73	291
Sucrose	173	691
Cellulose	50	0
Soybean oil	25	225
Lard	178	1598
Maltodextrin 10	100	400
Mineral mix	10	0
Vitamin mix	10	40
Choline bitartrate	2	0
L-cystine	3	12
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0

3.2.3 Treatment

In the present thesis, the study comprised of two parts- the first part deals with the initial screening of some medicinal plants for antidiabetic potential and the second part deals with the detailed analysis of some of the chosen plants from the initial screening in order to identify the active principles and understanding their mode of actions. Different animal group pattern, dose level, diabetic models and other related information will be described in respective sub-chapters of Chapter 4.

3.2.4 In-vivo study

3.2.4.1 Plasma Glucose Measurement

Blood glucose was measured using GOD-POD glucose estimation kit (Excel Diagnostics Pvt. Ltd. Mumbai, India) which is a colorimetric assay based on enzymatic reactions. In this experiment, glucose is firstly oxidized by glucose oxidase (GO) to produce hydrogen peroxide (H_2O_2) which in turn in the presence of 4-aminoantipyrineand phenol, is converted to a red-violet quinone complex under the action of peroxidase (PO). The intensity of the color development is measured at 510 nm which is proportional to glucose concentration.

For the reaction, all samples and standard were prepared in triplicates. Ten micro liter of sample or glucose standard was added to 1 ml of glucose colour reagent. Reaction mixture was then vortexed and incubated at 37°C for 5 min. The absorbance was measured with UV/Vis spectrophotometer (Perkin Elmer, USA) at 510 nm within 60 min.

3.2.4.2 Oral glucose tolerance test

The Oral glucose tolerance test (OGTT) is the 'gold standard' for the diagnosis of diabetes mellitus. It is a useful epidemiological tool and has been used to determine the

prevalence of diabetes. In this test following a standard glucose load, plasma and urine glucose levels are monitored at regular intervals.

OGTT was performed in all the groups over night fasted animals after completion of the treatment. During fasting, the animals were allowed to drink water. Oral administration of glucose load of 1 g/kg bw in 0.1 ml water was used for the study and the blood samples were collected form the tail vein at 30, 60, 90, 120 and 150 min. Blood glucose was measured using GOD-POD glucose estimation kit (Excel Diagnostics Pvt. Ltd., Ahmedabad, India) as described earlier. The results were expressed in terms of milligram per deciliter (mg/dl) of blood.

3.2.4.3Estimation of lipid profile in blood samples

On completion of the treatment, blood samples were collected and lipid profile i.e. total cholesterol, HDL cholesterol and TG levels in serum were determined according to the instruction of the manufacturer (Transasia Bio Medical limited Mumbai, India) .

3.2.4.3.1 Triglyceride estimation (Enzymatic/GPO Trinder method)

Quantitative determination of triglycerides concentration in serum was based upon a modified Enzymatic/GPO Trinder, Endpoint reaction method (Trinder, P., 1969). Triglycerides in the samples are hydrolyzed by lipase to glycerol and fatty acids. The glycerol is then phosphorylated by adenosine-5-triphosphate (ATP) to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate in a reaction catalyzed by glycerol kinase (GK). Glycerol-3-phosphate is then converted to dihydroxyacetone phosphate (DAP) and hydrogen peroxide by glycerophosphate oxidase (GPO). The hydrogen peroxide then reacts with 4-aminoantipyrine (4-AAP) and 3-hydroxy-2,4,6-tribomobenzoic acid (TBHB) in a reaction catalyzed by peroxidase to yield a red colored quinoneimine dye.

The intensity of the color produced is directly proportional to the concentration of triglycerides in the sample when measured at 540 nm.

For this reaction, all samples and standards were prepared in triplicates. Ten micro liter of sample or triglyceride standard was added to 1 ml of Triglycerides-GPO Reagent. Reaction mixture was then vortexed and incubated at 37°C for 5 min and absorbance was measured with UV/Vis spectrophotometer (Perkin Elmer, USA) at 540 nm within 60 min.

3.2.4.3.2 Cholesterol estimation (CHOD/PAP method)

Cholesterol estimation was based upon CHOD/PAP end point reaction method (Schettler and Nussel 1975)). Cholesterol esters in the sample are hydrolyzed by cholesterol esterase to cholesterol and fatty acids. The cholesterol is oxidized to cholestro-3-one and hydrogen peroxide by cholesterol oxidase. The hydrogen peroxide then reacts with 4-aminoantipyrine (4-AAP) and ρ-hydroxybenzene sulfonate (ρ-HBS) in a reaction catalyzed by peroxidase to yield a red colored quinoneimine dye. The intensity of the colour produced is directly proportional to the concentration of cholesterol in the sample when measured at 520 nm.

For this reaction, all samples and standards were prepared in triplicates. Ten micro liter of sample or cholestrol standard was added to 1 ml of cholesterol reagent. Reaction mixture was then vortexed and incubated at 37°C for 5 min and absorbance was measured with UV/Vis spectrophotometer (Perkin Elmer, USA) at 520 nm within 60 min.

3.2.4.3.3 HDL estimation

For the estimation HDL in serum sample, serum was mixed with the precipitating reagent consisting of 0.55 mmol/l phosphotungstic acid and 25 mmol/l magnesium

chloride. Serum (0.2 ml) was then mixed in a tube containing the precipitation reagent (0.5 ml) and incubated for 10 min at room temperature. This was then centrifuged for 10 min at 2,000 rpm. The clear supernatant was separated and HDL cholesterol content was determined by the CHOD/PAP method as described above.

3.2.4.3.4 LDL and VLDL estimation

For the determination of VLDL and LDL cholesterol Friedwald's formula was used which states:

VLDL cholesterol= Triglyceride/5

and

LDL cholesterol = Total Cholesterol-(VLDL+ HDL cholesterol).

3.2.4.4 Tissue level of glycogen content

Glycogen content of liver and skeleton muscle was measured according to earlier established method (Maiti et al., 2004; Sadasivam and Manickam, 1996). Samples were homogenized separately in warm 80% ethanol at the concentration of 100 mg/ml and than centrifuged at 10,000 rpm for 20 min. The residue was collected and allowed to dry over a water bath. To each residue 5 ml of distilled water and 6 ml of perchloric acid was added and extracted further at 0°C for 20 min. The collected extract was centrifuged at 10,000 rpm for 15 min and the supernatant was collected. From the supernatant, 0.2 ml was transferred to a graduated test tube and the volume was made up to 1 ml with distilled water. To each tube added 4 ml of anthrone reagent and incubated at 95°C in a boiling water bath for 10 min. The absorbance of the sample was measured with UV/Vis spectrophotometer (Perkin Elmer, USA) at 630 nm after cooling the tube at room

temperature. The amount of glycogen in tissue sample was expressed in microgram of glucose per milligram wet weight of tissue.

3.2.4.5 Serum insulin

For the determination of serum insulin, the blood was collected by cardiac puncture from all intact animals on completion of the treatment and allowed to clot at 4°C overnight. The serum was then aspirated, centrifuged at 2,000 rpm for 10 min and the clear supernatant was used for the assays. The assays were performed using the commercial enzyme immunoassay kits as per manufacturer's instructions (Cayman chemicals; USA).

3.2.4.6 Biochemical estimation of enzyme activity

3.2.4.6.1 Glucose 6 phosphatase activity in liver

Glucose-6-phosphatase (G6Pase) catalyzes the conversion of glucose-6-phosphate to glucose. G6Pase activity was measured by the method of Baginsky et al. (1974). Briefly, the tissue samples were homogenized in ice cold sucrose solution (250 mM). For the reaction, 0.1 ml of sucrose/EDTA buffer (0.25 M / 0.001 M, sucrose/EDTA solution), 0.1 ml of 100 mM glucose-6-phosphate, 0.1 ml of imidazole buffer (100 mM pH 6.5) and 0.1 ml of liver homogenate were added and thoroughly mixed. The reaction mixtures were than incubated at 37°C for 15 min and the reaction was terminated by the addition of 2 ml of TCA/Ascorbate (10% : 3%w/v). The reaction mixture was then centrifuged at 3,000 rpm for 10 min and the supernatant was separated. To 1 ml of clear supernatant 0.5 ml of ammonium molybdate (1% w/v) and 1 ml of sodium citrate (2% w/v) were added

and the absorbance was measured with UV/Vis spectrophotometer (Perkin Elmer, USA) at 700 nm. The enzyme activity was expressed as unit per gram tissue.

3.2.4.6.2 Hexokinase activity in liver

The hexokinase activity was tested based on the reduction of NAD through a coupled reaction with glucose-6-phosphate dehydrogenase (Brandstrupt et al., 1957). The excised liver tissue homogenate was prepared in normal saline. To 0.1 ml homogenate 22.8 ml of Tris magnesium chloride buffer (200 mM Tris and 20 mM $MgCl_2$, pH 8) was added along with 0.5 ml of 0.67 M glucose , 0.1 ml of 16 mM ATP, 0.1 ml of 6.8 mM NAD and 0.01 ml of 300 U/ml of gluose-6-phosphate dehydrogenase. The solution was mixed thoroughly and the absorbance was measured with UV/Vis spectrophotometer (Perkin Elmer, USA) at 340 nm.

3.2.5 In vitro analysis

3.2.5.1 Estimation of glucose uptake by mouse psoas muscle tissue

Psoas muscles, immediately after isolation was placed in a krebs ringer bicarbonate (KRB) buffer (115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4) containing 11.1 mM glucose. The muscle tissue after isolation were processed and incubated following the method described by Gupta et al. (2005). The tissues were cut in to pieces of about equal mass (100 mg) and preincubated for 5 min in CO_2 incubator. The tissues were than incubated with or without insulin (25 µg) in the presence of 100 µg of test sample up to 150 min. Aliquots of 25 µl were removed from incubation mixture at various time points and glucose concentration was determined using GOD-POD glucose estimation kit (Excel Diagnostics Pvt. Ltd., Ahmedabad, India) as described earlier.

3.2.5.2 Induction of insulin release from pancreatic islet cell

Isolation of pancreatic islets cells was made according to the method reported earlier (Xia and Laychok 1993; Gupta et al., 2005) with slight modifications. Briefly, after removing the pancreas they were perfused with Hank's balanced salt solution (HBSS) (pH 7.4) for about 15 min to remove blood and endogenous insulin. This was followed by finely mincing the tissue and then they were incubated for 30 min at 37°C with rapid magnetic stirring in a solution of crude collagenase (4 mg/ml) in HBSS containing 0.3% glucose and 1% bovine serum albumin (BSA) pH 7.4. In the next step, separation of the islets from acinar tissues was done with ficoll (type-400) (Himedia, Mumbai, India) with varying gradient followed by centrifugation. The islets cells were picked up from the interphase of 20–11% gradient by Pasteur pipette. The purity of the islet cells were checked by Gomorois chromium hematoxylin phloxin stain (Gomoroi, 1941). After dividing 10 islets per batch they were pre-incubated with KRB buffer along with 0.2% $NaHCO_3$, 0.38% HEPES, 0.1% insulin- free BSA and 11.1 mM glucose, for 5 min at 37°C in a CO_2 incubator. The induction was continued for another 1 h after adding various concentrations of test samples or buffer for controls. Aliquots of 50 µl were removed from the incubation media at the end of the incubation (i.e. 1 h) and were stored at -20°C till insulin assay. Insulin was measured using commercially available ELISA kit.

3.2.5.3 Cell culture and reporter gene bioassay for peroxisome proliferator activated receptor (PPAR)

HepG2 and 3T3-L1 cell lines used in this study were obtained from National Center for Cell Sciences, Pune, India. The HepG2 cells and preadipocyte 3T3-L1 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (GIBCO-BRL,

Rockville, USA). All the media in addition to the regular antibiotics (penicillin and streptomycin) were also supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids and 1 mM sodium pyruvate as per the culture condition described by Shen et al. (2006).

The plasmids pcMX-mPPARα, pcMX-mPPARγ and PPREx3-tk-Luc were a kind gift of Dr. Ronald M. Evans of The Salk Institute for Biological Studies, California, USA. The reporter gene bioassays were performed as previously described (Shen et al., 2006). Briefly, cells were seeded at 40,000 cells /well in 24-well microtiter plates and incubated for 24 h before transfection. Twenty five nanogram of full length PPAR expression plasmids and/or 250 ng of reporter-gene plasmid (PPREx3-tk-Luc) were cotransfected in HepG2 cells or differentiated 3T3-L1 preadipocytes using transfection reagent (Qiagen Inc.-USA). Transfected cells were exposed to test samples in charcoal stripped medium for 24-36 h. On completion of the treatment the cells were lysed with the lysis buffer (0.6 M NaCl, 0.1 M EDTA, 0.2 M $MgSO_4$, 0.2 M DTT, Triton X-100, 0.08 M Tricine) and the luminescence was measured using luciferin as substrate using a luminomter (BMG, Germany). Luciferase inductions in response to each treatment group were expressed as percent transactivation with respect to reference drugs.

3.2.5 4. Adipocyte differentiation assay and triglyceride estimation

The 3T3-L1 cells were cultured and maintained as previously described by Shen et al. (2006). Cell cultures were propagated at 37°C in a humidified atmosphere of 5% CO_2 in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin (100 U/ml) and 1% streptomycin (100 µg/ml). Induction of differentiation was performed two days post

confluence stage. Cells were maintained in differentiation medium containing DMEM, 10% FBS, 1% penicillin and streptomycin, and inducer cocktail (0.25 mM dexamethasone, 0.5 mM 1-isobutyl-3-methylxanthine (IBMX), and 1 μg/ml insulin) for another four days, with changing of medium every two days. On day 4, the dexamethasone and IBMX were removed with insulin remaining on the cells for an additional two days. Differentiation was allowed to continue in DMEM supplemented with 10% FBS. The test samples were then tested at day 9-14 post-induction. Medium was replenished with appropriate test sample every 2 days. On completion of incubation, the cells were washed with phosphate buffered saline (PBS), lysed with the lysis buffer and cellular triglyceride content was determined using the commercially available kit (Transasia Biomedical Limited, Mumbai India).

3.2.5.5 Oil Red O Staining

For Oil Red O staining the method described by McNeel and Mersmann (2003) was followed. On completion of the treatment the 3T3-L1 cells were washed gently with PBS and fixed with 4% paraformaldehyde (pH 7.4) for 30 min. Lipid droplets were then stained with 0.5% Oil Red O in 60% isopropanol for 20 min and afterwards, the Oil Red O was aspirated from plates and the plates were rinsed with water and dried at 37°C. Finally, isopropanol was used to extract the dye from the cells and the absorbance was measured at 510 nm.

3.2.5.6 PTP-1B inhibitor activity

PTP-1B is an enzyme that is known to dephosphorylate insulin receptor resulting in its inactivation. The inhibitor for this enzyme is one of the lead targets for many pharmaceutical industries for anti-diabetic molecules.

PTP-1B activity was measured by the method of Tjernberg et al. (2004), using Para- nitrophenyl phosphate (pNPP) as substrate. The reaction mixture contained 50 mM NaCl, 1 mM EDTA, 50 mM HEPES, pH 7.2, 1.25 mM pNPP, 1.5 nM recombinant PTP-1B (Biomol, USA) and test samples. The reaction mixture was incubated for 60 min followed by terminating the reaction by the addition of 100 ul NaOH. The absorbance was measured with UV/Vis spectrophotometer (Perkin Elmer, USA) at 405 nm. For the determination of the IC_{50} value, various concentrations of inhibitor and relative percentage inhibition was estimated and final IC_{50} was calculated using GraphPad Prism® software. Parameters were set for competitive inhibition with built-in analyses along with nonlinear regression and one-site competitive binding equation.

3.2.5.7 α-glucosidase inhibition assay

α-glucosidase inhibition activity was measured using mice intestinal α-glucosidase (Juntheikki and Tiitto, 1999 and Tadera et al., 2005). Intestinal tissue was homogenized for 1 min in K_2HPO_4/ KH_2PO_4 buffer (0.1 M, pH 7.0) and then centrifuged for 20 min (10,000g, 48°C). Protein was precipitated from the supernatant with 40-80% saturated $(NH4)_2SO_4$, and the pellet was dissolved in 0.1 M MES (2-(N-Morpholino)ethanesulfonic acid) buffer (pH 5.5) and loaded on a Sephadex G25 column (BioRad, USA) (1.6 × 5 cm) for desalting. Protein was determined by the Bradford

method (Bradford, 1976) with bovine serum albumin (BSA) as a standard. Concentrated crude extract was used for the assay.

A reaction mixture consisting of 300 µl of 56 mM maleate buffer (pH 6.0), 600 µl of 2% of maltose in the buffer and 300 µl of test compound in dimethylesulfoxide (DMSO) was incubated at 37°C for 5 min and than 600 µl of crude enzyme solution was added to the mixture, after further incubation at 37°C for 30 min, reaction was terminated by boiling the reaction mixture in boiling water bath. For the estimation of librated glucose reaction mixture was passed through a short column of basic alumina (Sigma-Aldrich, USA) and the amount of liberated glucose was measured by the glucose oxidase method using a commercial test kit.

3.2.5.8 Adipocyte glucose uptake assay

The 3T3-L1 cells were cultured and differentiated as described in previous section (*3.2.5.4*). After 9 days of induction of differentiation, test compounds were added for an additional 3 days. Since the compounds used in the study were solubilized in dimethysulfoxide (DMSO), control cells were treated with matching concentrations of DMSO and the final concentration of DMSO was kept below 0.1%. At day twelve of differentiation, the cells were rinsed with serum-free DMEM and incubated for 2 h in serum-free DMEM. Then the cells were washed twice with freshly prepared Krebs Ringer Phosphate HEPES (KRPH) buffer (136 mM NaCl, 20 mM HEPES (pH 7.4), 5 mM sodium phosphate buffer (NaH_2PO_4-$Na_2HPO_4 \cdot 7H_2O$, pH 7.4), 4.7 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$). The buffer was removed and the cells were incubated with or without 100 nM insulin in KRPH buffer at 37°C for 30 min. Then the buffer was replaced with 10 µCi/ml of [^{3}H]-2-deoxy-d-glucose in KRPH buffer supplemented with 100 µM 2-

deoxy-D-glucose and was incubated at 37°C for 10 min. After that, the cells were rinsed carefully with ice-cold PBS three times to stop the reaction. Plates were drained briefly and cells were lysed with 250 µl/well 0.2 N NaOH and mixed by pipetting. Two hundred microliters of lysate was added to a scintillation vial and 2 ml of scintillation fluid was added. The vials were mixed and counted in a liquid scintillation counter (Beckman LS 3801). The rest of the lysate was used to determine the protein concentration using the BCA protein assay with bovine serum albumen (BSA) as standard. The glucose uptake values are expressed as "pmol radioactive 2-deoxyglucose taken up per minute/per mg protein."

3.2.5.9 MTT assay

The effects of test compound on cells viability were estimated by MTT assay. In this assay cell reduction of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, a yellow tetrazolium salt) to a blue formazan product by the viable cells was measured (West et al, 2001). For the assay, cells which were previously incubated for 2 h with different concentrations of test compounds and extracts were treated with 100 µl fresh medium containing 0.5 mg/ml MTT for 1 h. Following one hour, the medium was removed and the reduced formazan was dissolved in 100 µl acidified (0.04 N HCl) isopropanol at room temperature for 25 min. The dissolved formazan concentration was then measured in a OASYS plate reader (OASYS UVM 340) at 562 nm wavelength. Control (blank) wells contained only isopropanol.

3.2.6 Histopathology of various tissues

For histopathological staining, the basic protocol by Mukherjee et al. (2003) was followed with modifications according to our laboratory conditions. Briefly, the liver,

adipose and pancreatic tissues were separated and fixed in Bouins solution (saturated aqueous solution of picric acid, 75%; formalin, 25%, glacial acetic acid, 25%) for 4 h. Following the fixation, sections were washed thoroughly in 30% alcohol until all the color of picric acid disappeared. This was followed by gradual dehydration of the samples by placing them in ascending series of alcohols (each change for 1 h) viz. 30% - 50% - 70% - 80% (2 changes) - 95% (2 changes) - 100% (3 changes) -xylene (3 changes). The tissues were then placed in wax, made into blocks and finally cut into sections of 5 micron thickness. Sections were adhered on the glass slide treated with Mayer's solution (per 100 ml having 50 ml egg albumin, 50 ml glycerin, 1 gm sodium salicylate), stretched at approximately 40°C temperatures and preserved at room tempereture. The sections were then stained in haematoxylin and eosin by placing the slide in different solutions in a sequential manner as described below:

Xylene (2 changes, each 5 min) followed by 1 min change each in 1:1 (v/v) xylene and 100% alcohol - 100 % alcohol (2 changes) - 90% alcohol - 80% alcohol - 70% alcohol - washing in tap water followed by gentle tapping of slide - haematoxylin solution (5 min) - washing in running water - 3 to 4 dipping of slide in 0.5% HCl - wash in distilled water (1 min) - several dipping in ammonia water (section changes to blue colour) - wash in distilled water (1 min) - 80% alcohol (1 min) - 95% alcohol (1 min) - eosine staining (2 to 3 min) - 70% alcohol - 95% alcohol - 100% alcohol (1 min each) - xylene (two changes, 1 min each). This was followed by the mounting of tissues with canada balsam and preservation with cover slip.

3.2.7 Western blot analysis

The adipose tissues from various treatment groups were isolated according to the method of Joost and Schurmann, (2001). Adipose tissues were homogenized in Tris-EDTA-Sucrose (TES) buffer (20 m*M* Tris-HCl, 1 mM EDTA, 8.7% sucrose) pH 7.4, and centrifuged at 14,000 rpm for 15 min at 4°C. Solidified fat was discarded and supernatant was transferred to a fresh tube. Supernatants were then quantified for their protein content by Bradford assay using commercially available kit (Bangalore Genei, Bangalore, India). An equal quantity of protein was loaded in 12% polyacrylamide gel to visualize any changes in total protein profiling in the treated rats/ cells as compared to control.

For the prepration of plasma membrane and low density microsomal fraction; homogenized cells were centrifuged at 12,000 rpm for 1 min at room temperature, then for 15 min at -3°C. Solidified fat was discarded and the supernatant was spin at 20,000 rpm for 30 min at 4°C. Supernatant was separated and the pellet containing the high-density microsomes was resuspend and centrifuged again at 20,000 rpm. The supernatant obtained was further centrifuged for 75 min at 200,000*g* (4°C) to pellet the low-density microsomes.

The pellet of the first centrifugation (12,000 rpm,) was resuspended in TES buffer and loaded on the sucrose cushion (38.5% (1.12 *M*) sucrose, 20 m*M* Tris-HCL, 1 m*M* EDTA, pH 7.4, at 4□C) and centrifuged for 60 min at 100,000*g* (4□C) in a swing-out rotor. Plasma membrane rich fraction was collected from interphase and resuspended in TES buffer again followed by 20 min centrifugation at 40,000g (4°C).The resulting pellet contains plasma membranes. Total protein was quantified using Bradford assay

(Bangalore Genei, Bangalore, India) and equal amount of protein content was loaded on gel.

For preparation of 5 ml of 12% resolving agarose gel, 2 ml of 30% acrylamide mix (acrylamide, 29% w/v in warm water and N-N'–methylene-bis-acrylamide, 1% w/v in warm water) was mixed with 1.3 ml of 1.5 M Tris (pH 8.8), 50 µl of each 10% sodium dodecyl sulfate (SDS) (w/v) and 10% ammonium per sulphate (APS) (w/v) and volume was made with deionised water. Twenty micro liter of N,N,N',N'-Tetramethyl-Ethylenediamine (TEMED) (Sigma, USA) was added to the solution which was then rapidly poured (with continuous swirling) into the gel casting tray. Once the resolving gel was cast, the top of the resolving gel was washed several times with deionised water to wash off any unpolymerised acrylamide. A 5% stacking gel (containing 170 µl of 30% acrylamide mix, 130 µl of 1 M Tris buffer, 10 µl each of 10% SDS and 10% APS, 1 µl of TEMED per 1 ml of solution) was overlaid above the resolving gel. For sample preparation, the samples were mixed with 1x SDS gel loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS, w/v; 0.1% bromophenol blue, 10% glycerol, v/v) in 1:1 proportion and heated at 100°C for 3 min to denature the proteins. About 50 µg of the sample was loaded in the gel and separated using Tris-glycine buffer (25 mM Tris base, 250 mM glycine, 0.1% SDS).

After the proteins of different sizes were separated by polyacrylamide gel electrophoresis (PAGE), they were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) (pre wetted for about 30 min in methanol and then transferred to 1x blotting buffer until ready to use). The blot was electro-transferred using transfer buffer (25 mM Tris base, 0.2 M glycine, 20% methanol, pH 8.5) for 2 h at 200

volts. The membranes were then immersed in blocking buffer having 1x TBS (2.42 g Tris base and 8 g NaCl/ l of water), 0.1% Tween-20 and 5% w/v nonfat dry milk and blocked overnight at 4°C to obstruct non specific membrane binding sites. Blotted membranes were then probed with various antibodies depending on the experiments (for example, PPARα, PPARγ and Glut-4) of various dilution (dilutions 1:500) in blocking buffer followed by washing with 3 x 10 min with 0.05% Tween 20 in TBS. Goat anti-rabbit IgG antibodies (Bangalore Genei, Bangalore, India) conjugated to alkaline phosphatase was then used to probe the primary antibodies (1:1000 in blocking buffer) and again washed 3 x 10 min with 0.05% Tween 20 in TBS. Colour development was performed in 30 ml AP-buffer (100 mM Tris Cl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), with 200 µl nitroblue tetrazolium (NBT), 50 mg/ml and 100 µl 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), 50 mg/ml. Finally, the developed blots were subjected to densitometry using the β-actin as internal control.

3.2.8 RNA Extraction

This was performed in highly aseptic conditions and all the glassware used in the experiment was baked at 250°C for 6 h and then treated with DEPC treated water before autoclaving. The RNA was extracted by the methods described earlier (Chomczynski and Sacchi, 1987). Tissue samples were transferred from the liquid nitrogen to mortar and pulvarized in presence of liquid nitrogen. The powdered tissue was transferred to polypropylene tube containing 3 ml of denaturation solution (Solution-D; 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroylsarcosine and 0.1 M 2-mercaptoethanol) and was homgenised for 1 min. The homogenate was transferred to a fresh tube and 0.1 ml of 2 M sodium acetate (pH 4.0), 1 ml of water saturated phenol and

0.2 ml of chloroform-isoamyl alcohol (per ml of solution-D used) were sequentially added to the tube with through mixing (by inversion) with each addition. The homogenate was vortexed vigorously for 10 seconds and incubated on ice for 15 min (to permit the complete dissociation of nucleoprotein complexes) followed by centrifugation at 10,000 rpm for 20 min (4°C) and the upper aqueous phase containing extracted RNA was transferred to a fresh tube. An equal volume of isopropanol was added to the extracted RNA, the solution was mixed well and allowed to precipitate for 1 h at -80°C. The precipitated RNA sample was collected by centrifugation carefully decanted and was dissolved in 0.3 ml of solution-D for every 1 ml of this solution used in step 1. The precipitated RNA sample was again collected by centrifugation at maximum speed for 10 min and pellet was washed twice with 75% ethanol and any remaining ethanol was removed with a disposable pipette tip. The tube was stored at room temperature for few min to allow the ethanol to evaporate. The RNA pellet was then dissolved in 50 µl of DEPC treated water and was stored at -70°C till use.

3.2.9 Semiquantitative RT – PCR analysis

Total extracted RNA from the tissue sample of all the animals were quantified and tested and an equal amount of it was reverse transcribed. The reaction was carried out in two steps: cDNA synthesis and PCR amplification.

3.2.9.1 First strand cDNA synthesis (reverse transcription)

As the first step, total RNA was reverse transcribed to form cDNA by adding approximately 100 ng of RNA sample form all the groups in individual 0.2 ml tubes and sterile water was added to bring volume to 9 µl. To this, 1 µl of Oligo $(dT)_{18}$ primer was

added and the vial was placed at 65°C for 10 min and then at room temperature for another 2 min (to remove any secondary structure).

Finally the vial was spun briefly and following agents were added sequentially in the following order-

1.0 µl RNAse inhibitor (10 U/µl)

1.0 µl DTT (0.1 M)

4.0 µl RT Buffer (5x)

2.0 µl dNTP mix (30 mM)

0.5 µl M-MuLV Reverse Transcriptase (50 U/µl)

1.0 µl Sterile water

The solutions were mixed well and incubated at 37°C for 1 h and followed by incubation at 95°C to dentaure RNA-cDNA hybrids. The samples were then spun briefly and quickly placed on ice.

3.2.10.2 PCR Amplification

PCR amplification was performed in a 25 µl reaction volume with the desired number of cycles for each product. The reaction mixture consisted of following:

14.2 µl Sterile water

2.5 µl 10x PCR buffer (Bangalore Genei, Bangalore)

1.0 µl 30mM dNTP mix

1.0 µl Forward Primer (100ng/µl)

1.0 µl Backward Primer (100ng/µl)

0.3 µl Taq Polymerase (0.3U)

4.0 µl cDNA strand (as obtained above)

PCR was performed by denaturing at 94°C for 60 seconds, annealing for 30 seconds at various temperatures depending upon the primer pairs used (as shown in Table 3.3) and by extension at 72°C for 60 seconds. Primer sequence, gene bank accession number, annealing temperature and number of cycles used for amplification for each gene are mentioned in the table 3.3. The primer sequences were designed with the help of Primer3 software (Steve Rozen, Helen J. Skaletsky, 1998, Primer3) and standardized in the lab, except for Glucokinase (GK), PEPCK and G6pase which were adopted from Jung et al. (2005).

On completion of the PCR reaction the samples were ran in 2% agarose gels. Agarose gel was prepared by melting 2 gm agarose in 100 ml of 1x TAE (Tris acetate EDTA) buffer (50x stock having 242 gm of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA). The gel was allowed to cool up to a temperature of approximate 55°C and ethidium bromide was added to a final concentration of 0.5 µg/ml. The solution was poured into a tray and gel was allowed to set and finally dipped with sufficient amount of 1x TAE buffer. The PCR product were mixed with 0.2 volumes of 6x gel loading buffer (0.25% bromophenol blue, 40% sucrose w/v in water), loaded on the gel and electrophoresis was carried out. The intensity of the bands on gels was converted into digital image with a gel analyzer and β-actin gene was used as internal standards.

Table- 3.3 Primers used in RT-PCR.

Gene	Primer Sequence	Cycle used	Anne. temp	Pro.. size	Gene bank accession no
GK-F GK-R	5′-TTCACCTTCTCCTTCCCTGTAAGGC-3′ 5′-TACCAGCTTGAGCAGCACAAGTCG-3′	28	55	490	L38990
G6Pase-F G6Pase-R	5′-AAGACTCCCAGGACTGGTTCATCC-3’ 5′-TAGCAGGTAGAATCCAAGCGCG-3′	28	55	607	U00445
AR-F AR-R	5’GGTGCAAGTTCCATGACAAGAGC3’ 5’ CGTCCAAGTGTCCACACAATCG 3’	28	56	190	AF035156
PEPCK-F PEPCK-R	5′-TGCTGATCCTGGGCATAACTAACC-3 5’-TGGGTACTCCTTCTGGAGATTCCC-3′	30	61	489	BC037629
Glut-4- F Glut-4-R	5’-TGGCCATCTTCTCTGTGGGTGC-3’ 5’-GGCATTGGCTAGGCCATGAGG-3’	30	58	140	AF098634
β-Actin-F β-Actin-R	5'-TCACCCACACTGTGCCCCATCTACGA-3' 5'-CAGCGGAACCGCTCATTGCCAATGG-3'	30	59	297	M12481
TNF-α-F TNF-α-R	5’-GGTGCAGTGGAGAGCCTTCC 3’ 5’-CAGTGATGTAGCGACAGCC-TCG 3	30	57	307	NM013693
PPAR-γ-F PPAR-γ-R	5′-AGGGCCCTGTCTGCTCTGTG-3′ 5′-TACCAGCTTGAGCAGCACAAGTCG-3′	30	57	421	NM011146
PPAR-α-F PPAR-α-R	5′- GCTCTACAACAGGCCTCATG -3’ 5′-GCCAACAGCTTCTCC-TTCTC-3′	30	58	421	NM001113418

3.2.11 Reversed phase HPLC analysis of plant extracts

HPLC analysis of the plant extracts was performed to identify and establish the presence of the active principles from plant's extract using commercial reference standards. Compounds were separated on a 150 mm x 4.6 mm, i.d., 5-µm particle, nova pack column (Waters, USA). The HPLC system consisted of Agilent (Palo Alto, CA,

USA) G1312A binary gradient pump with dual λ absorbance detector (water 2487). Column oven temperature was 30°C. Each sample was injected in triplicate (n = 3).

3.2.11.1 Analysis of flavonoids rich fraction

The phenolic compounds of natural origin have the positive property of being soluble in polar solvents. This leads to the possibility of using reversed phase HPLC (RP-HPLC) in their analysis, sufficient retention being achieved by using acidic conditions in order to avoid the presence of ionized forms of the analytes.

HPLC grade methanol and milli Q water (pH 3.0 adjusted with HPLC grade phosphoric acid) were used as mobile phase. The injection volume was 25 µl and column was washed with milli Q water. For elution, methanol and milli Q water was used in 1:1 ratio at a flow rate of 1 ml/min. Mixture of authentic samples of rutin, quercitine, myricetine and kaempferol were dissolved in HPLC grade methanol and injected under same conditions as for sample. Flavonoids were detected at 265 nm.

3.2.11.2 Analysis of guggulsterone

For the identification and verification of the active principles from the isolated fractions of *Pterocarpus marsupium* showing antidiabetic activities, HPLC analysis was performed using the reference standard. Sample was filtered through 0.2 micron syringe filter prior to use. Column was equilibrated with 5% acetonitrile for 9 min at the beginning of each run. A gradient consisting of water (A) and HPLC grade acetonitrile (B) at a flow rate 600 µl/min was used. The gradient was as follows: 5% B held for 2 min, increased linearly to 65% by 12 min and held for 20 min, and stepped to 100% and held for 2 min to bring the total run time per sample to 45 min. *Pterostilbene* from Calbiochem was used as a reference standard. *Pterostilbene* was detected at 308 nm.

3.2.11.3 Analysis pterostilbene

Purified *Commiphora mukul* fraction was subjected to HPLC analysis for the verification of guggulsterone E/Z as the active ingredients. The injection volume was 25 µl and column was washed with milli Q water. For elution methanol and milli Q water was used in 46:54 ratio at a flow rate of 1.2 ml/min and UV detection at 245 nm.

3.3 STATISTICAL ANALYSIS

Data are expressed as mean ± S.E.M. Analyses were carried out with the Statistical Package Origin (Origin® 6.1, v6 1052, [B232], Northampton USA). Differences among groups were determined by one-way ANOVA followed by the post hoc analysis using, Tukey's Honestly Significant Difference or HSD test.. The differences were considered statistically significant if $p<0.05$.

CHAPTER- IV

CHAPTER IV

STUDY ON THE ANTIDIABETIC POTENTIAL OF SOME OF THE MEDCINAL PLANTS

4.1 COMPARATIVE ANALYSIS OF ANTIDIABETIC EFFECTS OF SOME INDIAN MEDICINAL PLANTS ON DIABETIC MICE

4.1.1 Introduction

Under normal physiological conditions a complex and non-linear cross talk between different metabolic pathways and neuro-hormonal regulation maintains a normal glycemic status. In diabetic condition, either lack of insulin or resistance of body towards the action of insulin leads to an improper regulation of carbohydrate and lipid metabolism resulting in hyperglycemia and diabetes related complications. Due to complex pathophysiological processes involved in fostering diabetes the unidirectional and single drug therapeutic approach in the management of diabetes does not appear to be the way to address this problem (Tiwari and Madhusudana, 2004). On the other hand the therapeutic approach of traditional medicines are more holistic which uses variety of herbal and non-herbal ingredients that are thought to act on a variety of targets by various modes and mechanisms. In recent years, the plant medicines are gaining a new thrust for the treatment of different diseases since the herbal drugs are mostly free of side effects (Dubey et al., 1994; Prince et al., 1998; Ladeji et al., 2003; Rao et al., 2003; Maiti et al., 2004). Also, these products have been found to be non-toxic in animal studies done so far. In the last century, roughly 121 pharmaceutical products were formulated based on the traditional knowledge obtained from various sources. In theory, the activity shown by a mixture, for example an extract, is due to the sum of the activities of the individual

constituents. Thus fractionation, leading to the isolation of individual compounds, may have two types of effects, either having a higher activity than the original extract or a lower activity than the original extract (Houghton et al., 2007). While the former can be explained in terms of competition for the enzyme active sites between various components in the extract, the latter effect may be attributed to two reasons: decomposition of the active components during fractionation and/or presence of synergy between various components in the extract (Houghton et al., 2007).

Although current scientific tools are trying to elucidate their fundamental mechanisms still there are several unexplored and unexplained gems of immense relevance for acquisition. In this study series a comparative analysis of different medicinal plant extracts were performed in order to evaluate their antidiabetic potential. In order to evaluate their efficiency based on some preliminary data, aqueous and ethanolic extracts of nine medicinal plants based on some preliminary data were administrated to STZ induced diabetic mice along with tolbutamide as positive control. After 15 days of treatment, body weight (bw), FBG, OGTT, glycogen content, lipid profile, G6Pase activities, PTP-1B inhibition activity, α-glucosidase inhibition were studied. This preliminary screening system thus provided a platform where the potential plants demonstrating antidiabetic activities could be selected to be further analyzed in detail and understand their mechanism of action.

4.1.2 Animals groups and treatment

The mice were randomly divided into four groups (n = 6) as follows:

Group I - Normal mice (control);

Group II - STZ treated (diabetic)

Group III- Diabetic mice treated with different plant extracts (listed in Table 3.1) (treated);

Group IV- Diabetic mice treated with tolbutamide (tolbutamide).

The mice belonging to group III and IV were treated with respective plant extracts as listed in Table 3.1 at a dose of 100 mg/kg bw and tolbutamide (15 mg/kg bw) respectively for another 15 days, while those from group I and II received only 0.01% ethanol as vehicle control.

4.1.3 Results

4.1.3.1 Antidiabetic effects of different plant extracts in STZ induced diabetic mice

4.1.3.1.1 Effects on body weight

Table 4.1.1. Pre- and post-treatment body weights in various groups of animal.

Group	Initial body weight (g)	Final body weight (g)
Control	34.6 ± 1.4	35.2 ± 1.4
Diabetic	35.2 ± 1.4	29.8 ± 1.3[a]
Diabetic + *TC*	34.8 ± 1.2	35.4 ± 1.3[b]
Diabetic + *PE*	34.4 ± 1.3	32.8 ± 1.3
Diabetic + *MK*	34.6 ± 1.2	35.4 ± 1.4[b]
Diabetic + *CD*	34.0 ± 1.4	38.2 ± 1.4[b]
Diabetic + *EJ*	35.9 ± 2.1	35.1 ± 1.5[b]
Diabetic + *AM*	34.1 ± 1.9	32.2 ± 1.7
Diabetic + *PM*	35.6 ± 2.2	36.7 ± 1.8[b]
Diabetic + *EG*	36.2 ± 1.8	31.0 ± 1.4
Diabetic + *CM*	35.2 ± 1.5	35.2 ± 1.6[b]
Diabetic + *Tol*	34.3 ± 1.6	36.3 ± 1.4[b]

Each value denotes mean ± S.E.M. of n = 6

a and b, Significantly different from the final body weight of control and diabetic groups respectively at $p<0.05$.

TC, Tinospora cordifolia; *PE, Phyllanthus emblica*; *MK, Murraya koenigii*; *CD, Capparis decidua*; *EJ, Eugenia jambolana*; *AM, Aegle marmelos*; *PM, Pterocarpus marsupium*; *EG, Eucalyptus globulus*; *CM, Commiphora mukul*; *Tol, Tolbutamid.e*

STZ caused a significant reduction (15%) in body weight of diabetic mice in comparison of control group. On the other hand, the groups treated with different plant extracts could resist against reduction in body weight in response to most of the plant extracts as shown in Table 4.1.1. However some plant extracts (*P. emblica, A. marmelos and E. globules*) failed to have a significant effect on the change in body weight albeit to a lesser extant

4.1.3.1.2 Fasting blood glucose levels and oral glucose tolerance test

Reduced insulin level due to STZ induced destruction of pancreatic β-cells resulted in significantly elevated fasting blood glucose (FBG) level in diabetic mice in comparison of control. Treatment with different plant extracts for 15 days demonstrated comparatively lower FBG (Table 4.1.2). Reduction in FBG level was more significant in case of *M. koenigii* (28%), *C. decidua* (36%), *E. jambolana* (22%), *C. mukul* (29%) *and P. marsupium* (27%) than others and was comparable to tolbutamide treated group which showed a reduction of about 40% (Table 4.1.2). For the determination of glucose tolerance, after completion of 15 days of treatment with different plant extracts, over night fasting mice were fed with 1 g/kg bw of glucose and the blood glucose level was determined up to 150 min afterwards. Acute elevation of blood glucose level after glucose load was comparatively less in case *A. marmelos, C. decidua, E. jambolana,* and *C. mukul* in comparison to groups treated with other plant extracts or diabetic group (Fig. 4.1.1). At the same time blood glucose level also reduced significantly ($p<0.05$) during 150 min of glucose tolerance test in *C. decidua, A. marmelos, E. jambolana, and C. mukul* extract treated mice (Fig. 4.1.1).

Table 4.1.2. Pre and post-treatment fasting blood glucose levels in various groups of animals.

Group	Initial FBG (mg/dl)	Final FBG (mg/dl) (15 days)
Control	75 ± 3.6	79 ± 5.2
Diabetic	148 ± 4.7	170 ± 4.5[a]
Diabetic + *TC*	146 ± 3.6	155 ± 3.6
Diabetic + *PE*	142 ± 2.1	139 ± 5.1[b]
Diabetic + *MK*	150 ± 2.9	143 ± 3.9[b]
Diabetic + *CD*	150 ± 3.6	109 ± 5.3[b]
Diabetic + *EJ*	147 ± 3.9	123 ± 5.2[b]
Diabetic + *AM*	163 ± 4.2	146 ± 4.7
Diabetic + *PM*	154 ± 3.4	121 ± 4.1[b]
Diabetic + *EG*	152 ± 3.4	146 ± 3.8
Diabetic + *CM*	147 ± 4.6	121 ± 3.9[b]
Diabetic + *Tol*	156 ± 2.3	93 ± 3.2[b]

Each value denotes mean ± S.E.M. (n = 6)

a and b, represents significantly different from final FBG of control and diabetic group, respectively at $p<0.05$.

TC, Tinospora cordifolia; *PE, Phyllanthus emblica*; *MK, Murraya koenigii*; *CD, Capparis decidua*; *EJ, Eugenia jambolana*; *AM, Aegle marmelos*; *PM, Pterocarpus marsupium*; *EG, Eucalyptus globulus*; *CM, Commiphora mukul*; *Tol, Tolbutamide.*

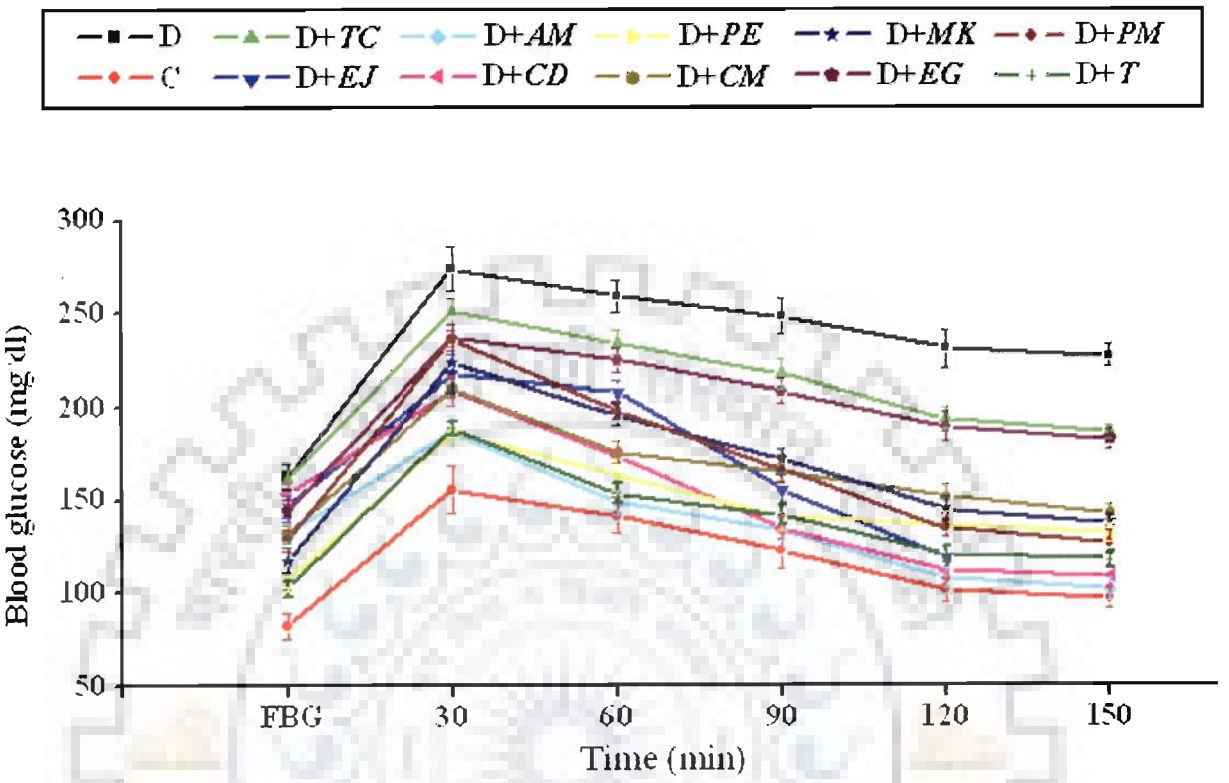


Figure 4.1.1. Oral glucose tolerance test in STZ induced diabetic mice in response to various plants extracts as described in materials and methods. Results are means ± S.E.M. of n = 6.

D, Diabetic; TC, Tinospora cordifolia; PE, Phyllanthus emblica; MK, Murraya koenigii; CD, Capparis decidua; EJ, Eugenia jambolana; AM, Aegle marmelos; PM, Pterocarpus marsupium; EG, Eucalyptus globulus; CM, Commiphora mukul; Tol., Tolbutamide.

4.1.3.1.3 Effects on plasma lipid profile

Various parameters of blood lipid profiles were tested in STZ induced diabetic mice after 15 days of treatment with various plant extracts. The elevated levels of TC, LDL cholesterol and TG in diabetic mice reduced significantly ($p<0.05$) in plant extracts treated groups.

Table 4.1.3. Plasma lipid profile after the administration of various plant extract in STZ treated mice.

Groups	Plasma lipid level (mg/dl)				
	TC	HDL	LDL	TG	VLDL
Control	96.6±4.8	25.4±1.9	53.3±7.6	89.6±4.2	17.9±0.8
Diabetic	175.6±5.6[a]	18.7±1.5[a]	130.4±5.1[a]	132.4±4.9[a]	26.5±0.9[a]
Diabetic + *TC*	152.6±4.3	19.5±2.8	108.±6.2	125.2±4.1	25.0±0.8
Diabetic + *PE*	164.2±3.3	22.2±1.6[b]	121.3±5.1	103.5±5.6[b]	20.7±1.1[b]
Diabetic + *MK*	121.6±3.2[b]	22.7±1.1[b]	76.6±5.7[b]	111.5±9.0	22.3±1.1
Diabetic + *CD*	152.6±3.9	22.9±2.1[b]	107.2±5.3	112.5±9.5	22.5±1.1
Diabetic + *EJ*	135.5±4.3[b]	20.8±1.8	91.8±3.2[b]	135.2±3.1	23.0±2.1
Diabetic + *AM*	134.5±2.7[b]	23.6±2.3[b]	87.3±3.9[b]	94.5±3.7[b]	18.8±3.5[b]
Diabetic + *PM*	124.2±3.3[b]	21.7±1.6[b]	82.6±5.1[b]	101.6±5.6[b]	19.9±1.1[b]
Diabetic + *EG*	167.2±3.2	27.2±1.6[b]	121±5.6	94.8±5.1[b]	18.96±1.0[b]
Diabetic + *CM*	125.4±4.2[b]	20.4±1.8	82.0±7.7[b]	114.8±5.5	22.9±1.1
Diabetic + *Tol*	127.2±3.1[b]	32.2±1.6[b]	80.1±5.6[b]	74.8±5.2[b]	14.9±1.0[b]

Each value denotes mean ± S.E.M. (n = 6)

a and b, significantly different from control and diabetic group respectively at $p<0.05$.

TC, Tinospora cordifolia; *PE, Phyllanthus emblica*; *MK, Murraya koenigii*; *CD, Capparis decidua*; *EJ, Eugenia jambolana*; *AM, Aegle marmelos*; *PM, Pterocarpus marsupium*; *EG, Eucalyptus globulus*; *CM, Commiphora mukul*; *Tol, Tolbutamide.*

There was about 25-30% fall in TC level in case of *C. mukul, M. koenigii,* and *P. marsupium* treated groups (Table 4.1.3). HDL level was improved by 30-40% in case of *P. emblica, M. koenigii* and *A. marmelos*. LDL cholesterol levels were also decreased significantly in case of *C. mukul, M. koenigii* and *P. marsupium.* TG level was decreased by 27% and 28% respectively in case of *E. globulus* and *A. marmelos* (Table 4.1.3).

4.1.3.1.4 Effects on Glucose-6-phosphatase activity in liver

After 15 days of treatment with different plant extracts, there was a significant reduction in the hepatic G6Pase activity in treated groups with respect to diabetic group. The decrease in the level of enzyme concentration was 45%, 31%, 39% and 35% respectively in case of *P. marsupium, E. jambolana, C. mukul* and *C. decidua* and it was found to be almost at the level of non-diabetic control mice (Fig. 4.1.2).

4.1.3.1.5 Glycogen level in tissues

Glycogen content of the liver and skeletal muscles was estimated on completion of the treatment in all the groups. In diabetic group, both liver and muscle glycogen content dropped significantly by 28% and 35% respectively as compared to non-diabetic control ($p<0.05$) (Fig. 4.1.3 A and B). Treatment with *A. marmelos, T. cardifolia* and *P. marsupium* extract led to about 35%, 52% and 35% increase respectively in liver glycogen content (Fig. 4.1.3A) and 48%, 33% and 33% increase in muscle glycogen content in case of *E. jambolana, C. mukul* and *C. decidua* respectively (as compared to diabetic control) (Fig. 4..1.3B).

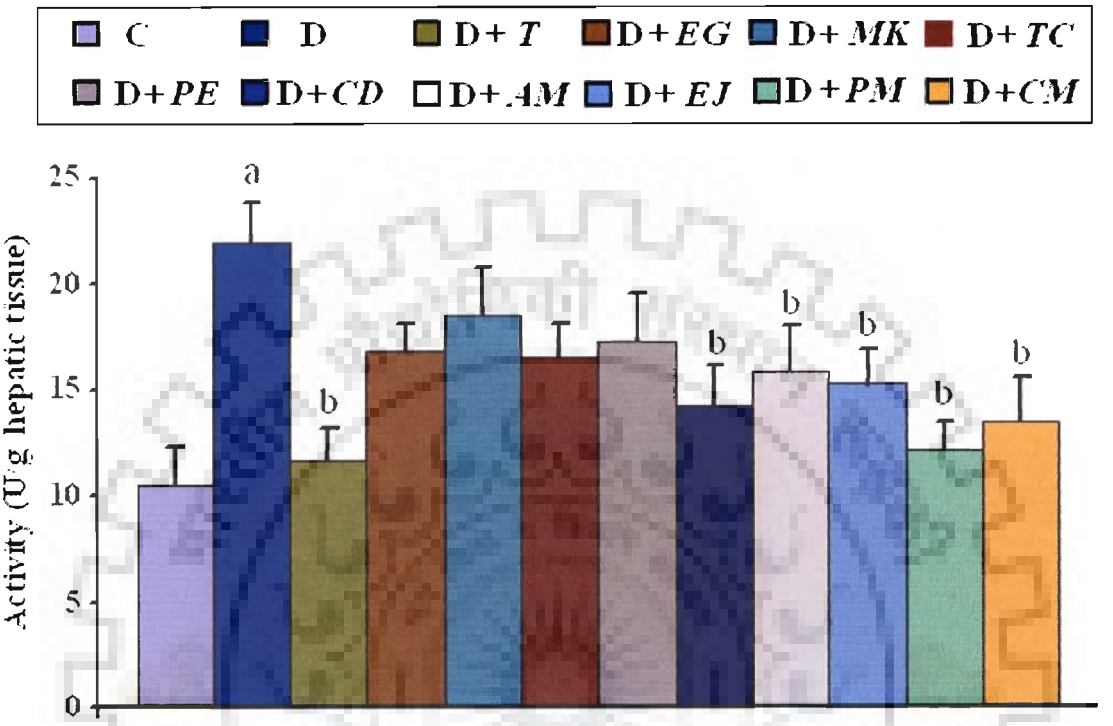


Figure 4.1.2. Effects of various plant extracts on hepatic levels of G6Pase in STZ induced diabetic mice. Data are expressed as means ± S.E.M. of n = 6. a and b, indicates the significant levels of difference in enzyme activities as compared to control (non-diabetic) and untreated diabetic mice, respectively ($p<0.05$).

C, Control; D, Diabetic; TC, Tinospora cordifolia; PE, Phyllanthus emblica; MK, Murraya koenigii; CD, Capparis decidua; EJ, Eugenia jambolana; AM, Aegle marmelos; PM, Pterocarpus marsupium; EG, Eucalyptus globulus; CM, Commiphora mukul; T, Tolbutamide.

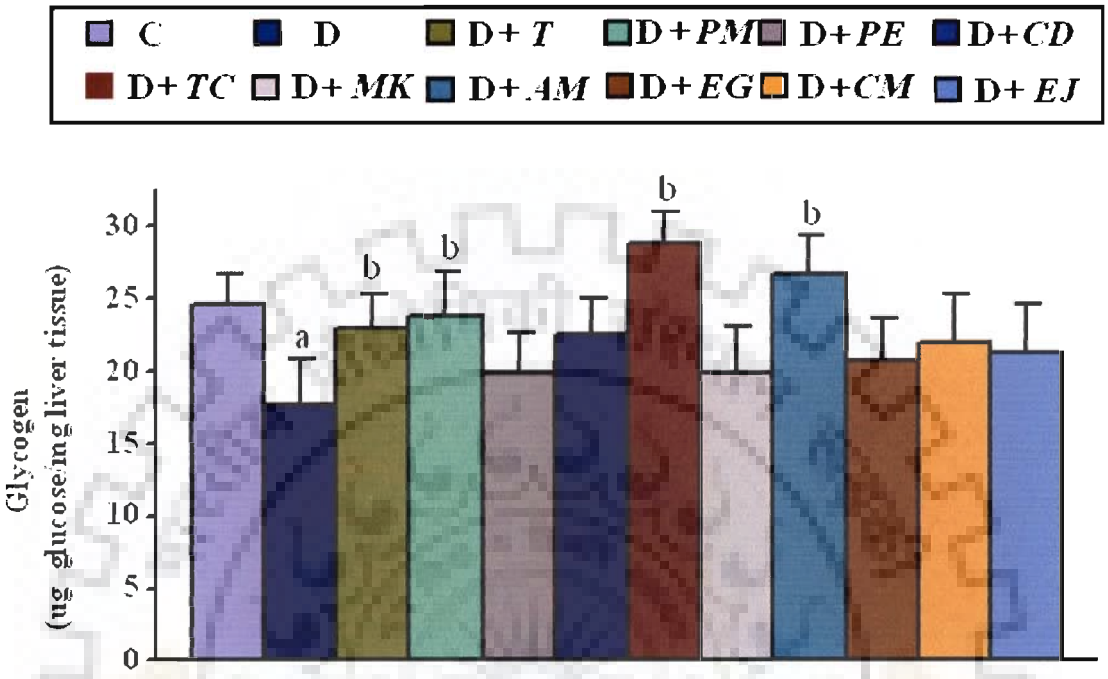


Figure 4.1.3A. Effect of various plant extracts on liver glycogen contents in STZ induced diabetic mice. Data are expressed as means ± S.E.M. of n = 6. a and b, indicates the significant levels of difference in glycogen content as compared to control (non-diabetic) and untreated diabetic mice, respectively ($p<0.05$).

C, Control; D, Diabetic; TC, Tinospora cordifolia; PE, Phyllanthus emblica; MK, Murraya koenigii; CD, Capparis decidua; EJ, Eugenia jambolana; AM, Aegle marmelos; PM, Pterocarpus marsupium; EG, Eucalyptus globulus; CM, Commiphora mukul; T, Tolbutamide.

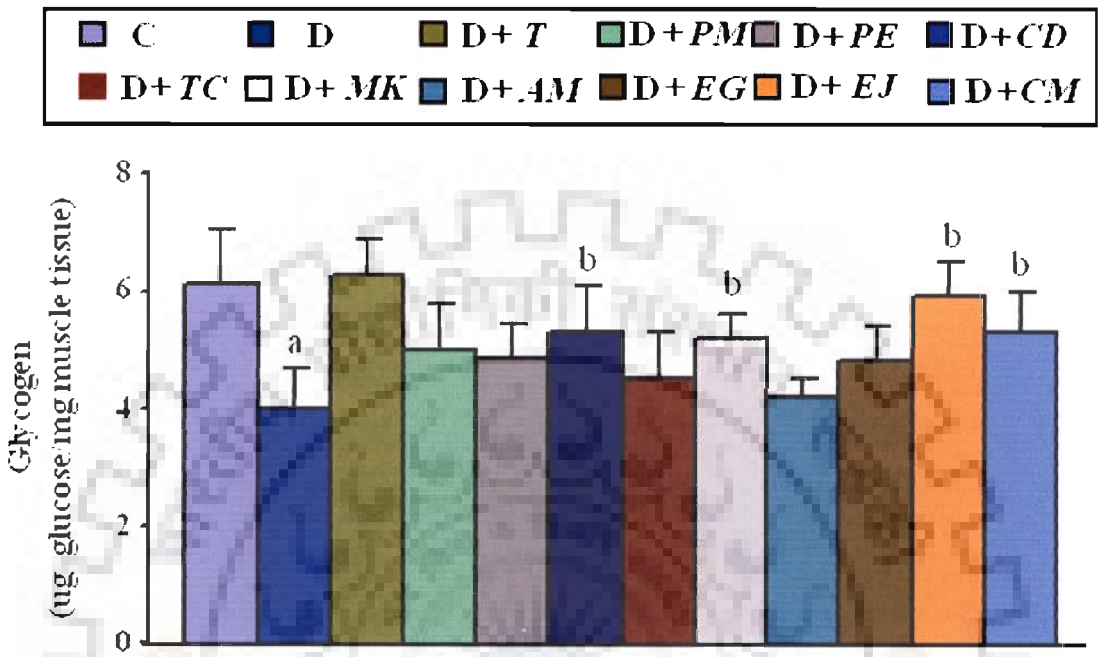


Figure 4.1.3B. Effect of various plant extracts on muscle glycogen contents in STZ induced diabetic mice. Data are expressed as means ± S.E.M. of n = 6. a and b, indicates the significant levels of difference in glycogen content as compared to control (non-diabetic) and untreated diabetic mice, respectively ($p<0.05$).

C, Control; D, Diabetic; TC, Tinospora cordifolia; PE, Phyllanthus emblica; MK, Murraya koenigii; CD, Capparis decidua; EJ, Eugenia jambolana; AM, Aegle marmelos; PM, Pterocarpus marsupium; EG, Eucalyptus globulus; CM, Commiphora mukul; T, Tolbutamide.

4.1.3.1.6 α-Glucosidase inhibition activity

α-Glucosidase inhibition activity was estimated in all the extracts with respect to acarbose used as positive control (IC_{50} value of $7x10^{-6}$ mg/ml). Ethanolic extraction of *P. marsupium* and *E. globules* showed significant dose dependent α-glucosidase inhibitory activities, with IC_{50} value of 3.3 mg/ml and 5.0 mg/ml respectively (Table 4.1.4). Extracts from *A. marmelos* and *C. mukul* were comparatively lesser effective with IC_{50} value of 4.75 mg/ml and 11.7 mg/ml. However others did not show any significant inhibition even at a higher dose (25 mg/ml and above) and were taken as no inhibition activity.

Table 4.1.4. IC_{50} values for the inhibition of α-glucosidase by different plant extracts.

Plant extracts	IC_{50} value (mg/ml)
T. cordifolia	no activity
C. decidua	19.5 ± 0.34
P. emblica	no activity
E. globules	5.0 ± 0.27
E. jambolana	no activity
P. marsupium	3.3 ± 0.44
A. marmelos	4.75 ± 0.54
M. koenigii	no activity
C. mukul	11.7 ± 0.97

Values are presented as mean IC_{50} values (±S.E.M.) of three independent experiments each performed in triplicate. IC_{50} of acarbose as a reference $7x10^{-6}$ mg/ml.

4.1.3.1.7 PTP-1B inhibition activity

Plant extracts were tested for PTP-1B Inhibition activity using sodium orthovanadate as reference standard (IC_{50} values 0.0015 mg/ml). As shown in Table. 4.1.5 *P. marsupium* and *C. decidua* demonstrated the maximum effect with an IC_{50} value of 3.2 mg/ml, 3.2 mg/ml and 4.3 mg/ml respectively). Extract of *A. marmelos* and *E. globulus* did not show any significant effect even at a highest concentration of 25 mg/ml and above.

Table 4.1.5. IC_{50} values for the inhibition of PTP-1B by different plant extracts.

Plant extracts	**IC_{50} value (mg/ml)**
T. cordifolia	5.1 ± 0.35
C. decidua	4.3 ± 0.27
P. emblica	no activity
E. globules	no activity
E. jambolana	17.4 ± 1.29
P. marsupium	3.2 ± 0.37
A. marmelos	no activity
M. koenigii	4.5 ± 0.22
C. mukul	13.7 ± 1.38

Values are presented as mean IC_{50} values (±S.E.M.) of three independent experiments each performed in triplicate. IC_{50} of vanadate as reference 0.0015 mg/ml.

4.1.4 Discussion

Diabetes is a chronic metabolic disorder affecting a major population worldwide. A sustained reduction in hyperglycemia will decrease the risk of developing micro vascular diseases and reduce their complications (Kim et al., 2006). The conventional therapies for diabetes have many short comings like side effects and high rate of secondary failure. On the other hand herbal extracts are expected to have similar efficacy without side effects as that of conventional drugs. The present investigation reports the antidiabetogenic and antihyperglycemic effects of aqueous and alcoholic extracts of leaves, seeds, resin and fruits of nine medicinal plants on STZ induced diabetic mice. The preliminary data presented here could provide a base for understanding the exact molecular mechanism of action of this plant's active principles. STZ injection resulted in diabetes mellitus, which is probably due to the destruction of β-cells of islets of Langerhans as proposed by many authors (Maiti et al., 2004; Beppu et al., 2006). This effect is being depicted by the high level of blood glucose in animals.

Symptoms like loss of body weight, weakness, polyuria and polyphysia are some of the marked indicators for type I diabetes mellitus (Maiti et al., 2004). Weight loss was found very distinctly in our STZ induced diabetic mice also. After treatment of mice for 15 days with different plant extracts, the mice resisted against weight loss which was close to the control (non diabetic mice) level. This was further confirmed by the alteration in the fasting blood glucose levels in diabetic mice followed by its regeneration after the plant extract treatment. However, there were no significant alterations of fasting blood glucose in control mice. Further the extract of *C. decidua*, *A. marmelos, E. jambolana,* and *C. mukul* resulted in the significant reduction of peak level of sugar

within 2.5 h time and this fact further strengthens the antidiabetogenic potentiality of these plant extracts as reported by some authors earlier in rat models (Yadav et al., 1997; Khan et al., 1995).

Significant improvement in peripheral glucose tolerance in *P. marsupium, C. mukul* and *C. decidua* treated groups provides probable explanation for the reduction in acute elevation of blood glucose level during OGGT. These extracts might enhance glucose utilization since it significantly decreased the blood glucose level in glucose loaded mice (OGTT). Further this fact could be attributed to the potentiation of the insulin effect of plasma by increasing the pancreatic secretion of insulin from existing β-cells or its release from bound insulin. In this context a number of other plants have been observed to have hypoglycemic effects (Kasiviswanath et al., 2005; Eidi et al., 2006). Apart from this, PTP-1B inhibition activity of *P. marsupium* and *C. decidua* which in-turn is responsible for improved insulin sensitivity and enhances the insulin effects could explain the improvement in peripheral glucose utilization as has been described by previous researchers in marine sponge *Hyrtios erectus* (Sun et al., 2007; Wang et al., 2008).

The administration of *E. globulus, P. marsupium, P. emblica* and *A. marmelos* extract significantly decreased serum triglyceride levels while, cholesterol level was decreased in *C. mukul, A. marmelos, P. marsupium* and *P. emblica* treated diabetic mice. In consistence with the present data, other workers have reported that the administration of some of these plant extracts to alloxan induced diabetic rats improved the serum cholesterol levels as compared to control and this effect was also similar to insulin treatment. With respect to cholesterol lowering property of these extracts, it could be

suggested that the constituents of the extracts may act as inhibitors for some enzymes such as hydroxyl methyl glutaryl CoA reducatse, which participates in cholesterol synthesis as has been suggested for some plants earlier (Gebhardt and Beck, 1996; Eidi et al., 2006).

Glycogen is the primary intracellular storable form of glucose and its levels in various tissues especially in liver and skeletal muscles are a direct reflection of insulin activity since it regulates glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosporylase. Since STZ causes selective destruction of β-cells of islets of Langerhans resulting in marked decrease in insulin levels, it could be predicted that glycogen levels in tissues (muscle and liver) decreases as the influx of glucose in the liver is inhibited in the absence of insulin (Golden et al., 1979). However, this alteration in muscle and hepatic glycogen content is normalized by insulin treatment (Vats et al., 2004). Our results showed that upon supplementation of diabetic mice with different extracts resulted in significant elevation in both muscle and hepatic glycogen content. This point toward one of the possible ways atleast some of the plant extracts might act by improving the glycogenesis process in muscle and liver.

G6Pase, an enzyme, had also been reported to play an important role in glucose homeostatis in liver and kidney by activating the gluconeogenic pathway (Berg et al., 2001). In our study the G6Pase activity in the liver was elevated in STZ induced diabetic mice and this on par with the earlier report in diabetic animals and even human models (Gupta et al., 1999). *P. marsupium, A. marmelos, C. mukul* and *C. decidua* extract also resulted in significant reduction in the level of the enzyme activity close to control level and thus making way for proposing the antidiabetic activity of this plant by acting at

another target. This result is similar to that of reported earlier where several potential herbal plant extracts had been known to improve the diabetic condition (Gupta et al., 1999).

In conclusion, the data obtained from the present chapter of the thesis indicates that these extracts could have some bioactive molecules which may have beneficial effects as antihyperglycemic agents. Out of these nine plants as screened here although *Tinospora cordifolia*, *Commiphora mukul*, *Murraya koenigii*, *Eugenia jambolana*, *Pterocarpus marsupium* and *Aegle marmelos* plant extracts demonstrated overall potential antidiabetic activities, yet some of the remaining plants like *Eucalyptus globulus*, *Capparis decidua*, *Phyllanthus emblica* showed potential hypolipidemic activities. This conclusively proved that although these medical plants act as antidiabetogenic agents but their targets are variable. Naturally a two tiered study (initial screening followed by isolation of bioactive molecules) will enhance the process of understanding the mechanism of action of these plant molecules and development of new plant based drugs. Toxicity data (data not shown) has already proved that the used dose in this investigation is far below the LD_{50} dose of the extract and they did not show any change in the blood parameters. Considering all these, it was reasonable to take up further studies on possible usefulness of some of these extracts in the treatment of diabetes mellitus.

4.2 ANTIDIABETIC POTENTIAL OF ALKALOID RICH FRACTION FROM *Capparis Decidua* IN *STZ* INDUCED DIABETIC MICE

4.2.1 Introduction

A number of scientific investigations have highlighted the importance and the contribution of many plants in treatment of diabetes mellitus. Out of different medicinal plants screened for antidiabetic potential in first chapter *Capparis decidua* was selected and studied in detail as described in the present chapter. *Capparis decidua* (*CD*) is a xerophytic shrub (Fig. 4.2.1), found widely in the western part of India, Pakistan and some of the Asian countries. The dried fruits are used as an ingredient in antidiabetic compositions and its green berries are used in food preparations such as pickles due to the belief that it possesses antidiabetic properties. Different parts of the plant have been reported for several medicinal values. The bark is useful in the treatment of cough, asthma and inflammation, roots in fever, and buds in the treatment of boils (Rastogi and Malhotra, 1979). *CD* fruit has also shown hypolipidemic and hypotriglyceridemic effects in animal and human studies respectively (Purohit and Vyas, 2005). Studies on alloxan-induced diabetic rats have shown that *CD* fruit powder has hypoglycemic effect, decreases lipid peroxidation (LPO) and alters free radical scavenging enzymes such as superoxide dismutase (SOD) and catalase (CAT) in erythrocytes, liver, kidney and heart (Yadav et al., 1997). Different classes of phytochemicals such as alkaloids, flavonoids, fatty acids, glucosides, etc. with a vast variety of biological activities have been reported in different parts of this plant (Dhar, 1972; Gaind, 1970; Mukhamedova and Yunusov, 1969).

Figure 4.2.1 *Capparis decidua* plant; Inset shows the fruits of this plant.

On the basis of preliminary screening for different chemical groups of compounds showing antidiabetic properties from *CD* fruit extract (flavonoids, terpenes, tannins, steroids etc.), alkaloid rich (*AR*) fraction was chosen for further detailed study. To the best of our knowledge, this is the first ever reports wherein alkaloids from *CD* fruit extract were studied for their antidiabetic potential. Alkaloids are a group of nitrogenous substances in plants, derived from amino acids or from the transamination process as secondary metabolites and are known for their multiple medicinal properties (Aniszewski, 2007). Although alkaloids are known for their toxic effects on physiological system of human and other animals and are considered as plant toxicants in general (Affeltranger et al., 2007; Robert and Wink, 1998; Cheeke, 1989); various alkaloids with medicinal properties have also been isolated from several plant sources (Kang et al., 1995; Sgambato et al., 1987; Bobkiewicz-Kozłowska et al., 2007). Although a number of different alkaloids have been isolated from root and bark of this plant, no attempt has been made to study their biological significance (Ahmad et al., 1985, 1986, 1987, 1992). The data obtained from this study will provide the importance of alkaloids from this plant in the cure of diabetes which can further be taken up for the final isolation of the most active constituents.

4.2.2 Animal groups and treatment

Mice were randomly divided into three groups (n = 8 mice per group) as given below:

Group I – Normal (control)

Group II – STZ induced diabetic mice (diabetic)

Group III – STZ induced diabetic mice treated with alkaloids rich (*AR*) fraction (treated).

The mice belonging to group III were treated with *AR* fraction at a dose of 50 mg/kg body weight for another 28 days while, those from group I and II received only 0.01% ethanol as vehicle control.

4.2.3 Results

4.2.3.1 Effect of the treatment of alkaloids rich fraction of Capparis decidua in *STZ* induced diabetic mice.

4.2.3.1.1 Effect on fasting blood glucose levels and oral glucose tolerance

FBG levels were significantly high in diabetic mice as compared to control group ($p<0.05$). After the treatment with extract although marginal, a significant improvement in FBG levels was observed in treated group in comparison with that of diabetic group of animals ($p<0.05$) (Fig. 4.2.2, FBG data).

For determination of glucose tolerance, over night fasted mice were fed with 1 g/kg bw of glucose and the blood glucose levels were determined up to 150 min. Comparative acute elevation in the blood glucose levels after 30 min of glucose load was lesser in treated mice (48%) when compared to both diabetic and control groups which showed respective increase in blood sugar level of about 69% from their 0 min (FBG) point. Further decline in glucose level was also significant in comparison to diabetic counterpart and the final blood glucose level after 150 min of study was comparable to that of the control group (Fig. 4.2.1). ($p<0.05$)

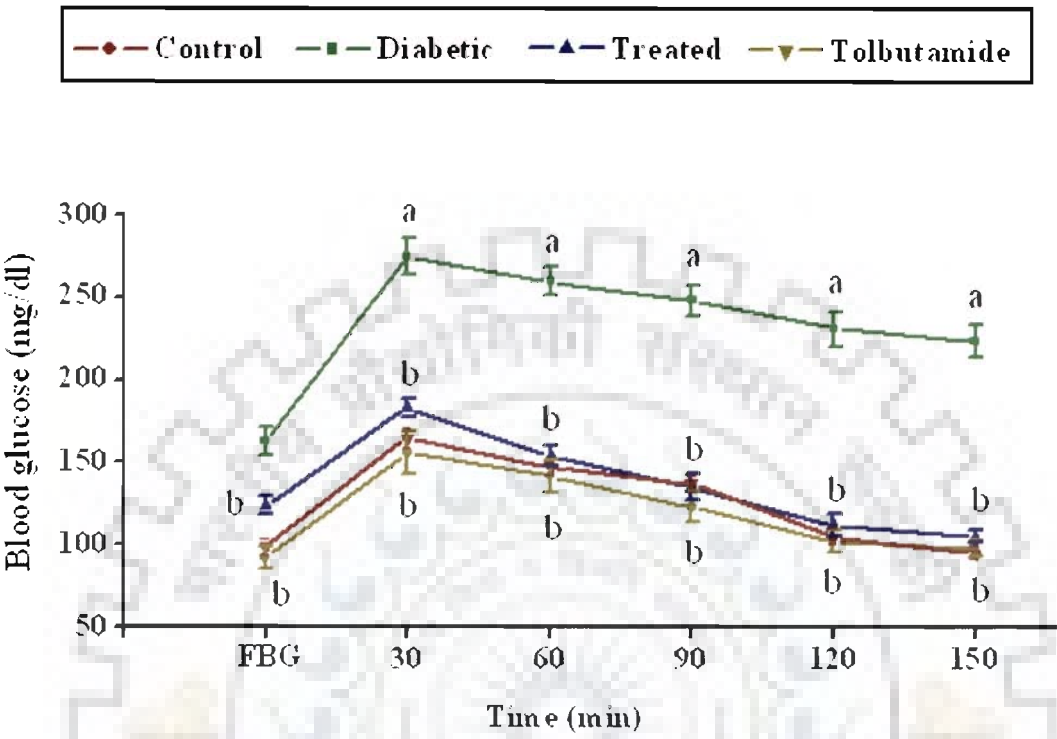


Figure 4.2.2. Oral glucose tolerance test (OGTT) in STZ induced diabetic mice in response to treatment with *AR* fraction of *CD* fruits. Results are mean ± S.E.M. of n = 8. FBG, Fasting blood glucose. a and b, represent the significant difference in comparison of control and diabetic groups, respectively at that particular time point ($p<0.05$).

4.2.3.1.2 Estimation of plasma lipid profile

Plasma lipid profiles were analyzed in control, diabetic and treated groups after 28 days of treatment. Levels of TC and LDL cholesterol and TG decreased about 25%, 32% and 27% respectively. On the other hand HDL levels improved by 28% (as compared to diabetic animals) in treated group (Table 4.2.1).

Table 4.2.1. Plasma levels of different lipids after the administration of *AR* fraction of *CD* fruits in STZ induced diabetic mice

Treatment group	Plasma lipid level (mg/dl)				
	TC	HDL	LDL	TG	VLDL
Control	96.6 ± 4.8	25.4 ± 1.97	53.3 ± 7.6	89.6 ± 4.2	17.92 ± 1 .84
Diabetic	175.6[a] ± 5.65	18.7[a] ± 1.46	130.42[a] ± 5.1	132.4[a] ± 4.9	26.48[a] ± 2.98
Diabetic+*CD*	132.6[b] ± 3.9	23.9[b] ± 2.1	89.3[b] ± 7.9	97.2[b] ± 5.3	19.4[b] ± 1.06

Each value represents the mean ± S.E.M. of n = 8. a and b, represent statistically significant as compared to control and diabetic groups respectively ($p<0.05$).

4.2.3.1.3 Effect on enzyme activity in liver

After 28 days of treatment, hepatic G6Pase activity was significantly reduced in treated group as compared to the diabetic group ($p<0.05$). The reduced level of enzyme activity (44%) was almost same as that of the control group same as tolbutamide treated group (Fig. 4.2.3). However, hepatic hexokinase activity did not improve significantly even on completion of the treatment (Fig. 4.2.3).

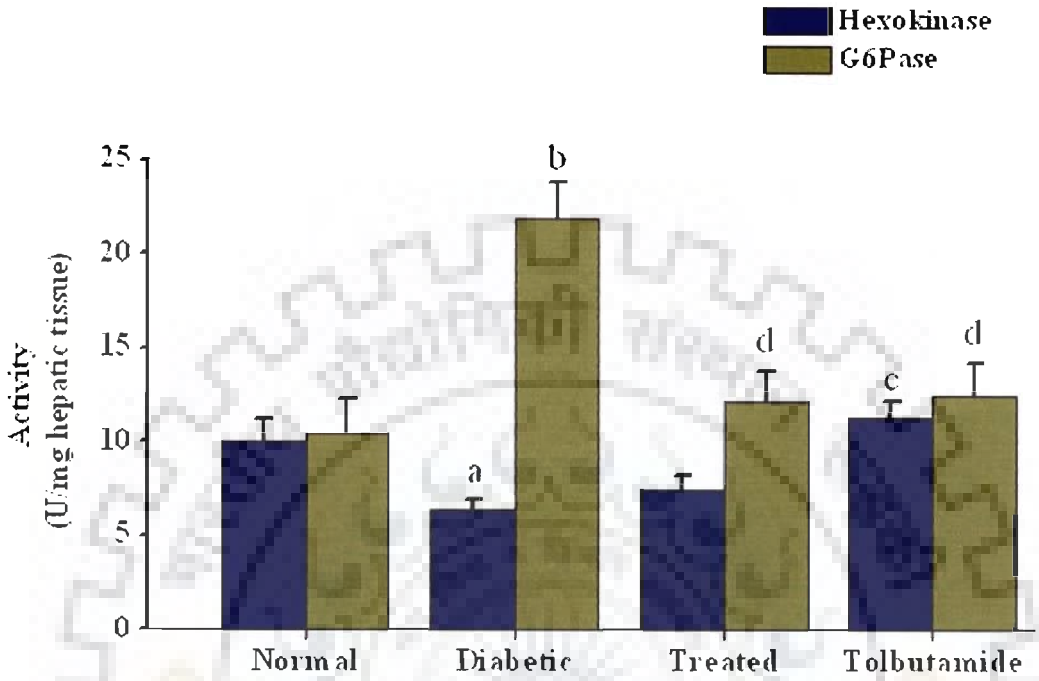


Figure 4.2.3. Effect of *AR* fraction of *CD* fruits on G6Pase and hexokinase activity in STZ induced diabetic mice. Data are expressed as mean ± S.E.M.; n = 8. a and c, indicate the significant level of differences in G6Pase; b and d, indicate the significant level of differences in hexokinase activity as compared to their respective normal and diabetic groups ($p<0.05$).

4.2.3.1.4 Effects on liver and muscle glycogen content

Glycogen content of skeletal muscles and liver was estimated in control, diabetic and *AR* fraction or tolbutamide treated groups on completion of the treatment period. Treatment with *AR* fraction of *CD* fruits improved the muscle and hepatic glycogen contents by 33% and 28% respectively in comparison to diabetic group ($p<0.05$) (Fig. 4.2.4).

4.2.3.1.5 Effects on serum insulin level

Induction of diabetes with STZ caused a severe effect on pancreatic islets, which was clearly observed in serum insulin profile. As shown in Fig. 4.2.5, there was a significant reduction in the level of serum insulin level in STZ treated mice which on treatment with *AR* fraction of *CD* fruits resulted in an improved condition (38%) ($p<0.05$).

4.2.3.1.6 Expression profile and of some gene involved in diabetes

Expression profile for various genes involved in glucose homeostasis from treated and diabetic groups further confirmed the antidiabetic potential of *AR* fraction. Expression of glucose regulatory genes, G6Pase and PEPCK, reduced clearly in treated group when compared to diabetic group (Fig. 4.2.6a). This was comparable with enzyme activity pattern of G6Pase observed in liver tissues as mentioned above. On the other hand, for hepatic GK, another key glucose homeostatic enzyme, similar to hexokinase, expression in treated group did not improve significantly in comparison to the diabetic group (Fig. 4.2.6a).

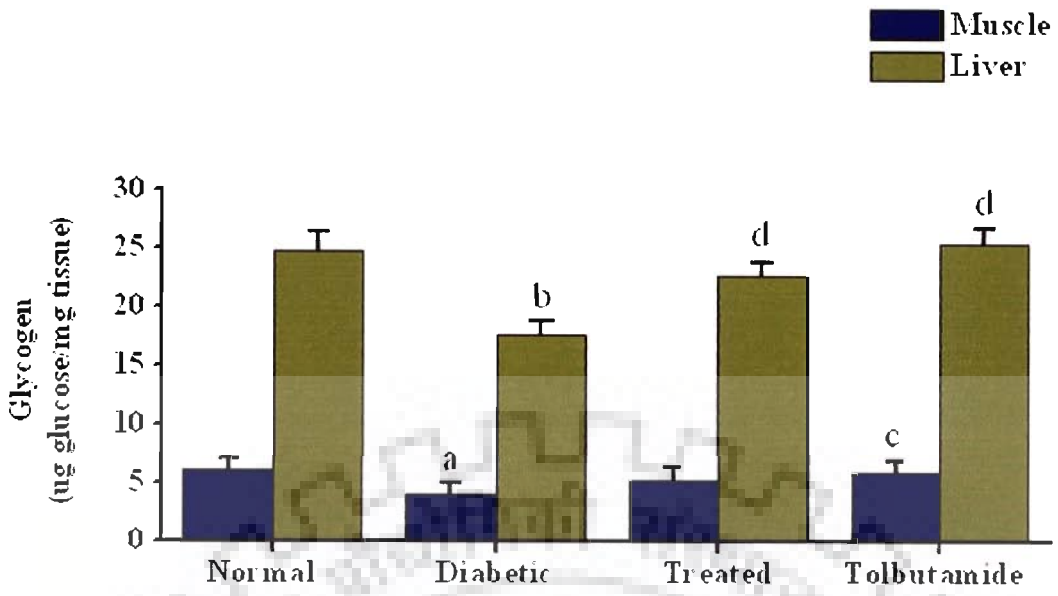


Figure 4.2.4. Effect of *AR* fraction of *CD* fruits on hepatic and muscle glycogen content in STZ induced diabetic mice. Data are expressed as mean ± S.E.M; n = 8. a and c, indicate the significant level of differences in hepatic glycogen; b and d, indicate the significant level of differences in muscle glycogen content as compared to their respective normal, and diabetic groups ($p<0.05$).

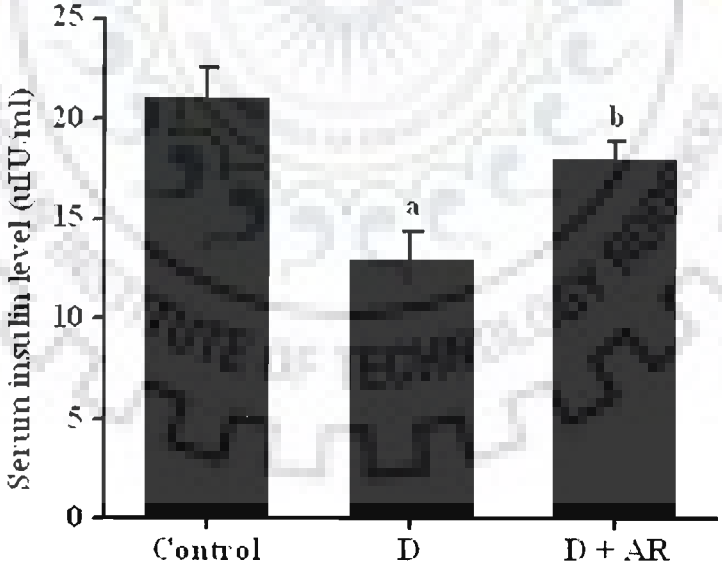


Figure 4.2.5. Effect of *AR* fraction of *CD* fruits on serum insulin levels. Data are expressed as mean ± S.E.M. of n = 8. a and b, indicate the significant level of differences as compared to control and diabetic group respectively ($p<0.05$). D, diabetic group; AR, alkaloid rich fraction.

Improvement in the expression of Glut-4, which is a receptor responsible for tissue specific glucose uptake in peripheral tissues like muscle and adipocytes, was observed in the treated animals as compared to the diabetic animals (Fig. 4.2.7). Tumor necrosis factor-α (TNF-α) is an important mediator of insulin resistance in obesity and diabetes through its ability to decrease the tyrosine kinase activity of the insulin receptor (IR) (Hotamisligil, 1999). The up-regulation of this factor in adipocytes is responsible for necrosis in adipose tissues. In our study, STZ induced up regulation of TNF-α in adipose tissue was significantly downregulated by the *AR* fraction of the *CD* fruits (Fig. 4.2.6b).

PPAR, a sub-family of the 48-member steroid and nuclear receptor super family, are ligand dependent transcription factors that control energy homeostasis by regulating carbohydrate and lipid metabolism (Shen et al., 2006). They exists in three different subtypes: PPARα, PPARβ/δ and PPARγ out of which PPARα is expressed in liver, kidney, heart, muscles and is involved in lipoprotein metabolism while PPARγ is predominantly expressed in adipose tissue and is involved in controlling insulin sensitivity, adipocyte differentiation and lipid storage. Due to their insulin receptor sensitizing activity the agonist for these receptors (thiazolidinediones) are used in treatment of type II diabetes mellitus. In the present investigation the *AR* extract also increased the transcription of PPARγ in the adipocytes of the treated animals as compared to diabetic group as shown by RT-PCR. However there was no significant change in the level of PPARα as compared to diabetic group (Fig. 4.2.6b). In addition, the expression of aldose reductase one of the key enzymes responsible for diabetes related complications in kidney reduced remarkably in treated group as compared to diabetic group (Fig. 4.2.7).

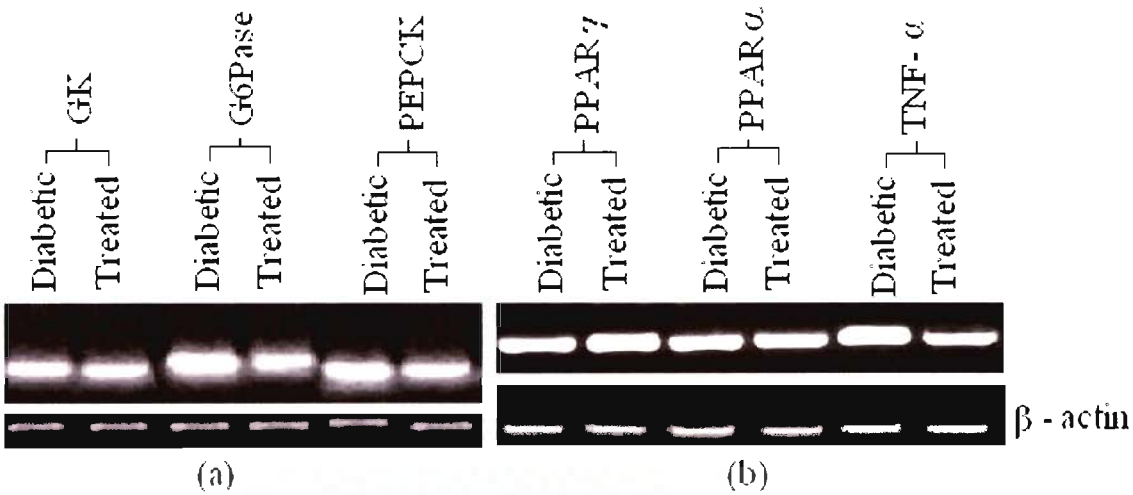


Figure 4.2.6. RT-PCR analysis of (a) liver mRNA expression of GK, G6Pase and PEPCK, (b) mRNA expression of PPARγ and α, and TNF-α genes in adipose tissue in diabetic and treated animal groups. GK, glucokinase; G6Pase, glucose-6-Phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PPARγ, peroxysome prolifirator activated receptor γ; PPARα, peroxysome prolifirator activated receptor-α; TNF-α, tumor necrosis factor-α.

Figure 4.2.7. RT-PCR analysis of Glut-4 and aldose reductase genes expression in adipose, muscle and kidney tissue in diabetic and treated animal groups.

4.2.3.1.7 Western blot analysis

The RT-PCR data for PPARγ was further confirmed by the elevated expression of this protein in treated group of mice as was demonstrated by western blot analysis (Fig. 4.2.8). Glut-4 gene was found to be over expressed in *AR* fraction treated group. The significant point was its accumulation in PM from LDM, showing the functional activity of this protein in response to *AR* fraction treatment (4.2.8b).

4.2.3.1.10 Histopathological studies

Histopathological sections of pancreas from control, diabetic and treated mice clearly indicated the protective effect of *AR* fraction. The pancreatic islets (shown by arrow) were larger in treated group (Fig. 4.2.9c), almost comparable to control group (Fig. 4.2.9a) but they were significantly damaged and reduced in size in STZ treated diabetic animals (Fig. 4.2.9b). In order to confirm if any toxic effects were caused by the alkaloids in the fraction, the histopathological analysis of the liver sections were performed which did not show any significant alteration in the architecture of the liver tissues as observed in control (Fig. 4.2.9d), STZ treated group (Fig. 4.2.9e) and *AR* treated group (Fig. 4.2.9f). This further proved the non-toxic nature of the alkaloids and confirmed that the effects were specific to the target sites. The TNF-α induced effect was also depicted in adipocyte sections which demonstrated a severe necrosis in STZ treated diabetic mice (Fig. 4.2.9h). However, this significantly improved in *AR* treated mice (Fig. 4.2.9i) and was almost comparable to control animals (Fig. 4.2.9g).

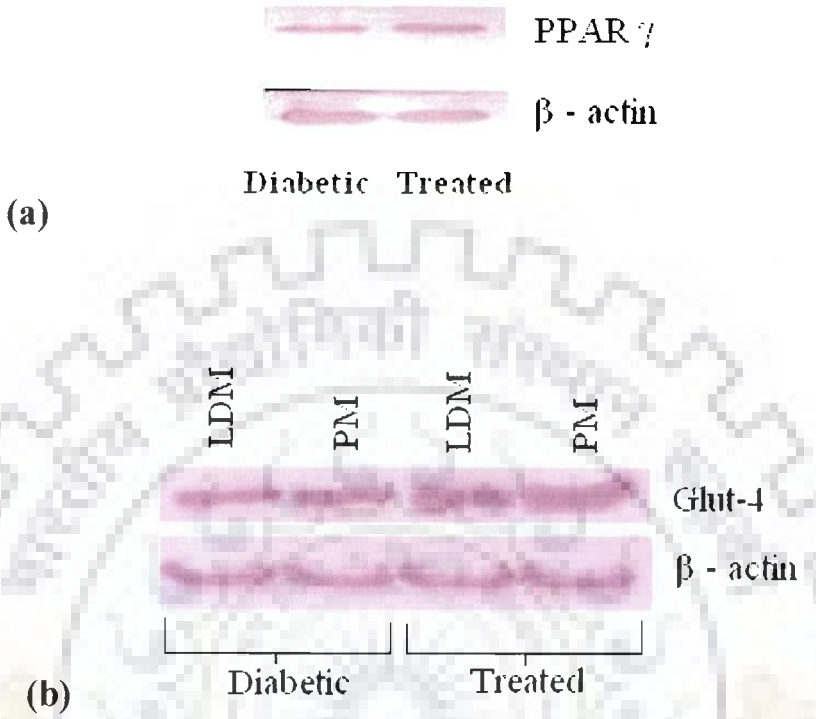


Figure 4.2.8. Immunoblot showing the expression of (a) PPARγ protein and (b) Glut-4 protein in adipose tissues in response to *AR* fraction treatment. The protein was extracted from adipose tissues of diabetic and treated animal and immunoblot analysis was performed as described in chapter 3. PPARγ, peroxisomal prolifirator activated receptor-gamma; Glut-4, glucose transporter-4; LDM, low density microsomes; PM, plasma membrane.

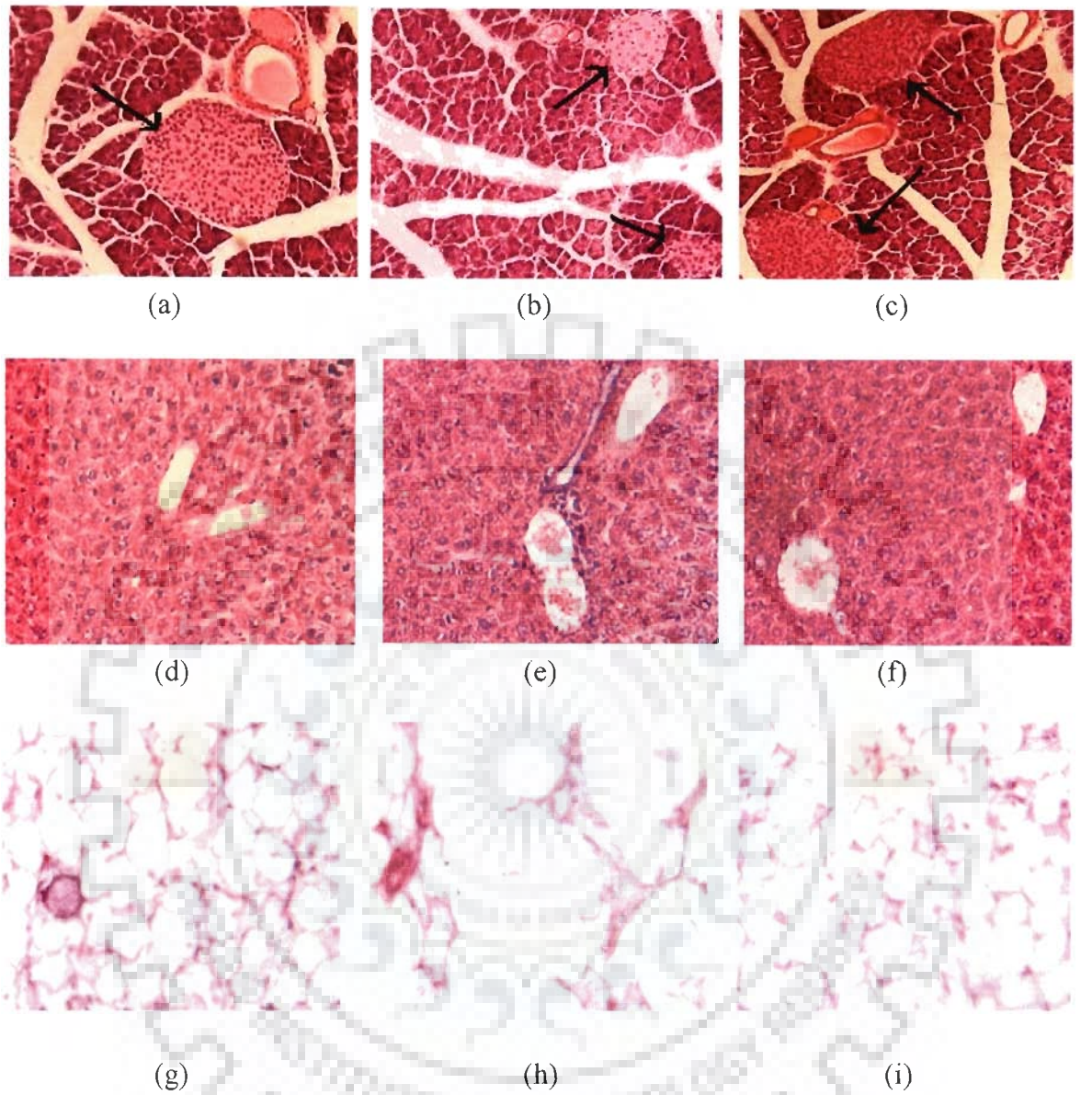


Figure 4.2.9. Photomicrograph of the sections from the pancreas (a-c), liver (d-f) and adipose tissues (g-i) of various groups of animals (a), (d), and (g) represent the respective tissues obtained from control group of animals. (b), (e), and (h) represent the respective tissues obtained from diabetic group of animals. (c), (f), and (i) represent the respective tissues obtained from *AR* fraction treated group of animals. The arrow indicates the islet region of the section. Haematoxylin and Eosin, 20X objective.

4.2.3.2 In vitro analysis of antidiabetic potential of alkaloid rich fraction of *C. decidua*

4.2.3.2.1 Effects on glucose uptake by the psoas muscle tissues

Incubation of muscle tissues with the *AR* fraction resulted in a gradual increase in cellular concentrations of glucose by transfer of glucose from media to cells (measured as decrease in glucose level in the medium). As shown in table 4.2.3, that 150 mg/l of the fraction alone by itself increased glucose uptake in muscle cell by about 20% in 30 min, after which the effect gradually decreased in another 120 min. However, in the presence of both fraction and insulin, further increase in glucose uptake was not significant when compared to insulin alone. This can be attributed to the fact that the AR fraction and insulin did not have any additive or synergistic actions.

Table 4.2.2. Effect of *AR* fraction on glucose uptake from medium by psoas muscle isolated from mice. The data are represented as increase in the cellular level of glucose with time, implying the equivalent reduction in glucose level in media by cellular uptake.

Treatment group	Glucose uptake (mg/dl)				
	30 min	60 min	90 min	120 min	150 min
MT	20±1.5	29±2.4	34±2.2	38±2.4	46±2.6
MT + E	24±1.3[a](20)[δ]	33±3.4[a](13)[δ]	41±3.4[a](20)[δ]	45±4.2[a](18)[δ]	52±3.3[a](13)[δ]
MT + I	32±2.6	43±3.8	56±3.7	65±4.1	75±2.7
MT + I + E	34±2.5(6)[γ]	48±2.9 (11)[γ]	59±2.9(5)[γ]	69±3.4(6)[γ]	78±3.6(4)[γ]

Values are mean ± S.E.M. of three independent experiments each performed in quadruplicates. MT: Muscle Tissue; E: Extract; I: Insulin.
Extract and insulin in the incubation was added at a concentration of 150 mg/l and 25 µg, respectively.
[δ] Values in the bracket indicate percent increase when compared with muscle tissue alone at that particular time point.
[γ] Values in the bracket indicate percent increase when compared with muscle tissue with insulin at that particular time point.
a $p<0.05$ when compared to control (MT).

4.2.3.2.2 PTP-1B inhibition activity

AR fraction from *CD* fruits was tested for PTP-1B inhibition activity *in vitro*. *AR* fraction demonstrated a dose dependent inhibition of PTP-1B activity with an IC_{50} value of 3.3 mg/ml. The inhibition potential of *AR* fraction was almost same as that of the crude extract indicating no significant improvement in the activity even after partial purification of the extract.

4.2.3.2.3 α-Glucosidase inhibition activity

AR fraction from *C. decidua* inhibited the α-glucosidase activity in a dose dependent manner with an IC_{50} value of 1.9 mg/ml. However, unlike that of PTP-1B inhibition activity, the inhibition effect was more prominent in case of *AR* fraction (IC_{50} value 1.9 mg/ml) than that shown by the crude ethanolic extract (IC_{50} value 19.5 mg/ml).

4.2.4 Discussion

Diabetes mellitus is known to be the leading cause of end-stage renal disease, blindness and amputation, and a major cause of cardiovascular disease resulting in premature death in the general population. Disturbed carbohydrate, fat and protein metabolism, resulting in hyperglycemia and deterioration of blood lipid profile is responsible for these problems. The present chapter of this thesis reports the antidiabetogenic activity of the *AR* fraction from *CD* fruits with special emphasis on antihyperglycemic and antihyperlipidemic effects of the extract in STZ induced diabetic mice.

The data obtained from OGTT strengthens the antidiabetogenic potential of this fraction and gives an idea that perhaps this fraction contains some active ingredients which inhibit the absorption of glucose from intestine as the acute elevation of blood glucose level after glucose load was lesser in comparison to both diabetic and control

group, similar pattern of activity is reported for other members of alkaloids also (castanospermine and chlorogenic acids) (Stegelmeier et al., 2008; Kang et al., 1995). Reduction in acute elevation of blood glucose was even more significant than that of crude ethanolic extract used in initial screening, which gives an idea that perhaps some alkaloids present in the isolated fraction inhibit glucose absorption from the intestine. This was further confirmed by estimation of α-glucosidase inhibition activity, where *AR* fraction was again more effective in comparison of ethanolic fraction used in initial screening. Similar pattern of phytochemical based inhibition of α-glucosidase inhibition activity has also been shown by brown alga (Ohta et al., 2002) plants like *Psidium guajava* leaves (Wang et al., 2007). Further, the decrease in blood glucose level was also significantly improved compared to that of the control group ($p<0.05$). The increase in glucose uptake by muscle tissues incubated in the presence of *AR* fraction alone (in the absence of insulin) indicated a potential antihyperglycemic action of this extract. However, on co-incubating the cells with insulin and *AR* fraction no significant synergistic effects were observed in terms of glucose uptake by the muscle cells. This result reflects two possibilities: either the alkaloids themselves have insulin like effects on psoas muscles or direct stimulatory effect on the enzymes involved in the metabolism of glucose (Sharma et al., 2007).

Hypertriglyceridemia and hypercholestremia is a major problem in patients with diabetes mellitus and is responsible for vascular complications. Improvement in lipid profile after administration of *AR* fraction proves the hypolipidemic nature of this fraction affecting the cholesterol metabolism (Fan et al., 2006; Babu et al., 2007). The cholesterol lowering property of this extract could be attributed to several factors out of which one factor tested by us was hydroxyl-methyl-glutaryl-CoA reductase, an enzyme which

participates in *de novo* cholesterol biosynthesis. This was also found to be down regulated by this fraction (data not shown). The regulation of this enzyme has also been reported by several authors earlier for some other plants (Gebhardt and Beck, 1996; Eidi et al., 2006).

Glycogen, the primary intracellular storable form of glucose gets significantly affected due to the defects in the glucose regulatory system. Glycogen levels in tissues (muscle and liver) decrease as the influx of glucose in the liver is inhibited in the absence of insulin (Golden et al., 1979; Vats et al., 2004). The treatment with *AR* fraction has shown a clear improvement in size and number of pancreatic islets indicating a healthier insulin profile for an improved endocrine control. This was further supported by the increased level of serum insulin in treated groups of animals as compared to diabetic animals, these results are in parallel with other authors where different alkaloids (like harmane, norharmane and pinoline) isolated from various plant sources have shown direct effect on insulin secretion (Sgambato et al., 1987; Bobkiewicz-Kozłowska et al., 2007; Kirtikar and Basu, 1993). The increase in serum levels of insulin could be solely attributed to the improvements in pancreatic islet cell number and functions by the extract since the *AR* fraction failed to release insulin from isolated pancreatic β-cells (data not shown). It further proved that this extract do not have any role as insulin mimetic. Rather it may indirectly facilitate the insulin release and improvement of other associated complications related with increased insulin sensitivity, for example inhibition of PTP-1B, which further improves insulin signaling and potentiates the metabolic effects of insulin signaling. Urosolic acid, another alkaloid isolated from some of the plants has already shown similar pattern of effect (Zhang et al., 2006). Here again the inhibition

potency of *AR* fraction was more prominent than that of ethanolic fraction of initial screening, which indicated the presence of some inhibitory complex in the extract.

The antidiabetogenic potential of *AR* fraction was further analyzed by observing the changes in the levels of some enzymes responsible for maintaining the glucose homeostasis. Out of several such enzymes, hepatic hexokinase type IV (also called glucokinase, GK), G6Pase and PEPCK are known to be some of the most crucial enzymes in this process. G6Pase and PEPCK direct the production of glucose from glycogen, the other enzyme i.e. GK, favors the production of glycogen from glucose (Nelson and Cox, 2005). In the diabetic condition the levels of these enzymes are altered resulting in the production of glucose from stored glycogen (Grover et al., 2002) and any antidiabetic molecules will restore or at least improve these enzyme statuses. In the present study the treatment with *AR* extract did not show any significantly change in the activity of hepatic hexokinase (i.e. GK) but at the same time it was able to decrease G6Pase activity in the treated groups of animals ($p<0.05$) and which was almost normalized to that of control. This effect was in parallel with the changed expression levels of GK and G6Pase the enzymes directly linked to glucose homeostasis and production of glycogen. Thus the improvement in some of the enzyme activities and expression patterns could be attributed to the alkaloids in the fraction which plays an effective role in improving carbohydrate metabolism. Further, the reduction in the expression of G6Pase and PEPCK enzymes which have been previously reported to play a role in improving the condition of insulin resistance, indicate that this fraction may help in combating the type II diabetic condition as well (Wu, 2007). This result is similar to that of reported earlier where several alkaloids (castanospermine and chlorogenic acid)

have been shown to improve the diabetic condition in a similar manner (Gupta, 1997; Jung, 2006; Stegelmeier et al., 2008; Kang et al., 1995; Kagami et al., 2008;).

Till this point, the possible mode of action of this extract appears to be mostly extra pancreatic since it did not show any insulin mimetic activity yet it improved the diabetic condition which is probably by increasing the pancreatic β-cell numbers and/or improving insulin sensitivity. In order to identify any possible involvement of this extract in insulin receptor sensitization process it was important to correlate their effects with expression profiles of some of the related genes and their effects on lipid profiles of diabetic animals. Weight loss in diabetic condition is a common phenomenon resulting due to osmotic diuresis and use of stored fat and cellular protein as fuel source. Increased lipolytic activity in adipose tissue causes increased serum triglyceride content and marked reduction in visible adipose depots (Terna and Kumar, 2004). These characteristics of diabetic conditions were improved in treated group as the serum triglyceride was reduced and also visible abdominal fat depots were significantly large and healthy. Histopathological examination of adipose tissue of diabetic animals also showed signs of marked necrosis in and it attained almost normalcy in *AR* fraction treated mice as was demonstrated by the sections from white adipose tissues of these mice which were compact and healthy.

Insulin receptor sensitizing activity of an antidiabetic molecule is generally attributed to the up regulation and activation of peroxisome proliferators activated receptor (PPAR) family members (PPARα and PPARγ). PPAR, a sub-family of 48-member steroid and nuclear receptor super family, are ligand dependent transcription factors that control energy homeostasis by regulating carbohydrate and lipid metabolism albeit with some prominent side effects like obesity (Shen et al., 2006). In the present

study the gene expression profile for PPARα and PPARγ were checked in adipocytes to confirm that the hypoglycemic activity of the extract is through these transcription factors. Although the expression profile of PPARγ showed dramatic improvement (about 30% over control), PPARα level did not improve significantly. This specific expression of PPARγ (not PPARα) indicated the direct involvement of this fraction in improving insulin resistant condition through PPARγ pathway. A similar pattern of activities has also been reported recently from several plant extracts rich in flavonoids, alkaloids, carotenoid, terpenoids (Takahashi et al., 2002; Shen et al., 2006; Huang et al., 2006; Sharma et al., 2008b). To the best of our knowledge no other studies provided any evidence regarding the role of alkaloids in the management of diabetes through PPARγ pathway.

The adipose tissue insulin resistance developed in type II diabetic condition is also associated with a pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α), the expression of which is also up regulated during this condition (Hotamisligil, G.S., 1999). A potential molecule demonstrating anti-diabetic (type II) activities may also regulate the expression of this protein, although its exact role in management of diabetes is not very clear as yet. In our study the *AR* fraction resulted in significant down regulation of TNF-α gene expression which further supports the antidiabetic potential of this fraction. Although, in case of Glut-4 although it's expression improved marginally both in muscle and adipose tissues (as shown by RT-PCR), the extract resulted in significant improvement in translation of this protein followed by its translocation to the plasma membrane from low density microsomes. This further conclusively proves the role of *AR* fraction in regulating glucose by peripheral tissues.

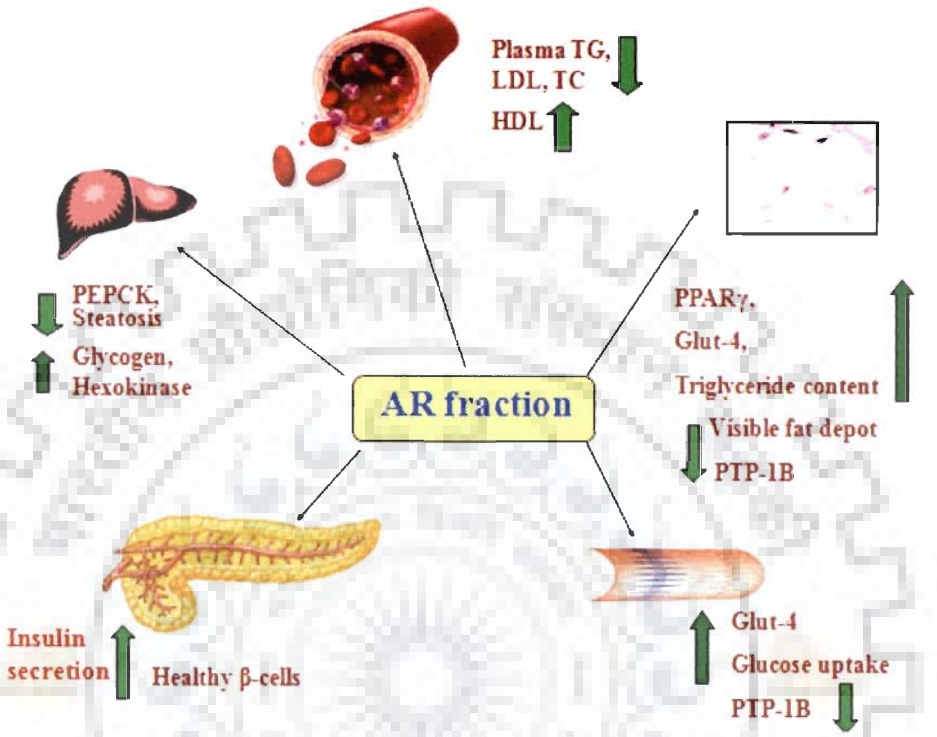


Figure 4.2.10. Effects of AR fraction from *C. decidua* plant extract on various targets.

Increased oxidative stress and high sorbitol accumulation in different organs is supposed to be the main cause of diabetes related complications such as retinopathy, neuropathy and nephropathy. Higher activity of aldose reductase is one of the major factors responsible for the accumulation of sorbitol in different organs (Nishimura, 1998). Therefore, inhibition of aldose reductase activity can provide a better prevention from diabetes related complications. A clear decrease in the expression levels of aldose reductase in the treated group when compared to the diabetic group thus confirmed that the alkaloid rich fraction has the potentiality for preventing complications associated with diabetes. Since alkaloids are generally suspected to be hepatotoxic when administered at high concentration, we checked the effect on liver of treated groups of animals. The doses used in the present study did not show any significant histopathological alteration in liver tissues thus confirming the dose to be non-toxic and this was also confirmed by LD_{50} analysis of the samples.

Results obtained from the present chapter demonstrate the action of alkaloids on various targets for diabetes treatment and might have multiple therapeutic effects. Improved FBG level and effective decline in blood glucose level following a glucose load indicates towards a clear improvement in peripheral glucose tolerance and insulin sensitivity which may be attributed to the inhibitory effect of *AR* fraction on PTP-1B activity. Increased serum insulin level and healthier histological profile might explain the antidiabetic and antihyperlipidemic effect of the extract.

Expression profile of different glucoregulatory enzyme i.e. G6Pase, PEPCK, PPARα and γ etc. proves the efficacy of *AR* fraction from *CD* for antidiabetic treatment. Increased amount of total fat depot and PPARγ activity which ultimately results in

sequenching of fat from blood to fat tissue can explain the improvement in plasma lipid profile. Fig. 4.2.10 shows the effect of the alkaloids at various target points in diabetic condition. Although alkaloids are known for their potential toxic effects on physiological system, *AR* fraction from *CD* fruits did not show any sign of toxic effect in the treated animals and validated their efficacy and safety for management of diabetes. In conclusion, the alkaloid rich fraction from *CD* fruits thus could be use for the management and cure of diabetes.

4.3 HYPOGLYCEMIC AND HYPOLIPIDEMIC EFFECTS OF FLAVONOIDS RICH FRACTION FROM *Eugenia jambolana* SEEDS ON STZ INDUCED DIABETIC RATS

4.3.1 Introduction

Eugenia jambolana (*EJ*) of family Myrtaceae (called black plum in English and Jamun in Hindi in India) had been widely used to treat diabetes by the traditional practitioners for many centuries (Nadkarni, 1954, Sharma et al., 2006). It is a large evergreen tree growing upto 30 m high found widely in India and many other countries of Asian subcontinent (Fig. 4.3.1). The fruits of this plant are oval to elliptical in shape, 1.5-3.5 cm long, dark purple or nearly black, luscious, fleshy and edible (Sharma et al., 2006).

The antihyperglycemic activity of seeds of *EJ* is well established (Shrotri et al., 1963; Bansal et al., 1981; Kohli, 1983; Archrekar et al., 1991; Grover et al., 2002; Vikrant et al., 2001; Sharma et al., 2003, 2006). However, there is not many information available on the effect of this plant extract in different types of diabetes and its role in improving lipid profiles except one by Sharma et al. (2003). They analyzed the effect of its ethanolic extract's effect on severe diabetic (SD) rabbits (Type I or IDDM) in which pancreas are almost or totally destroyed and mild diabetic (MD) rabbits (type II or NIDDM) having functional β-cells.

The aim of the present work was to explore the scientific basis of the utility of the flavonoids rich extracts of *EJ* seeds on biochemical and enzymatic parameters of SD and MD diabetic rats for correction of hyperglycemia and hyperlipidemia in diabetes. Though there are several reports on antihyperglycemic action of aqueous and ethanolic extracts of the *EJ* seed extracts, almost no information is available on the effects of the flavonoid rich extract from *EJ* plants on diabetic animal models.

Figure 4.3.1. *Eugenia jambolana* tree and inset showing the fruits of this plant

The term "flavonoid" is generally used to describe a broad collection of natural products that include a C_6-C_3-C_6 carbon framework, or more specifically a phenylbenzopyran functionality. Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes: **1)** the flavonoids (2-phenylbenzopyrans), **2)** isoflavonoids (3-benzopyrans), and **3)** neoflavonoids (4-benzopyrans). These classes are biogenetically and structurally related as they usually share a common chalcone precursor. The biochemical activities of flavonoids and their metabolites depend on their chemical structure and the relative orientation of various moieties in the molecule.

Over 4000 structurally unique flavonoids have been identified from plant sources (Harborne et al., 1975; Harborne, 1986). The flavonoids have long been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic activities. The flavonoids are typical phenolic compounds and, therefore, act as potent metal chelators and free radical scavengers. We extracted the flavonoid rich fraction from this plant and tested their efficacy on SD and MD rats representing two distinct categories of diabetes namely type I and type II, respectively.

4.3.2 Animal groups and treatment

Experimental rats were divided into five equal groups as follows:

(1) Control group: received intramuscular injection of citrate buffer.

(2) Mild diabetic group: (STZ, 4 mg/0.5 ml citrate buffer/100 g bw).

(3) Severe diabetic group: (STZ, 7 mg/0.5 ml citrate buffer/ 100 g bw).

(4) Mild diabetic + *EJ* supplement group: MD rats treated with flavonoids rich extract of *EJ* seed at the dose of 50 mg/kg bw

(5) Severe diabetic + *EJ* supplement group: SD rats treated with flavonoid rich extract of *EJ* seed at the dose of 100 mg/kg bw.

Flavonoids rich fraction was dissolved in 100 µl of 0.01% ethanol for oral gavaging to the animals of groups 4 and 5 for 21 days. At the same time, the animals of groups 1, 2, and 3 were subjected to 100 µl of 0.01% ethanol as vehicle control.

4.3.3 Results

4.3.3.1 In vivo analysis of antidiabetic potential of Flavonoids rich fraction

4.3.3.1.1 Fasting blood glucose levels and oral glucose tolerance test

There was a significant elevation in fasting blood glucose levels in SD and MD rats as compared to control group ($p<0.05$). However, supplementation of flavonoids rich extract of the *EJ* seeds to MD and SD rats for 21 days resulted in significant recovery of fasting blood glucose level and it resettled almost to the control level (Fig. 4.3.2, FBG data) ($p<0.05$).

The levels of blood glucose in control, MD, SD, MD plus *EJ* and SD plus *EJ* supplemented groups demonstrated a significant change in blood glucose level after administration of glucose (1 g/kg bw) in OGTT. The rats of MD and SD diabetic groups showed a significant elevation in blood glucose level throughout the total measurement period i.e. for 150 min with respect to control (Fig. 4.3.2) also it did not revert back to the initial value (FBG) even at the end of the period tested by us (150 min). However, in both the supplemented groups, although the blood glucose level did shoot up to a level higher than control animals, it started to decline gradually after 30 min of glucose administration and at 60 min time point it had almost resettled to the control level (Fig. 4.3.2).

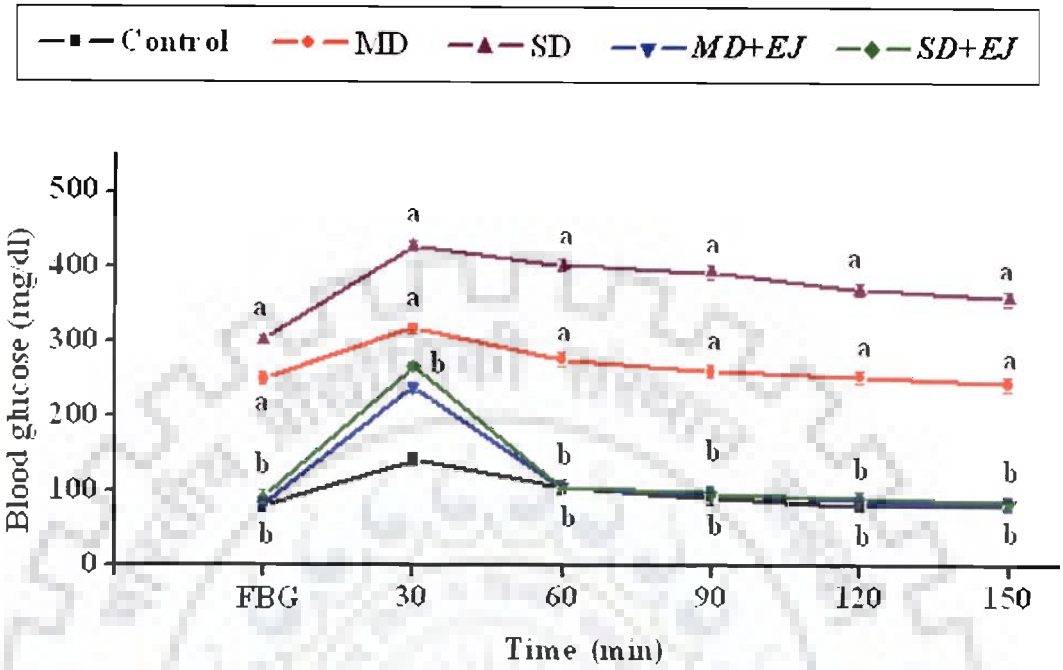


Figure 4.3.2. Oral glucose tolerance test in STZ induced mild and severe diabetic male albino rats in response to flavonoids rich fraction of *Eugenia jambolana*t. Results are mean ± S.E.M. of n = 10. MD, mild diabetic; SD, severe diabetic; FBG, Fasting blood glucose level; *EJ Eugenia jambolana.* a and b, indicates significant level of difference as compared to control and corresponding diabetic groups (MD and SD) respectively for each corresponding time point ($p<0.05$).

4.3.3.1.2 Effect on glycogen level in tissues

Glycogen content of the liver and muscle tissues in control, diabetic (MD and SD) and diabetic supplemented with *EJ* extract groups are shown in Fig. 4.3.3. Hepatic and skeletal muscle glycogen contents were significantly decreased in both MD and SD rats with respect to their corresponding control ($p<0.05$). However, treatment with flavonoid rich extract from *EJ* seeds led to an increase in liver (MD 35%, SD 67%) and muscle glycogen contents (MD 42% and SD 67 %) over control.

4.3.3.1.3 Enzyme activity in liver

After 21 days of treatment with flavonoid rich extract of *EJ* there was a significant reduction in liver G6Pase activity in both the diabetic groups compared to the untreated STZ induced diabetic rats. The reduced level of enzyme concentrations (MD 26 and SD 29%) were almost similar to that of the control rats (Fig. 4.3.4).

As compared to control group, the mean values of hepatic hexokinase activity decreased significantly ($p<0.05$) in diabetic rats (Fig. 4.3.4). Treatment of these diabetic (MD and SD) animals with *EJ* seed extract led to a rise in activity of this enzyme by 36% and 50% for MD and SD groups respectively ($p<0.05$), as compared to diabetic group.

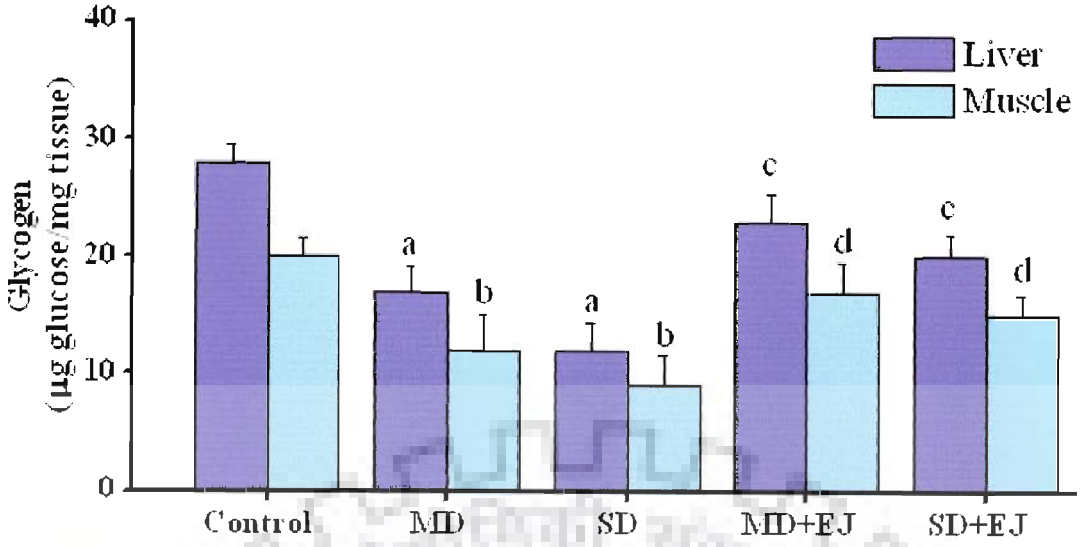


Figure 4.3.3. Effect of flavonoid rich fraction of *Eugenia jambolana* seed extract on liver and muscle glycogen contents in STZ induced diabetic MD and SD rats. Data is expressed as mean ± S.E.M; n = 10. a and c, indicates the significant level of differences in hepatic glycogen contents and b and d, indicates the significant level of differences in muscle glycogen contents as compared to control (non-diabetic), and treated group in respect to their corresponding diabetic groups (MD and SD) ($p<0.05$); MD, mild diabetic; SD, severe diabetic; *EJ*, *Eugenia jambolana*.

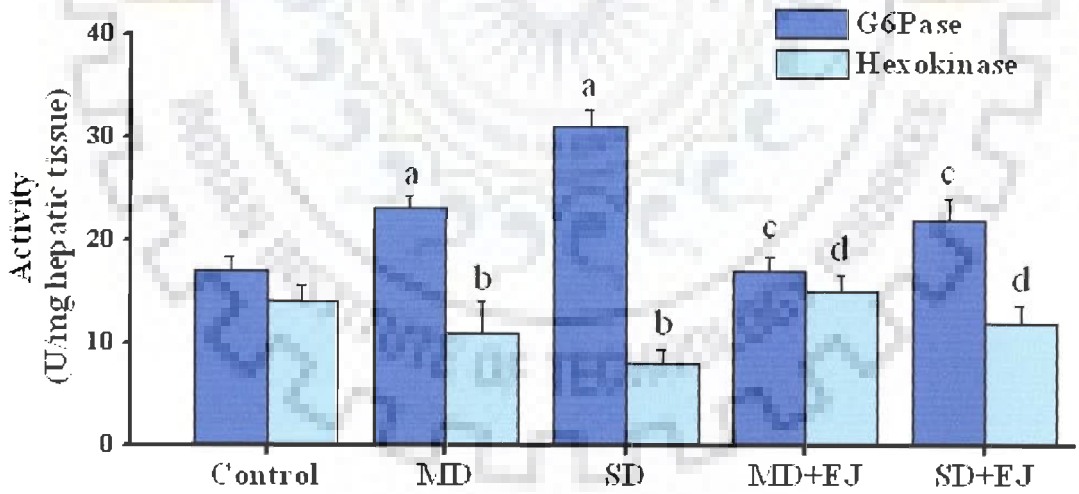


Figure 4.3.4. Effect of flavonoid rich fraction of *Eugenia jambolana* seed extract on G6Pase and hexokinase activity in STZ induced diabetic MD and SD rats. Data is expressed as mean ± S.E.M; n = 10. a and c, indicates the significant level of differences in G6Pase; b and d, indicates the significant level of differences in hexokinase activity as compared to control (non-diabetic), and treated group in respect to their corresponding diabetic groups (MD and SD) ($p<0.05$); MD, mild diabetic; SD, severe diabetic; *EJ*, *Eugenia jambolana*.

4.3.3.1.4 Estimation of plasma lipid profile

Plasma TC and TG levels were significantly elevated ($p<0.05$) in both diabetic groups in comparison to control (Table 4.3.1). Supplementation of *EJ* seed extract for 21 days to the MD and SD rats resulted in a significant diminution of these parameters ($p<0.05$) and the levels of these parameters resettled towards the control level (Table 4.3.1).

Other hyperlipidemic parameters like serum LDL and VLDL cholesterol were both elevated in MD (72% and 29%, respectively) and SD (102 and 83% respectively) groups in comparison to control (Table 4.3.1). However, both these parameters decreased significantly ($p<0.05$) in the extract supplemented groups of MD (27% and 21%, respectively) and SD (29% and 31% respectively), and were resettled towards the control level.

Levels of HDL cholesterol, a friendly lipoprotein, were decreased in both the diabetic groups in respect to the control (Table 4.3.1). After 21 days of treatment with the flavonoid rich extarct there was a significant elevation ($p<0.05$) of this lipoprotein level in both MD and SD group of rats (Table 4.3.1).

Table 4.3.1. Plasma level of different lipids after the administration of flavonoids rich fraction from Eugenia *jambolana* seed in STZ induced diabetic rats

Treatment Group	Plasma Lipid level (mg/dl)				
	TC	HDL	LDL	TG	VLDL
Control	75.2±5.1	69.2±4.0	64.3±6.0	29.1±4.0	97.5±3.6
MD	97.7±2.5[a]	48.5±4.5[a]	110.5±5.9[a]	37.6±2.7[a]	164.7±5.3[a]
SD	120.3±5.0[a]	38.0±2.6[a]	129.7±3.5[a]	53.3±3.5[a]	191.6±7.4[a]
MD + *EJ*	81.9±3.6[b]	58.0±2.9[b]	80.3±4.7[b]	29.7±1.5[b]	107.2±2.6[b]
SD + *EJ*	84.8±4.0[c]	51.6±4.7[c]	92.1±3.0[c]	36.7±2.0[c]	120.4±5.7[c]

Each value represents the mean ± S.E.M. (n = 10).
a, b and c represents statistically significant ($p<0.05$) as compared to normal control, MD and SD groups respectively among each class of lipids. MD, Mild diabetic; SD, Severe diabetic; *EJ*, *Eugenia jambolana*.

4.3.3.1.5 Effects of flavonoids rich EJ seed extract on insulin release in diabetic rats

Serum insulin level was significantly decreased in MD and SD rats with respect to control ($p<0.05$) (Fig. 4.3.5). After 21 days of *EJ* seed extract supplementation to the MD and SD groups of diabetic animals, though there was a significant elevation in serum insulin levels in SD rats as compared to their diabetic counterpart ($p<0.05$), the improvement was only marginal in case of MD rats (statistically not significant).

In order to further understand the role of the seed extract as an insulinotropic agent, insulin release *in vitro* from pancreatic islets of Langerhans of mild diabetic rats was investigated. The incubation of ten islets from these animals with 10 mM glucose in presence of *EJ* seed extract for 1 h resulted in significant stimulation of insulin ($p<0.05$), which was about 14.5 % of increase over the diabetic untreated control group (Table 4.3.2). However the level of released insulin failed to reach the level as shown by islet cell from control (normal rats) animals.

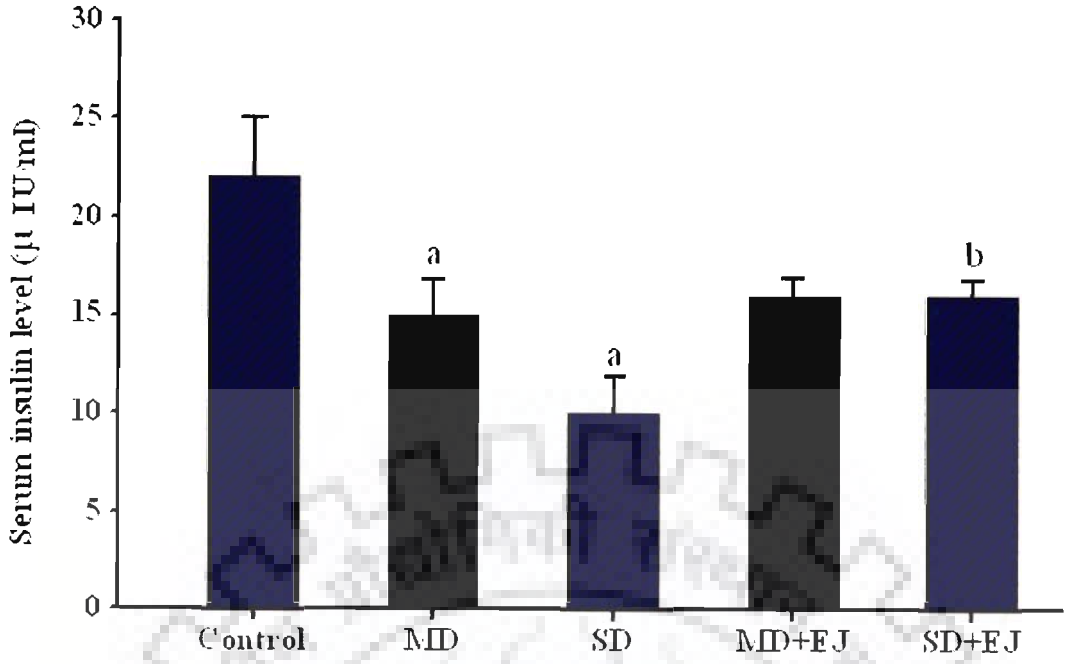


Figure 4.3.5. Effects of flavonoid rich fraction of *Eugenia jambolana* seeds on serum insulin levels in STZ induced MD and SD male rats. Data is expressed as mean ± S.E.M; n = 10. a and b, indicates the significant level of difference in serum insulin concentrations as compared to non-diabetic control and SD rats, respectively ($p<0.05$). MD, mild diabetic, SD, severe diabetic, *EJ, Eugenia jambolana.*

Table 4.3.2. Effect of flavonoid rich fraction of *Eujenia jambolana* seed on insulin release *in vitro* from isolated islet of Langerhans of mild diabetic rats

Treatment Group	**Insulin release (µIU/ten islets/h)**
Control	332.3 ± 17.6
MD	271.7 ± 7.6[a]
MD + EJ	311.0 ± 10.5[b]

Each value represents the mean ± S.E.M. of four independent experiments each performed in quadruplicates. a and b, represents statistically significant ($p<0.05$) as compared to control and MD groups respectively. MD, Mild diabetic; *EJ, Eugenia jambolana.*

4.3.3.1.6 PTP-1B inhibition activity

The flavonoids rich fraction from the *EJ* seeds showed a potent PTP-1B inhibition activity. The extract showed an IC_{50} value of 13.7 mg/ml on PTP-1B. The most surprising part of this activity was almost two folds decrease in the inhibition of PTP-1B activity by the flavonoid rich fraction over the crude extract (IC_{50} value of 6.8 mg/ml). This could be attributed to the loss of some active constituents during the fractionation process.

4.3.3.1.7 Gene expression profile

As shown in fig. 4.3.6, a marked change in expression profiles of mRNA of all the glucose regulatory enzymes studied in liver, muscle, adipose tissue, and kidney was observed in treated groups. The hepatic level of GK was increased by 2.5 folds in treated rats, at the same time there was a clear reduction in the expression patterns of gluconeogenic enzymes, G6Pase and PEPCK by almost 2 folds compared to diabetic group (Fig. 4.3.6a). Glut-4 is responsible for tissue specific glucose uptake in muscle and adipose tissues. There was a clear increase in the expression level of Glut-4 in treated group (about two folds as compared to diabetic control), more prominent in adipose tissue in comparison to muscle tissue (Fig. 4.3.6b). Further, the kidneys of the treated rats demonstrated a clear reduction of more than 50% in the expression level of aldose reductase, an enzyme which is responsible for diabetes related complications as compared to diabetic rats (Fig. 4.3.6b).

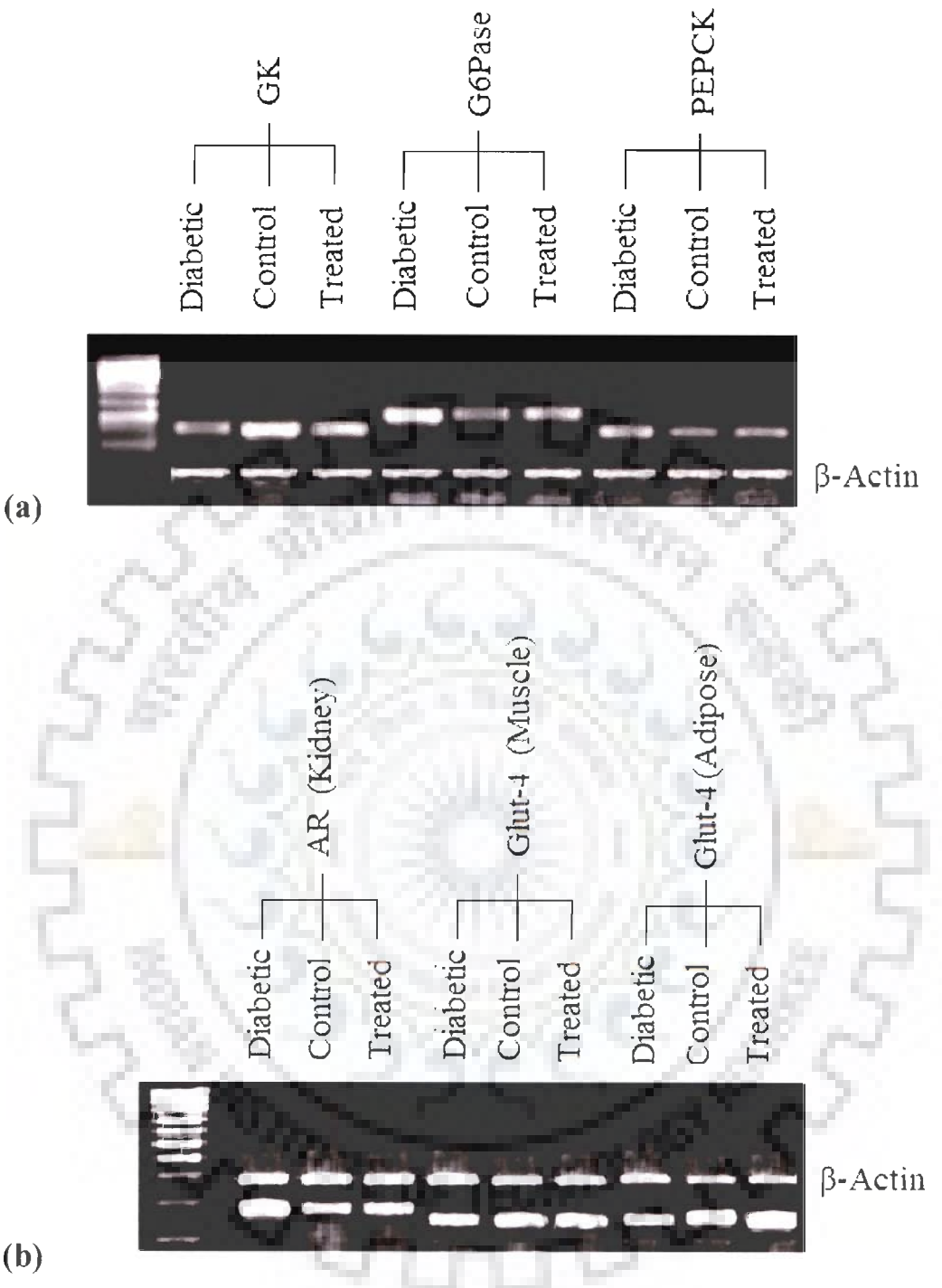


Figure 4.3.6. RT-PCR analysis of (a) liver mRNA expression of GK, G6Pase and PEPCK and (b) kidney, muscle and adipose tissue mRNA expression of AR and Glut-4 genes in diabetic, control and treated animal groups. The total RNA isolated was reverse transcribed and cDNA obtained was subjected to PCR. GK, Glucokinase; G6Pase, Glucose 6 Phosphatase; PEPCK, Phosphoenolpyruvate carboxykinase; AR, Aldose reductase; Glut-4, glucose transporter-4.

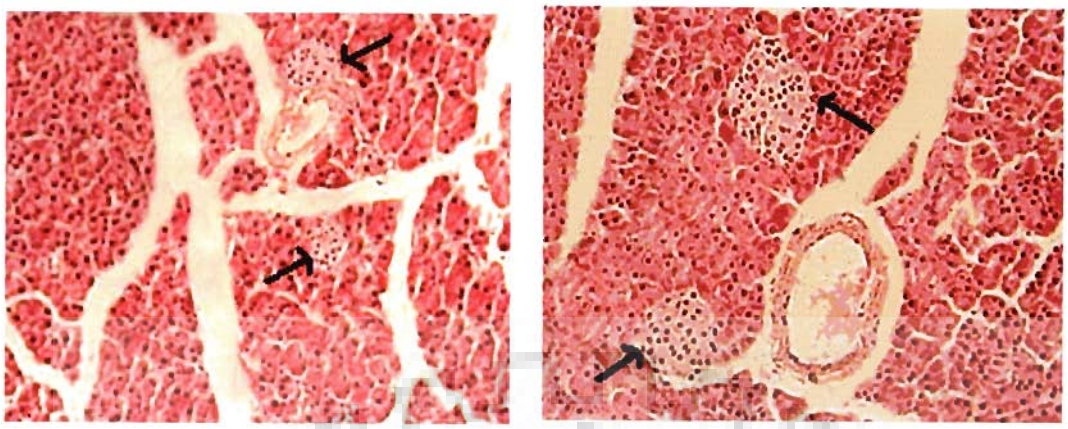
4.3.3.1.8 Histopathological studies

Sections of pancreas from diabetic and treated rats have clearly shown the protective effect of flavonoid rich extract. A clear decrease in the size of pancreatic β-cells in STZ induced rats was observed (Fig. 4.3.7a) which again normalized in flavonoid rich extract of *EJ* treated rats (Fig. 4.3.7b).

4.3.3.2. In vitro analysis of antidiabetic potential of flavonoids rich fraction

4.3.3.2.1 Effect of flavonoids rich fraction on cell viability

In order to check for the effect of flavonoid rich fraction on cell viability, the cells were incubated with increasing concentration of the fraction. As shown in fig. 4.3.8, no significant reduction in the cellular viability was detected till 500 mg/l concentrations of the extract and the cells appeared healthy even at the highest concentration tested by us.



(a) (b)

Figure 4.3.7. Photomicrograph of (a) pancreas from STZ induced diabetic rats and (b) diabetic rats treated with flavonoid rich extract. Haematoxylin and Eosin, 20X. The arrow indicates the islet region of the section.

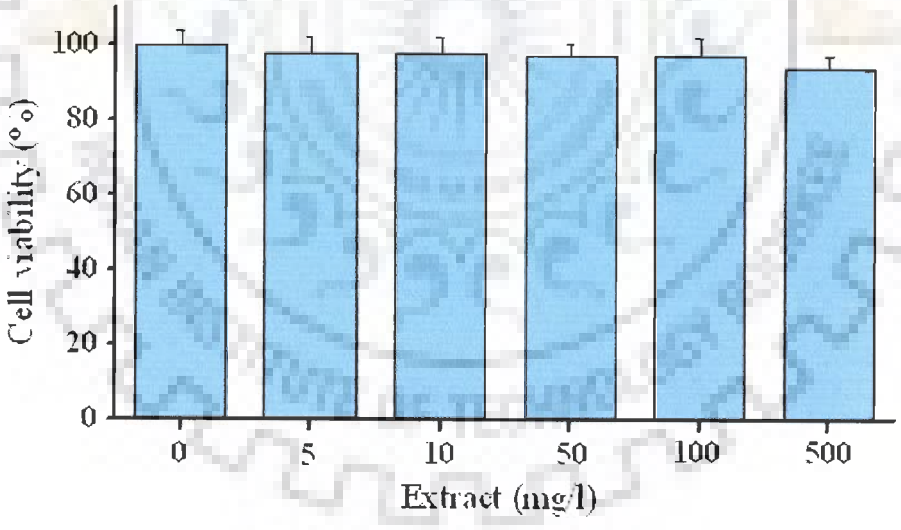


Figure 4.3.8. Dose dependent effect of flavonoid rich fraction from *EJ* seed on cell viability. Various concentrations of *EJ* extract were added and incubated for 1 h. Cell viability was expressed in comparison to vehicle treated control cells which given a value of 100%. Values represent means ± S.E.M. of three independent experiments each performed in triplicates.

4.3.3.2.2 Identification of PPAR activity for the EJ seed extracts

Flavonoids rich extract of *EJ* seeds were tested for PPAR activity using PPAR reporter-gene bioassay (Fig. 4.3.9). The extracts significantly stimulated both PPARα and PPARγ activities in a dose dependent manner ($p<0.05$). At a dose of 30 mg/l, the extract increased PPARα and PPARγ activity upto 3 and 4 folds over the vehicle control, respectively (Fig. 4.3.9). Thereafter though there was no significant increase in the *EJ* induced activity in PPARγ but PPARα activity continued to increase which stabilized at 500 mg/l concentrations (data not shown). Thus flavonoid rich *EJ* seed extract seems to demonstrate dual PPARα and PPARγ activity in a dose dependent manner.

4.3.3.2.3 Western blot analysis

The potential effect of Flavonoids rich fraction on PPARγ activity was further authenticated by immunoblot analysis of PPARγ in 3T3-L1 adipocytes. Western blot analysis with specific antibody for PPARγ indicated that the extremely low levels of endogenously expressing PPARγ in 3T3-L1 adipocytes are significantly elevated in response to rosiglitazone (as positive control) and flavonoids rich extract of *EJ* seed (Fig. 4.3.10).

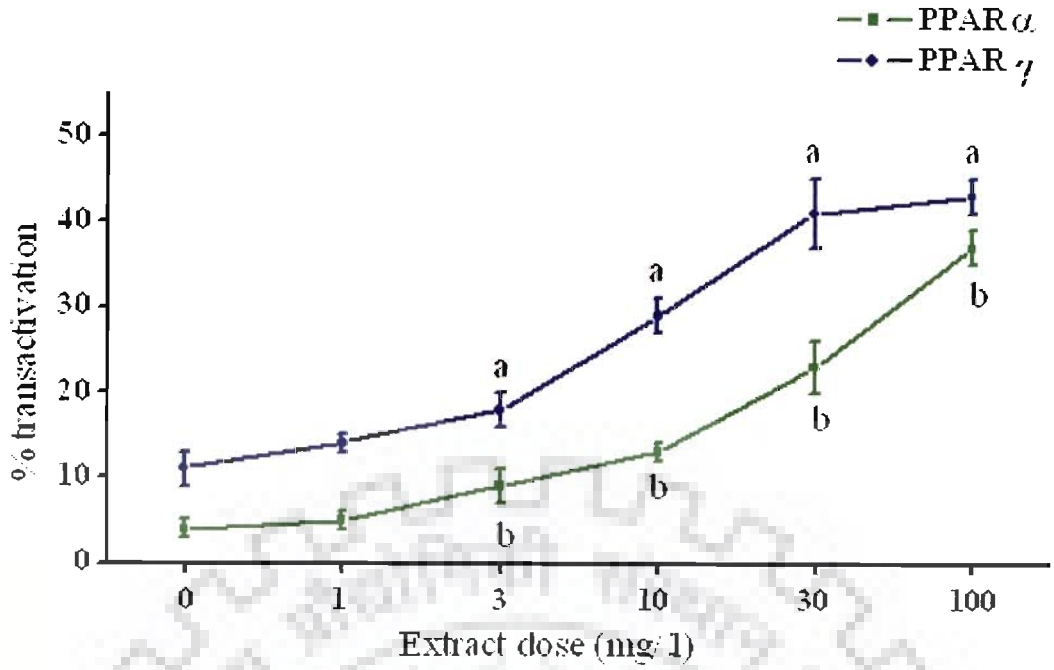


Figure 4.3.9. Flavonoid rich extract activated PPARs as determined by lucifersae assays using full length PPAR constructs. tk-PPREX3-Luc, pRL-CMV and pCMX-mPPARα or pCMX-mPPARγ were transfected to HepG2 cells and after 24 h of transfection, the cells were treated with flavonoid rich *EJ* extract of varying concentrations starting from 1 mg/l to 100 mg/l for 24 h. Values are mean ± S.E.M. of five independent experiments each performed in quadruplicates and are expressed as percentage of the positive controls for PPARα (Wy 14643, 16.2 mg/l) and PPARγ (Rosiglitazone 1.8 mg/l), which were given a value of 100%. a and b indicates the significant level of difference in values as compared to vehicle treated control incubation for PPARγ and PPARα, respectively ($p<0.05$).

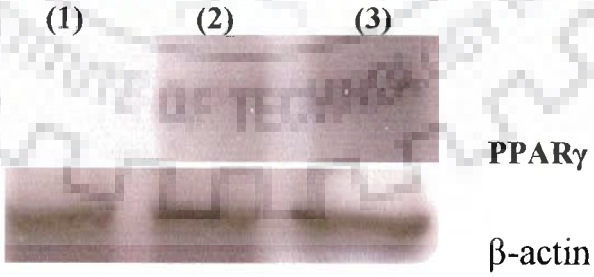


Figure 4.3.10. Immunoblot showing PPARγ in differentiated 3T3-L1 adipocytes in response to *EJ* extract and Rosiglitazone (as positive control). 3T3-L1 cells and were exposed to *EJ* extract (100 mg/l) and PPARγ protein was measured with an anti-PPARγ polycolonal antibody. 1, vehicle treated; 2, treated with 100 mg/l of *EJ* extract; 3, treated with 7 mg/l of rosiglitazone.

4.3.3.2.4 Adipocytes differentiation and triglyceride accumulation

Since adipocyte differentiation is marked by accumulation of lipid droplets and rise in the cellular TG content, we estimated the cellular triglyceride levels in differentiated 3T3-L1 preadipocytes after treating them with increasing concentration of *EJ* seed extract. Cellular TG levels showed significant rise at the concentration of 3 mg/dl and this continued upto 30 mg/dl ($p<0.05$). Thereafter the levels of TG begin to decline (Fig. 4.3.11). Maximum amount of TG accumulation (approximately 3 folds over vehicle control) was observed after the addition of 30 mg of *EJ* seed extract.

4.3.3.3 HPLC analysis of Flavonoid rich fraction

HPLC analysis of Flavonoids rich fraction of *Eugenia jambolana* was performed in comparison with known commercial standards. Chromatograph showed four major and five minor peaks representing different members of flavonoids family. Peak 1- 4 (at 6.24, 9.76, 20.32 and 25.12 min) represents rutin, myricitine, quercitine and kaempferol respectivey. Five more minor peaks designated as X^1-X^5 were also present but we failed to identify them.

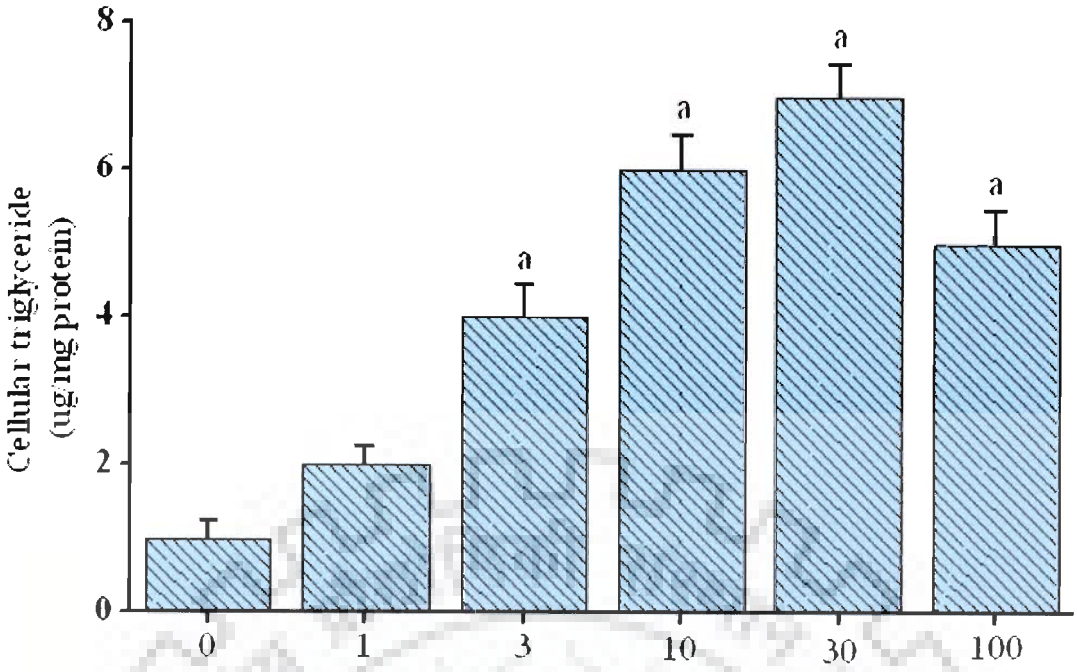


Figure 4.3.11. Dose dependent effect of flavonoid rich fraction from *EJ* seed on cellular triglyceride content in 3T3-L1 cells. Various concentrations of *EJ* extract were added from the induction stage. The cellular triglyceride content was determined as described in chapter 3 and expressed as µg/mg of protein. Values represent mean ± S.E.M. of five independent experiments each performed in quadruplicates. a indicate significance as compared to vehicle treated control ($p<0.05$).

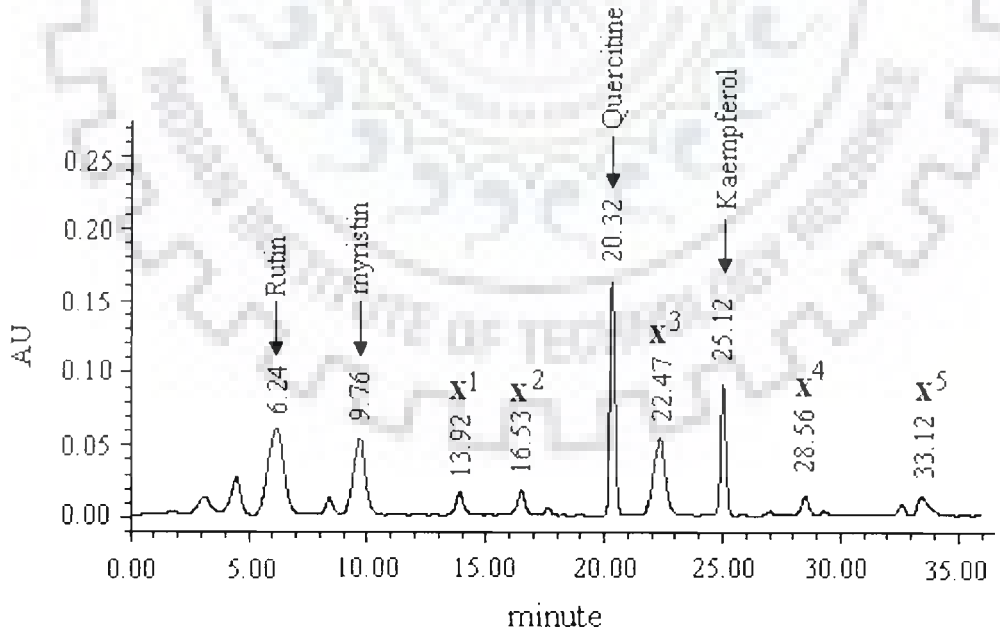


Figure 4.3.12. High performance liquid chromatograms of flavonoids rich fraction from *Eugenia jambolana*. The arrows indicate the flavonoids identified on the basis of similarity of retention time in comparison with their reference standards.

4.3.4 Discussion

Diabetes is a chronic metabolic disorder affecting a major proportion of the population worldwide. A sustained reduction in hyperglycemia will decrease the risk of developing microvascular diseases and reduce their complications (Kim et al., 2006). The conventional therapies for diabetes have many shortcomings like side effects and high rate of secondary failure. On the other hand herbal extracts are expected to have similar efficacy without side effects as that of conventional drugs. The present investigation reports the antidiabetogenic and antihyperglycemic effects of flavonoid rich fraction of *Eugenia jambolana* seed on STZ induced diabetic rats. It is generally accepted that SD is of type I diabetes and MD is of type II diabetes (Vessal et al., 2003; Sharma et al., 2003; Maiti et al., 2005). This effect is depicted by the high level of blood glucose in animals. The main aim of this study was to assess the multiple roles of *EJ* plant extract as an antidiabetic agent for correction of SD or type I diabetes where β-cells degeneration is dramatic and MD or type II diabetes where β-cell degeneration is partial.

Treatment with flavonoid rich extract resulted in the significant reduction of peak level of sugar within 2 h time and this fact further strengthens the antidiabetogenic potentiality of this plant extract in rodent models as reported by many authors earlier (Sharma et al., 2003; Sridhar et al., 2005; Grover et al., 2002). Antihyperglycemic potency of the flavonoid rich extract of *EJ* seeds in SD and MD rats has been indicated here by improvement in FBG levels which is also an important parameter for monitoring diabetes (Maiti et al., 2005). Further, the plant extract significantly decreased the blood glucose level in glucose loaded rats (GTT) and this fact could be attributed to the potentiation of the insulin effect of plasma by increasing the pancreatic secretion of insulin from existing β-cells or its release from bound insulin. In this context a number of

other plants have been observed to have similar pattern of hypoglycemic effects (Kasiviswanath et al., 2005). Results on the plasma insulin release from pancreas (both *in vitro* and *in vivo*) directly indicate that the antihyperglycemic activity of the flavonoid rich extract is partly due to the release of insulin from the pancreas i.e. it exerts a direct insulinotropic effect. Surprisingly, the insulinotropic efficacy of this extract is lower in MD rats *in vivo,* probably due to higher level of serum insulin as compared to SD rats. A similar effect has also been reported earlier by *Tarmarindus indica* seed extract *in vivo* (Maiti et al., 2005). Earlier studies by Achrekar at al. (1991) and Sharma et al. (2006) demonstrated that the water extract of pulp of *EJ* potentiates insulin release from pancreatic β-cells like some of the sulphoanylureas such as tolbutamide. Further, synthetic derivates of flavonoids have been shown to release insulin from insulinotropic INS-1 cells in culture which further supports our finding that flavonoid rich extract probably act as an insulinotropic agent as well (Bozdag-Dundar et al., 2001). Bansal et al. (1981) reported that the increase in plasma insulin brought by seeds of *EJ* may be attributed to proinsulin to insulin conversions, possibly by pancreatic cathapsin B, and/or its secretion. Another possible explanation of improved peripheral glucose clearance during OGTT come from the potential of flavonoid rich fraction to inhibit PTP-1B activity; which ultimately results in an improved peripheral insulin sensitivity and glucose clearance, earlier also several plants and phytochemicals has been shown to exert a similar effect (Zhang et al., 2006; Na et al., 2006; Jang et al., 2008). However, this plant extract failed to show any α-glucosidase inhibition activity, one of the factors responsible for postprandial glucose level regulations. The absence of this inhibitory function further strengthens the role of PTP-1B in the glucose clearance during OGTT as discussed earlier.

The administration of flavonoid rich *EJ* seed extract significantly decreased serum TG and cholesterol in diabetic rats. In consistence with the present data, other workers have reported that the administration of crude *EJ* plant extract to STZ induced diabetic rats improved the serum cholesterol and TG levels as compared to control and this effect was also similar to insulin treatment (Lopes-Virella et al., 1983; Ravi et al., 2005). Cholesterol lowering property of *EJ* seed extract could be attributed to hypocholesterolemic compounds that may act as inhibitors for relevant enzymes such as hydroxyl methyl glutaryl CoA reductase, which participates in cholesterol synthesis or reduce the absorption of cholesterol from intestine (Sharma et al., 2003) or the extract might stimulate the production of insulin which in turn inhibits lipoprotein lipase activity (Ravi et al. 2005) or reduces lipid peroxidation (Ravi et al., 2004).

Glycogen is the primary intracellular storable form of glucose and its level in various tissues especially in liver and skeletal muscles is a direct reflection of insulin activity since it regulates glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase. Since STZ causes selective destruction of β-cells of islets of Langerhans resulting in marked decrease in insulin levels, it could be predicted that glycogen levels in tissues (muscle and liver) decrease as the influx of glucose in the liver is inhibited in the absence of insulin (Golden et al., 1979; Weber et al., 1966; Vats et al., 2004). Our results showed that supplementation of diabetic rats with *EJ* extract resulted in significant elevation of both muscle and hepatic glycogen content. This point's towards one of the possible ways the plant extract might act by improving the glycogenesis process in muscle and liver. Further, the flavonoids were also found to improve hepatic hexokinase and G6Pase enzyme levels, two major enzymes involved in glucose homeostasis, in both MD and SD rats. On treatment with flavonoids from *EJ*

plants to diabetic rats, the levels of hexokinase and G6pase increased and decreased, respectively almost to control in those animals. This result is similar to those reported earlier where several potential herbal plant extract and pure flavonoids like quercetin have been shown to improve the diabetic condition (Gupta et al., 1999; Vessal et al., 2003; Maiti et al., 2005). However, the extract did not show any significant improvement in LDH activity (data not shown).

Effect on enzyme activities was further confirmed by expression analysis in which the changes in enzyme activity were in parallel with the changed expression level of GK, G6Pase and PEPCK, the enzymes directly linked to glucose homeostasis and production of glycogen in treated rats as compared to diabetic rats. Reduction in the expression of latter two enzymes (G6Pase and PEPCK) in response to extract treatment provides an additional clue for its role in carbohydrate metabolism. Further, reduction of these two enzymes has been linked to improved insulin resistant condition by some authors, indicating this extract's involvement in attenuating type II diabetic conditions too (Wu et al., 2007). This result is similar to that of reported earlier where several potential herbal plant extracts have been shown to improve the diabetic conditions (Gupta et al. 1999; Jung et al., 2006).

Retinopathy, neuropathy and nephropathy are main complications which make diabetes more severe. Accumulation of sorbitol in different organs due to a higher activity of aldose reductase is one of the major causes of these complications (Nishimura, 1998). Therefore, inhibition of aldose reductase activity can provide a better management of diabetes related complications. In this respect, aldose reductase expression level was observed in kidney and there was a clear decrease in its expression in treated rats when compared to diabetic animals. This result is similar to those reported earlier where several

potential herbal plant extracts has been shown to improve the diabetic conditions (Gupta et al., 1999; Maiti et al., 2005).

Till this point, the possible mechanism of action of this extract appears to be both pancreatic and extra pancreatic that have been supported by several assays as described earlier. For example, in serum insulin assay though the increase in serum insulin level in MD rats supplemented with *EJ* extract did not increase significantly as compared to untreated MD group, yet these supplemented rats demonstrated a significant improvement in its G6Pase activity and glycogen storage capacity. The extra pancreatic activity may be by inhibiting insulinase activity in both liver and kidney. Water extract of pulp of *EJ* seed extract have already been reported to have insulinase inhibiting activity (Achrekar et al., 1991). However, to the best of our knowledge there is no report on the effect of *EJ* plant extract on sensitization of insulin receptors. PPAR, a sub-family of the 48-member steroid and nuclear receptor superfamily, are ligand dependent transcription factors that control energy homeostasis by regulating carbohydrate and lipid metabolism (Shen et al., 2006). It has been reported earlier that small phenolic molecules presenting some "antidiabetic" botanical foods may activate the PPAR-signaling system (Shen et al., 2006). The flavonoid rich extract from *EJ* seeds also demonstrated a dual regulator function for PPARα/γ which could be attributed to some polyphenolic constituents within the extract. Although there have been reports on the hypolipidemic constituents in the *EJ* seed extract, there was almost no information on the activation of the PPAR dependent action of these constituents (Sharma et al., 2003; Ravi et al., 2005). A similar pattern of PPAR activation and antihyperlipidemic effect has been reported earlier from ethanolic extracts of licorise (Mae et al., 2003), purified isoflavones from *Astragalus membranaceus* and *Pueraria thomosonii* (Shen et al., 2006), carotenoids, terpenoids and fenofibrates (Takahashi et al., 2002), ethyl acetate extract of *Punica granatum* flowers (Huang et al., 2005), CM 108, a flavone derivative, (Guo et al., 2006), flavonoids isolated

from Hawthorn leaves (Fan et al., 2006), kaempferol and quercetin isolated from *Euonymus alatus* (Fang et al., 2008). In our study the *EJ* seed extract was also found to be involved in the differentiation of adipocytes from preadipocytes which was shown by cellular accumulation of triglycerides on treating the cells with rosiglitazone (as positive control) and the extract. Further the extract could also up regulate the expression of PPARγ protein which has also been shown earlier in response to various other plant extracts (Park et al., 2005; Shen et al., 2006).

The HPLC analyis of Flavonoid rich fraction showed that the flavonoid rich fraction of *Eugenia jambolana* seed contains different types of flavonoids: rutin, quercetin, myricetin and kaempferol etc. This is supported by earlier reports by several authors where extract *of EJ* leaves were reported to be rich in substances like triterpenoids, anthocianins, essential oils, oleic acid, saponins, glycosides, several members of flavonoids like rutin, quercetin, myricetin, myricitrin, myricetin 3-O-(4''-acetyl)-α-L-rhamnopyranosidase (Timbola et al., 2002). Hence it may be concluded that the hypolipidemic effect produced by the extract may be due to the presence of any or a combination of them having direct or indirect effects on this mechanism (Ravi et al., 2005).

The experimental evidences and observations obtained in the present chapter provide a clear idea about the efficacy of flavonoid rich fraction to improve carbohydrate and lipid metabolism. Improvement in insulin levels both *in vivo* and *in vitro* shows the direct effect of this fraction on pancreatic cells, also evident from histological analysis of pancreatic β-cells, which ultimately results in an improved glucoregulation.

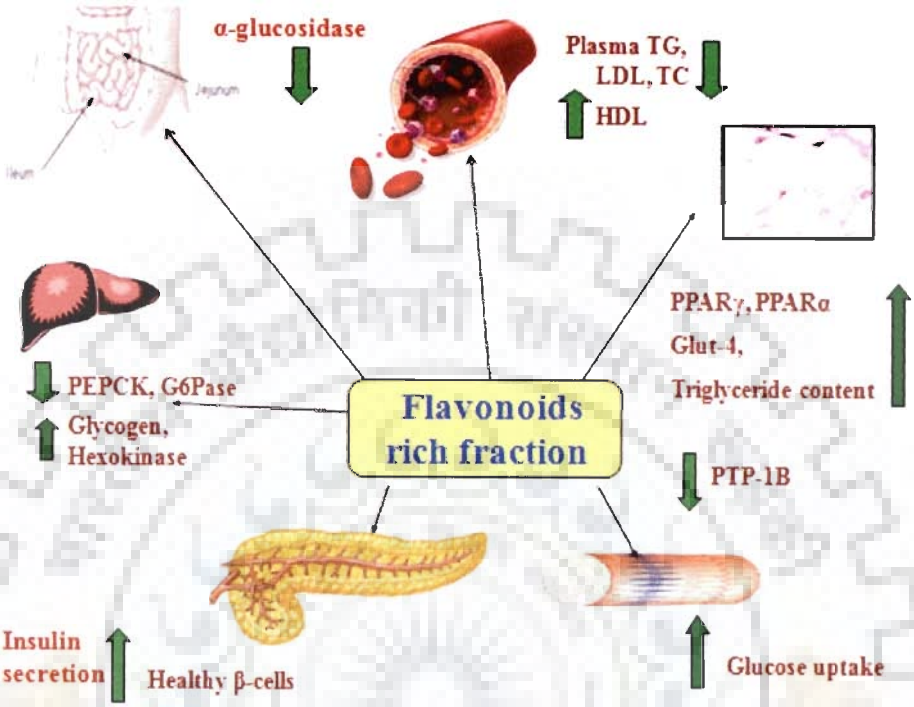


Figure 4.3.14. Effects of flavonoid rich fraction on different organ systems

Hypolipidemic effect of flavonoid rich extract can be attributed to its direct effect on adipose tissue, where the treatment with extract resulted in an increased level of PPARγ. Dual regulation of PPARα and γ is the one of the most interesting properties of the extract and explains the efficacy of extract for the improvement of lipid profile and glucoregulation. Fig. 4.3.14 summarizes the cross talk between various targets in glucose regulation by this flavonoid rich extract. In brief, the present study gives the proof of concept for the fact that the flavonoid rich *EJ* seed extract has the potential to act at multiple sites not only in type II diabetes but also in type I diabetes.

4.4 EFFECTS OF *GUGGULSTERONE* ISOLATED FROM *Commiphora mukul* ON HIGH FAT DIET INDUCED DIABETIC RATS

4.4.1 Introduction

In the present era of science and technology, treatment of any disease involves both traditional as well as conventional approaches each with its own positive or negative impacts. In case of conventional drug therapy, symptom or a specific target for that problem is considered (e.g. the tumor cell or the pathogenic microorganism) and not the root cause of a particular disease, whereas traditional medicines follows a holistic approach, its prime focus on activation of the body's defense, protective and repair mechanisms (Wagner, 2005). In spite of having enormous potential to cure many diseases traditional medicine could not achieve the popularity it deserves in the scientific community, which demands scientific validation of any drug for the treatment of a particular disease. The reason behind this is the lack of authentication, identification and qualitative as well as quantitative standardization of these traditional medicines. At the same time the presence of heavy metal contaminants and undesired compounds which impose hazardous effect on the physiological system makes the issue more complicated (Gilbert, 2008; Samy and Gopalkrishnakone, 2007).

One of the major ways to surmount these problems is to adopt the method of initial purification of medicinal products so as to eliminate any kind of detrimental components before using in herbal preparation. This has been described as "*shodhan*" in ancient medicinal literature (Tiwari, 2005). This can be achieved following a sequential activity based analysis along with the identification as well as standardization of active principles using certain modern analytical procedures. The strategy stated above was adopted to study the antidiabetic potential of a well known herbal resin known as gum

resin, gum guggul or oleo resin from *Commiphora mukul* commonly known as guggul (Fig 4.4.1). The gum resin is yellowish, collected when the sap is tapped and a pale yellow, aromatic fluid flows out that turns into an "agglomerate of tears or stalactic pieces" that are reddish brown, golden brown, or dull green. The gum resin has been used in Ayurvedic medicine for centuries to treat a variety of ailments, including obesity, bone fractures, arthritis, inflammation, cardiovascular disease, and lipid disorders (Satyavati, 1991; Dev, 1987; Sinal; Gonzalez, 2002; Urizar and Moore, 2003).

Two stereoisomers *E*- and *Z-guggulsterone* (*cis*- and *trans*-4, 17(20) - pregnadiene- 3, 16-dione, respectively) are the most important constituents from *C. mukul*, studied in detail for their therapeutic potential (Fig. 4.4.2). Various studies has been conducted to understand and illustrate the mechanism of action and potential of *guggulsterone* as an therapeutic agent using synthetic E and Z isomers (Urizar et al., 2002; Wu et al., 2002, Bramlett et al., 2000; Cui et al., 2003; Sinal and Gonzalez, 2002; Urizar and Moore, 2003) (Stayrook et al., 2005; Rizzo et al., 2006; Brobst et al., 2004; Burris et al., 2005; Ding and taudinger, 2005; Shishodia and Aggarwal, 2004; Cheon et al., 2006; Ichikawa and Aggarwal, 2006).

Although numerous investigation reports are available on the hypolipidemic nature and its plausible relation with the type II diabetes treatment, but in most of the cases in place of natural *guggulsterone* their synthetic counterparts have been used. In these studies with synthetic products, investigators mainly used either *E* or *Z* forms instead of the combination of them, as they exist naturally in guggul lipids along with other components as mentioned and recommended in traditional medicinal literature.

Figure. 4.4.1. *Commiphora mukul* tree and the inset shows the resin of this plant.

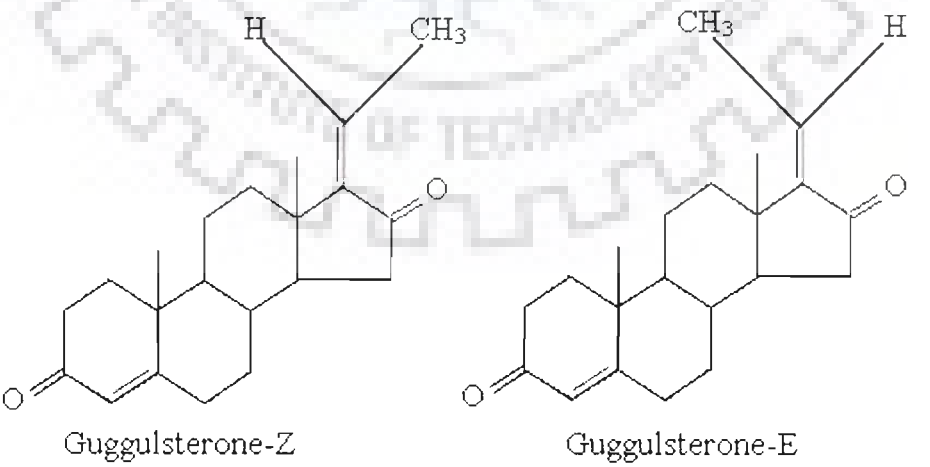


Figure 4.4.2. Isomers of *guggulsterone*

These synthetic isomers do not represent the exact form of the traditional extract from *C. mukul* and have not been approved either by Indian Pharmacopoeia or Food and Drug Administration (Majeed, 2004).

In order to address the role of natural *guggulsterone* the present chapter deals with the study of hypolipidemic and antidiabetic potential of the natural *guggulsterone E/Z* isolated from *C. mukul*, for the treatment of high fat diet induced insulin resistance and type II diabetes in rats. This was based on some earlier reports and preliminary screening from other laboratories, where this compound demonstrated hypolipidemic and antihyperglycemic activities (Urizar et al., 2002 and Urizar and Moore, 2003). The major aim was to explore any possible link between these compounds with the improvement of type II diabetic condition. To the best of our knowledge this is the first ever report on any holistic approach towards the effect of *guggulsterone* (isolated from *C. mukul*) for the management of type II diabetic conditions.

4.4.2 Animal group and treatment

The animals were randomly divided into four groups as given below:

Group I - Normal palate diet fed (control);

Group II - High fat diet fed rats (diabetic);

Group III - High fat diet fed rats treated with 200 mg/kg bw *guggulsterone E/Z* (treated).

Group IV - High fat diet fed rats treated with 20 mg/kg bw Metformin (metformin)

Rats from group II, III and IV were fed modified high fat diet (Table 3.2) for 16 weeks and rats from group I were fed normal palate diet. On completion of 16 weeks, the rats from group III and IV were treated with *guggulsterone* and metformin respectively for another 8 weeks. Rats from group I and II were gavaged 0.01% ethanol as control.

Guggulsterone E/Z isolated from *C. mukul* have been represented as *guggulsterone* in the present thesis.

4.4.3 Results

4.4.3.1 Effects of guggulsterone isolated from C. mukul in diet induced diabetic animal models

4.4.3.1.1Effect on fasting blood glucose level and glucose tolerance

Blood glucose profile of fasting rats of high fat diet fed diabetic group showed remarkable elevation in comparison to their control and treated counterparts. However, this level was reduced almost to controls upon treatment with 200 mg/kg bw of the *guggulsterone* (FBG data, Fig. 4.4.3). Systemic insulin resistance due to high fat diet caused a marked glucose intolerance in diabetic group as evident by poor glucose clearance after glucose load compared to normal diet fed control group. Treatment with *guggulsterone* improved the condition appreciably, as the rate of glucose clearance although not same but was comparable with that of metformin, an established agent for improvement of systemic insulin resistance (Fig. 4.4.3).

4.4.3.1.2 Effect on plasma lipid profile

Hypertriglyceredemia and elevated LDL level along with reduced HDL is the key feature of high fat diet induced insulin resistance. Treatment with *guggulsterone* showed significant improvement in lipid profile as reported by other authors (Singh et al., 1998; Chander et al., 1996). The level of harmful lipids like TC, LDL, VLDL cholesterol and TG were significantly decreased in the *guggulsterone* treated animals (About 25%, 25% a and 47%, respectively with respect to diabetic group) ($p< 0.05$). In addition, HDL level was found to be increased by 43% (as compared to diabetic control) in diabetic rats after treatment with *guggulsterones* (Table 4.4.1).

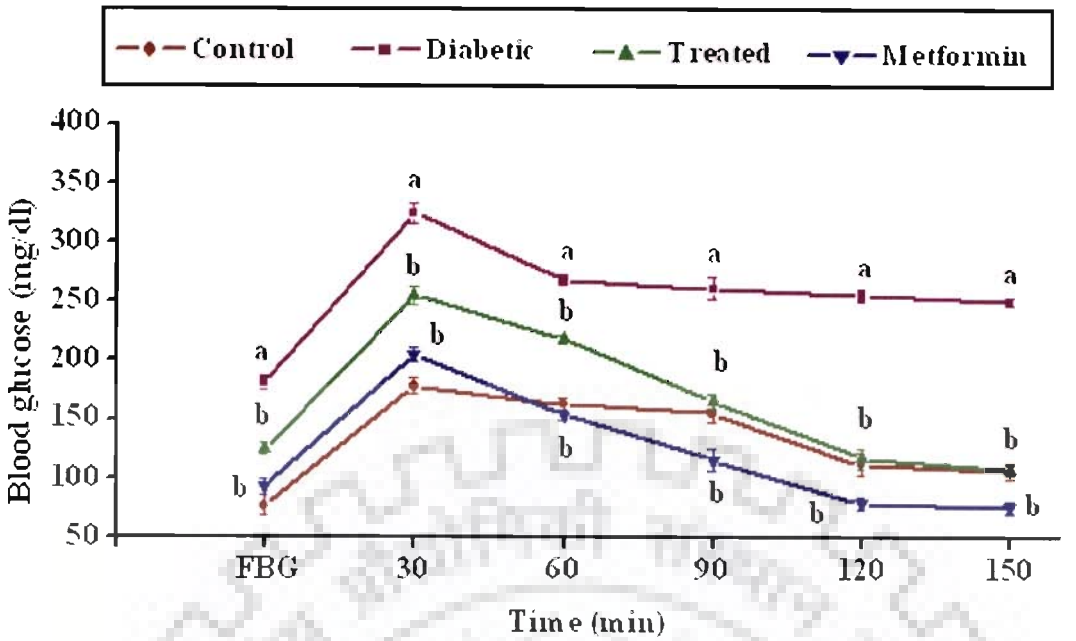


Figure 4.4.3. Oral glucose tolerance test (OGTT) in high fat diet induced diabetic rats in response to *guggulsterone*. Results are mean ± S.E.M. of n = 10; FBG, Fasting Blood Glucose. a and b, represent significant level of difference with respect to control and diabetic group respectively at each corresponding time point ($p<0.05$).

Table 4.4.1. Plasma level of different lipids after the administration of *guggulsterone* in high fat diet induced diabetic rats

Treatment Group	Plasma Lipid level (mg/dl)				
	TC	HDL	LDL	TG	VLDL
Control	154±9.3	44.1±2.4	76.6±8.8	33.1±2.1	165±10
Diabetic	220±8.2[a]	28.5±.3.2[a]	111.8±10.7[a]	79.7±1.4[a]	398.3±6.8[a]
Treated	166±4.2[b]	40.7±1.7[b]	83.6±7.9	42.2±2.3[b]	210.7±11.3[b]
Metformin	73.2±5.9[b]	45.7±3.3[b]	89.5±6.7[b]	38.0±1.5[b]	190.1±7.3[b]

Each value represents the mean ± S.E.M. (n=10).
a and b, represents statistically significant ($p<0.05$) as compared to normal control and diabetic groups respectively among each class of lipids.

4.4.3.1.3 Enzyme activity in liver

G6Pase is an important target for antidiabetic agents as its activity increases during type II diabetic condition. Treatment with *guggulsterone* resulted in a significant reduction (22%) in its activity in the present study. On the other hand, the activity of hexokinase, another major glucose regulatory enzyme did not improve significantly (19%) as compared to diabetic counterpart in response to *guggulsterone* treatment.

4.4.3.1.4 Effect on glycogen level in tissues

Glycogen content of the liver and muscle tissues in control, diabetic, *guggulsterone* and metformin treated groups are shown in fig. 4.4.5. Glycogen contents hepatic and skeletal muscles were significantly decreased in diabetic rats (63 and 45%) with respect to control ($p<0.05$). Improvement in glycogen content of liver and muscle (45%) also proved the effectiveness of *guggulsterone* for the management of diabetes.

4.4.3.1.5 Gene expression profile of some genes involved in diabetes

Type II diabetes and insulin resistance develops from the complex interplay of various genes, interrelated with carbohydrate and lipid metabolism. Expression profiles of different target genes in liver, muscle, adipose tissue and kidney were studied in control, diabetic and treated groups. As shown in fig. 4.4.6a, the expression of GK did not improve significantly by *guggulsterone* although metformin showed a positive effect on this gene. However, there was a clear reduction in the expression of other gluconeogenic enzymes like G6Pase and PEPCK, which were well supplemented by activity data analysis of these enzymes as shown earlier.

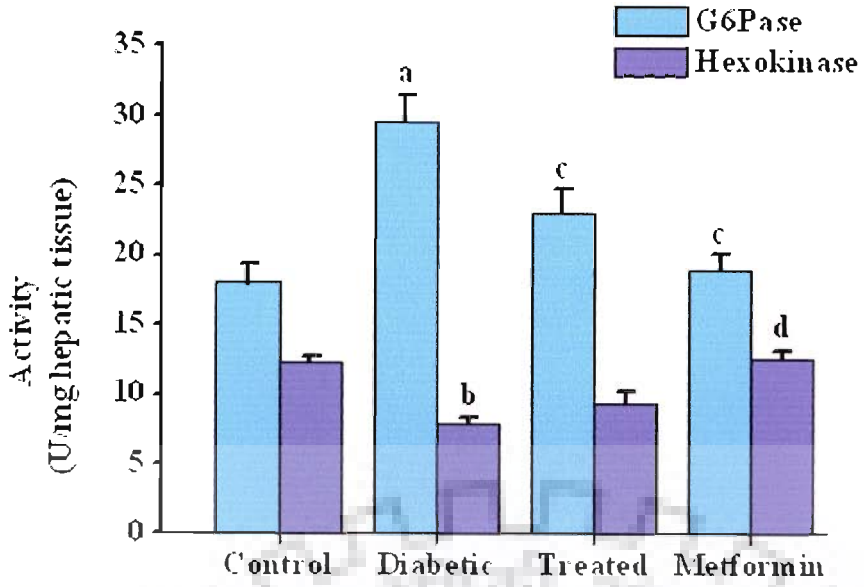


Figure 4.4.4. Effect of *guggulsterone* on hepatic G6Pase and hexokinase activity in control, diabetic and treated rats. Data is expressed as mean ± S.E.M; n = 10. a and c, indicates the significant level of differences in G6Pase b and d, indicates the significant level of differences in hexokinase levels, as compared to control and diabetic groups respectively ($p<0.05$).

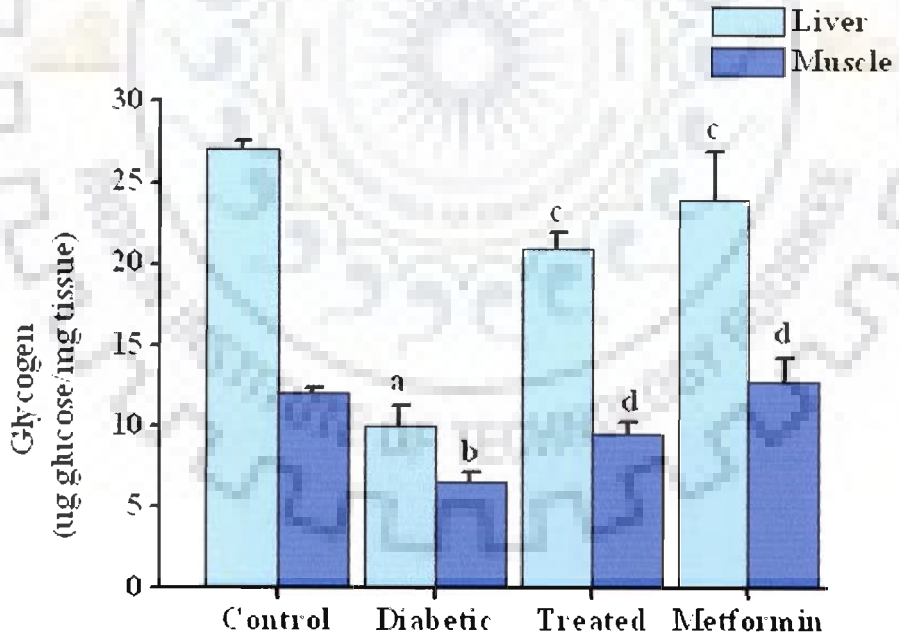


Figure 4.4.5. Effect of *guggulsterone* on hepatic and muscle glycogen content. Data is expressed as mean ± S.E.M; n = 10. a and c, indicate the significant level of differences in liver glycogen, b and d, indicate the significant level of differences in muscle glycogen levels, as compared to control and diabetic groups respectively ($p<0.05$).

Insulin resistance is a causal factor of slow systemic blood glucose clearance and defect in Glut-4 expression or translocation could be attributed to this effect. Under similar conditions, in our study, also expression of Glut-4 was significantly low in adipocyte and muscle tissues of diabetic animal groups as compared to control group and treatment with *guggulsterone* resulted in a profound increase in its expression (Fig. 4.4.7). TNF-α is a multifunctional cytokine implicated as a pathogenic factor in the development of insulin resistance because of the multitude of effects it exerts on adipose tissue metabolism. Expression of TNF-α was upregulated in diabetic rats and treatment with *guggulsterone* led to a significant decrease, which proved the efficacy of *guggulsterone* as antidiabetic agent (Fig. 4.4.6b).

PPARγ showed increased expression after the treatment with *guggulsterone* in adipose tissues (Fig. 4.4.6b). However, PPARα did not showed any significant improvement in its expression as compared to that of diabetic rats. Further in kidney, the treated rats demonstrated a clear reduction in the expression level of aldose reductase, an enzyme which is responsible for diabetes related complications when compared to diabetic rats (Fig.4.4.7).

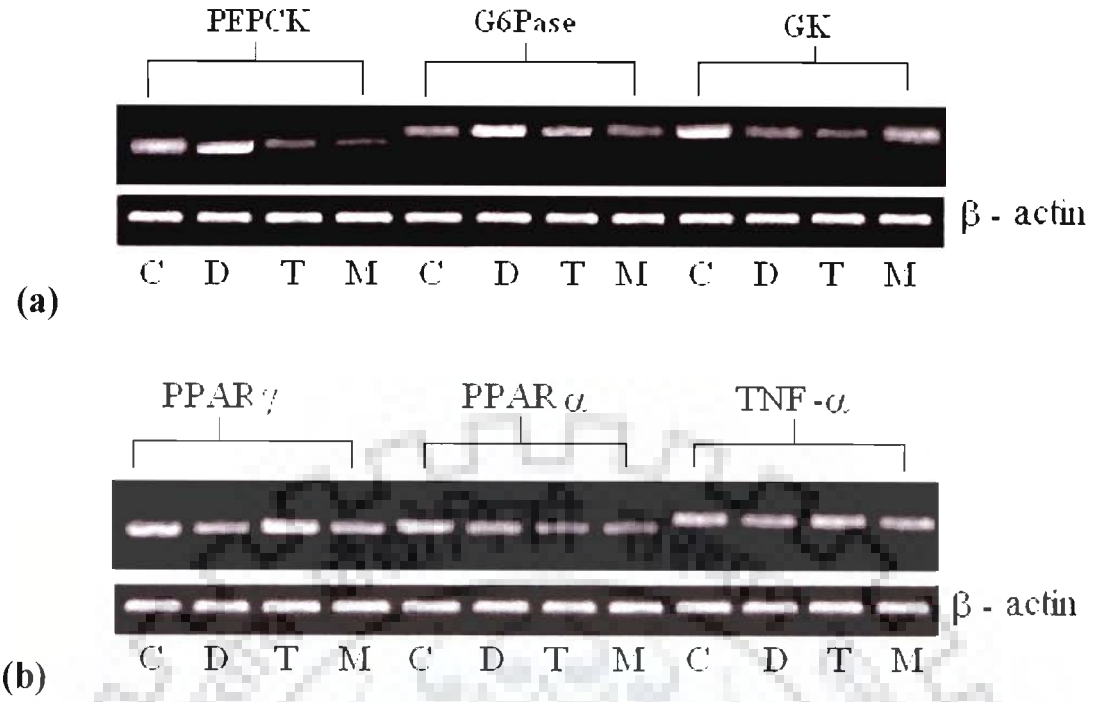


Figure 4.4.6. RT-PCR analysis of (a) liver mRNA expression of GK, G6Pase and PEPCK, (b) adipose tissue mRNA expression of PPARγ, PPARα and TNF-α genes in control, diabetic and treated groups respectevely. The total RNA isolated from the foresaid tissues were reverse transcribed and cDNA obtained was subjected to PCR. GK, Glucokinase; G6Pase, Glucose 6 Phosphatase; PEPCK, Phosphoenolpyruvate carboxykinase; PPARγ, Peroxysome prolifirator activated receptor γ; PPARα, Peroxysome prolifirator activated receptor α; TNF-α, tumor necrosis factor-α.

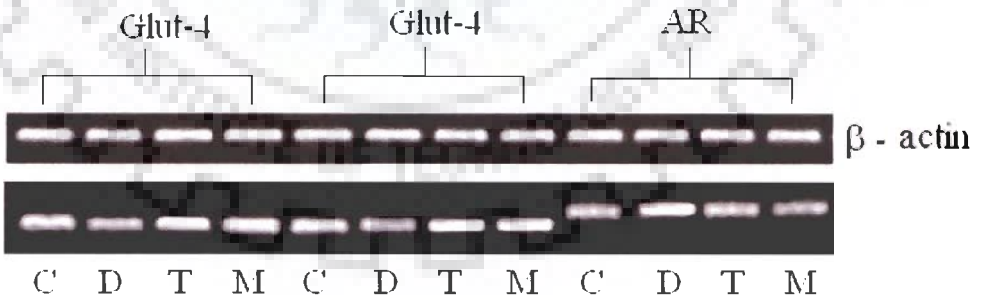


Figure 4.4.7. RT-PCR analysis of adipose, muscle and kidney tissue; mRNA expression of Glut-4 and aldose reductase genes in control, diabetic, *guggulstreone* and metformin treated groups. The total RNA isolated from the foresaid tissues of the control, diabetic and treated groups of animals, was reverse transcribed and cDNA obtained was subjected to PCR. Glut-4, glucose transporter-4; AR, aldose reductase.

4.4.3.1.6 Western blot analysis

Changes in the expression profiles of PPARγ and PPARα were further confirmed by western blot analysis of these proteins. Western blot clearly showed a significant reduction in PPARα, whereas PPARγ was significantly upregulated in treated rats as compared to diabetic counterpart (Fig. 4.4.8a).

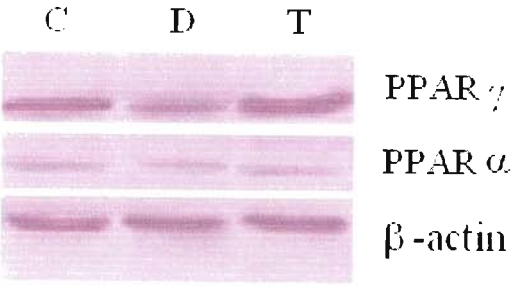
Further treatment with *gugulsterone* resulted in an increased translocation of Glut-4 from cytoplasmic fraction to plasma membrane, as evident from the western blot analysis of the LDM and PM fractions of adipose tissue from diabetic and treated rats (Fig. 4.4.8 b).

4.4.3.1.7 PTP-1B inhibition activity

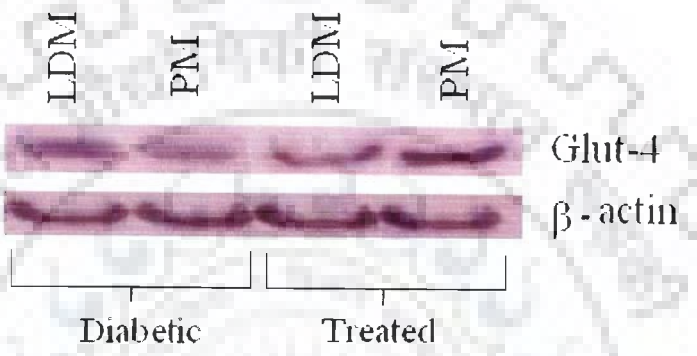
Guggulsterone treatment resulted in the inhibition of PTP-1B activity in a dose dependent manner. The inhibition potential of purified *guggulsterone* (as shown by IC_{50} values) was significantly improved (2.5 mg/ml) as compared to crude ethanolic fraction (17.4 mg/ml) obtained during the initial screening.

4.4.3.1.8 α-Glucosidase inhibition activity

α-Glucosidase inhibition activity was quanitified for *guggulsterone* where it failed to show any significant inhibition effect. *Guggulsterone* did not show any response even at a highest concentration (25 mg/ml) as tested by us, although the crude extract showed inhibition effect (11.7 mg/ml).



(a)



(b)

Figure 4.4.8. Immunoblot showing the expression of (a) PPARα and PPARγ protein in adipose tissues from control, diabetic and treated animals (b) Glut-4 expression in adipose tissue from diabetic and treated group, in response to *guggulsterone* treatment. The protein was extracted from adipose tissues and immunoblot analysis was performed as described in chapter 3. C, control group; D, diabetic group; T, treated group, LDM, low density microsome; PM, plasma membrane.

4.4.3.1.9 Histopathological studies

Sections of pancreas, liver and adipose tissue from diabetic, control and *guggulsterone* treated rats clearly demonstrated the protective effect of *guggulsterone*. A clear reduction in the size of pancreatic β-cells in high fat diet fed rats was observed (Fig. 4.4.9b) which was again normalized in rats further treated with *guggulsterone* (Fig. 4.4.9c) almost to the level of control rats (Fig. 4.4.9a). Histopathology of adipose tissue sections from diabetic rats showed significant increase in adipocytes size (Fig. 4.4.9h) as compared to normal control group (Fig. 4.4.9.g). While on treatment with *guggulsterone* there was a significant reduction in the size of adipocytes (Fig. 4.4.9i). Liver tissue from diabetic rats showed marks of severe steatosis i.e. accumulation of lipid droplets which was evident in the form of vacuoles (Fig. 4.4.9e) as compared to the control animal liver.(Fig. 4.4.9d). However treatment with *guggulsterone* not only reversed this deformity but at the same time did not show any sign of toxicological effects (Fig. 4.4.9f).

4.4.3.2. Effect of guggulsterone in vitro assays

4.4.3.2.1 Effect of guggulsterone on cell viability

Guggulsterone did not show any significant effect on cell viability upto the dose of 50 mg/l and cells were healthy when observed under microscope (Fig. 4.4.10). However, further increase in this phytochemical concentration resulted in marginal decrease in cell viability.

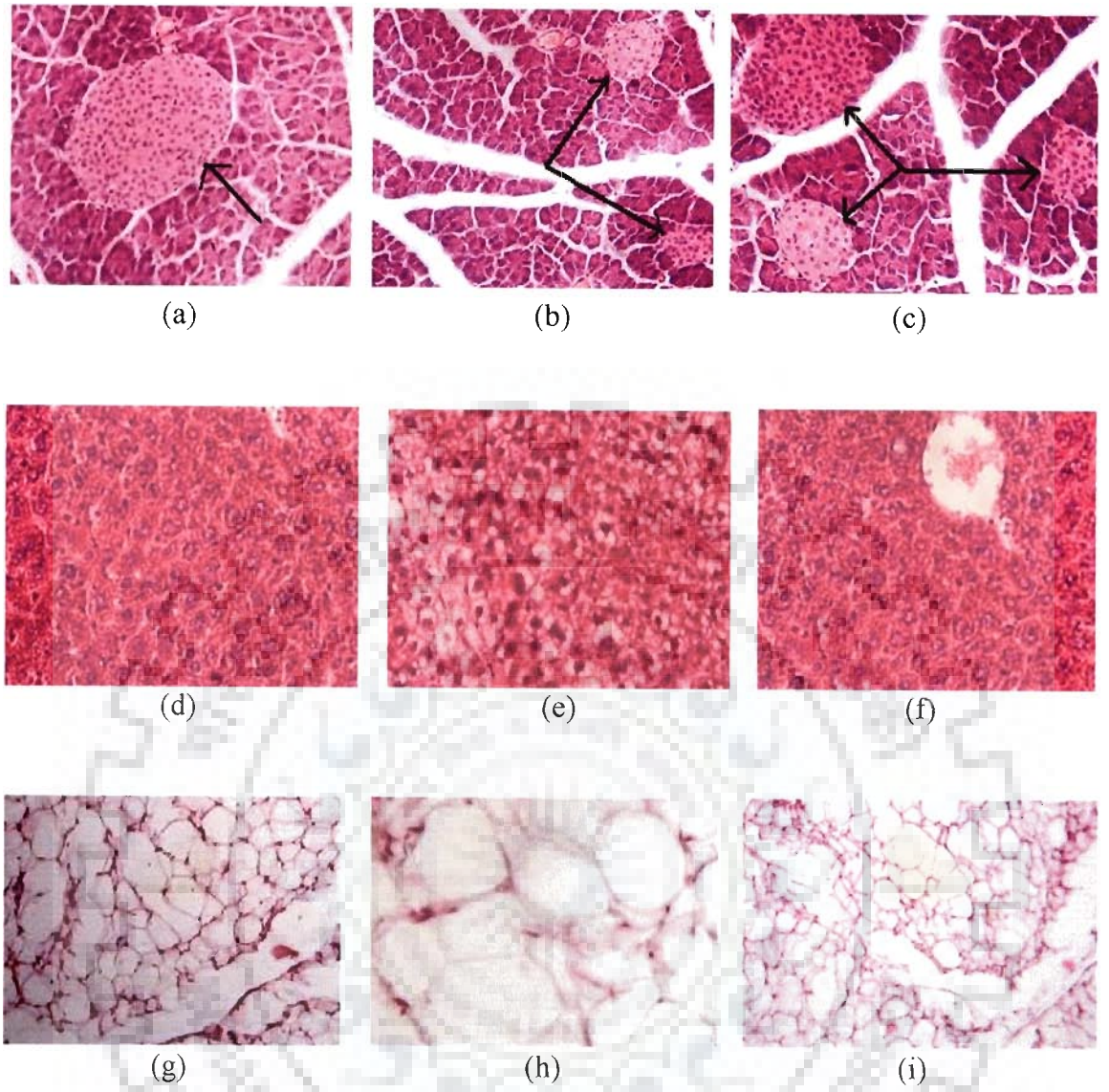


Figure 4.4.9. Photomicrograph of the sections from the pancreas (a-c), liver (d-f) and adipose tissues (g-i) of various groups of rats (a), (d), and (g) represents the respective tissues obtained from control group of animals. (b), (e), and (h) represents the respective tissues obtained from diabetic group of animals. (c), (f), and (i) represents the respective tissues obtained from *guggulsterone* treated group of animals. The arrow indicates the islet region of the section. Haematoxylin and Eosin, 20X objective.

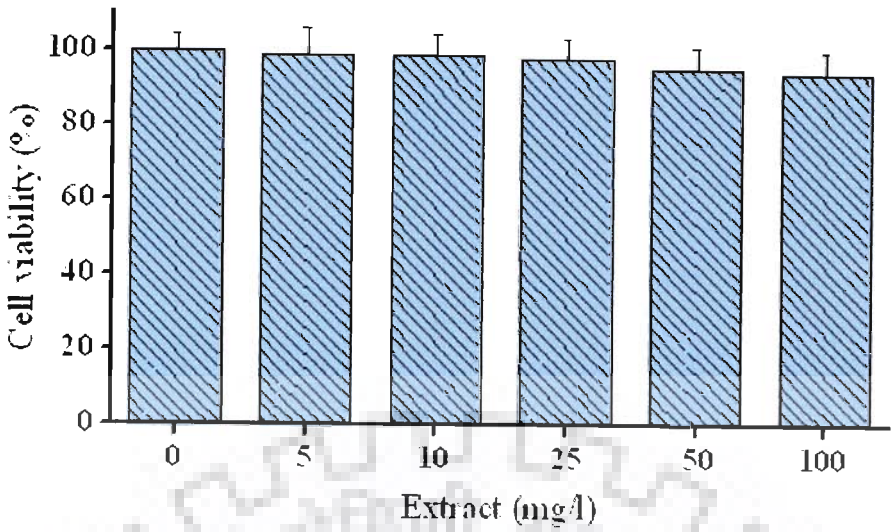


Figure 4.4.10. Dose dependent effect of *guggulsterone* on cell viability. Cells were incubated for 1 h with various concentrations of *guggulsterone*. Cell viability was expressed in comparison to vehicle treated control cells which was given a value of 100%. Values represent mean ± S.E.M. of three independent experiments each performed in triplicates.

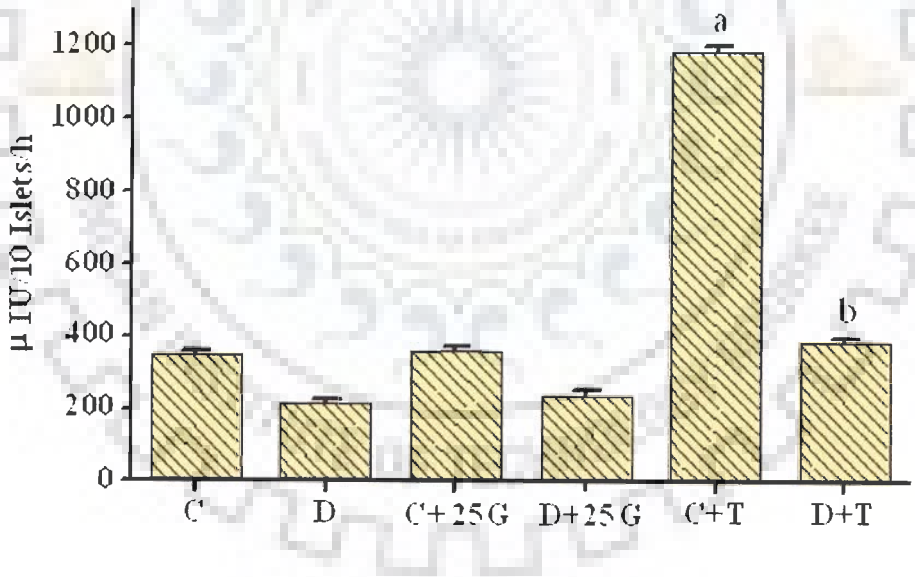


Figure 4.4.11. Levels of insulin released in the cell culture media on treatment of rat pancreatic islet cells with *guggulsterone*. Data is expressed as mean ± S.E.M. of three separate experiments each performed in quadruplicates. C, islet cells isolated from control animals; D, islet cells isolated from diabetic animals; T, Tolbutamide; 25G, *guggulsterone* 25 mg/l. a and b, represent significant difference in level of insulin secretion in comparison of islets isolated from control and diabetic groups respectively ($p<0.05$).

4.4.3.2.2 Effect on insulin release from pancreatic islets

The effect of *guggulsterone* on insulin release from pancreatic islets cells was determined in the presence and absence of *guggulsterone* from isolated islet cells from control and diabetic rats. Fig. 4.4.11 shows that the isolated islets cells from control and diabetic rats on treatment with *guggulsterone* (25 mg/l) for 1 h, did not show any significant improvement, even at the highest concentration (100 mg/l) tested by us (data not shown). This fact could be probably attributed to the absence of insulin secretagogues activity in this compound.

4.4.3.2.3 Determination of transactivation of PPAR

Guggulsterone was tested for PPAR transactivation activity using reporter based transactivation assay. The extracts significantly stimulated PPARγ activity in a dose dependent manner with lowest effective dose being 2 mg/l ($p<0.05$) (Fig. 4.4.12). This increase continued till 8 mg/dl of *guggulsterone* concentration, after which there was no further improvement in transactivation of PPARγ activity. However in contrast with PPARγ, *guggulsterone* failed to transactivate PPARα gene even at the highest dose 10 mg/l tested by us. This confirmed the PPARγ specific activity of this phytochemical. Significant level of difference in values was found as compared to vehicle treated control incubation for PPARγ ($p<0.05$).

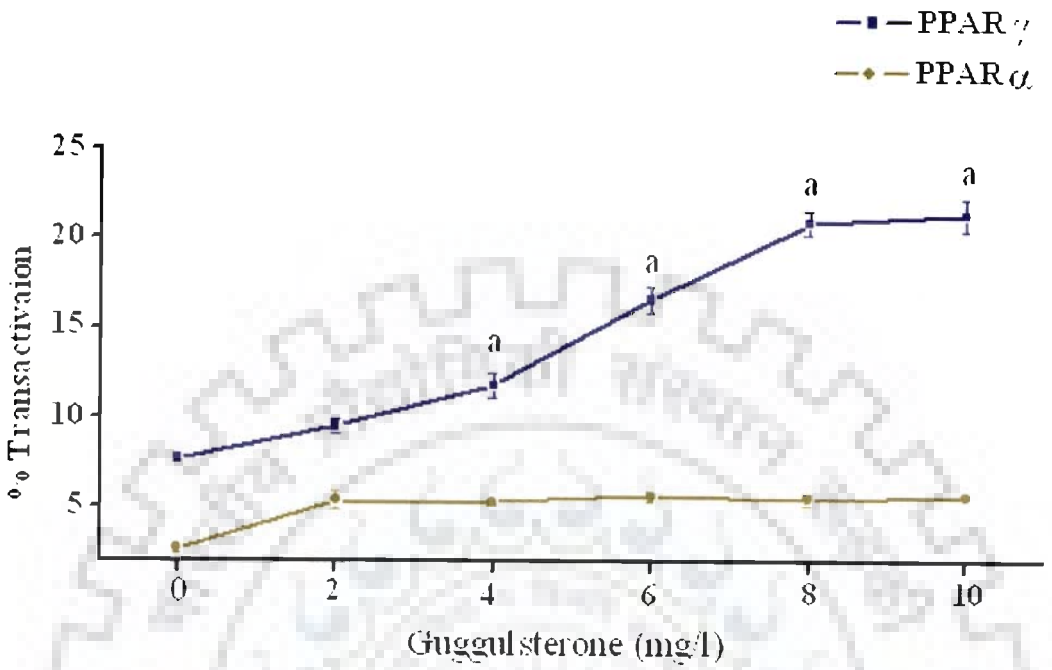


Figure 4.4.12. Transactivation assay to determine the PPAR activation. Values are means ± S.E.M of five independent experiments each performed in quadruplicates and are expressed as percentage of the positive controls for PPARα (Wy 14643, 16.2 mg/l) and PPARγ (Rosiglitazone 1.8 mg/l) which were given a value of 100% . a, indicates the significant level of difference in values as compared to vehicle treated control incubation for PPARγ ($p<0.05$).

4.4.3.2.4 Effects on adipocyte differentiation

Incubation of 3T3-L1 preadipocytes with *guggulsterone* resulted in a significant inhibition of adipocyte differentiation at higher concentrations as was evident from the decrease in TG accumulation in the adipocytes. Although the compound did not show any marked affect on preadipocyte differentiation at 6 mg/l of concentration, it demonstrated significant inhibition of 3T3-L1 cell's differentiation at 10 mg/l concentration and above (Fig. 4.4.13a). This result correlated with changes in the Oil Red O staining of lipid droplets in cells treated under similar conditions (Fig. 4.4.13b). These findings underlined the capacity of *guggulsterone* to inhibit the differentiation of 3T3-L1 preadipocyte at a concentration of 6 mg/ and above.

4.4.3.2.5 Glucose uptake by the muscle tissues

Stimulation of psoas muscle glucose uptake in rats by *guggulsterone* in the presence or absence of insulin was studied by measuring the decrease in glucose concentration in the incubation medium with time. Incubation in presence of (10 mg/dl) *guggulsterone* resulted in an (50%) increased glucose uptake up to 30 min by itself, which continued further till 120 min as tested by us. However, in the presence of insulin, increase in glucose uptake did not improve beyond the effect shown by the phytochemical alone (4%). As per the data the presence of insulin did not stabilize the rate of glucose uptake further by muscle tissue in the presence of *guggulsterone* which is indicative of absence of any synergetic activity between insulin and *guggulsterone*.

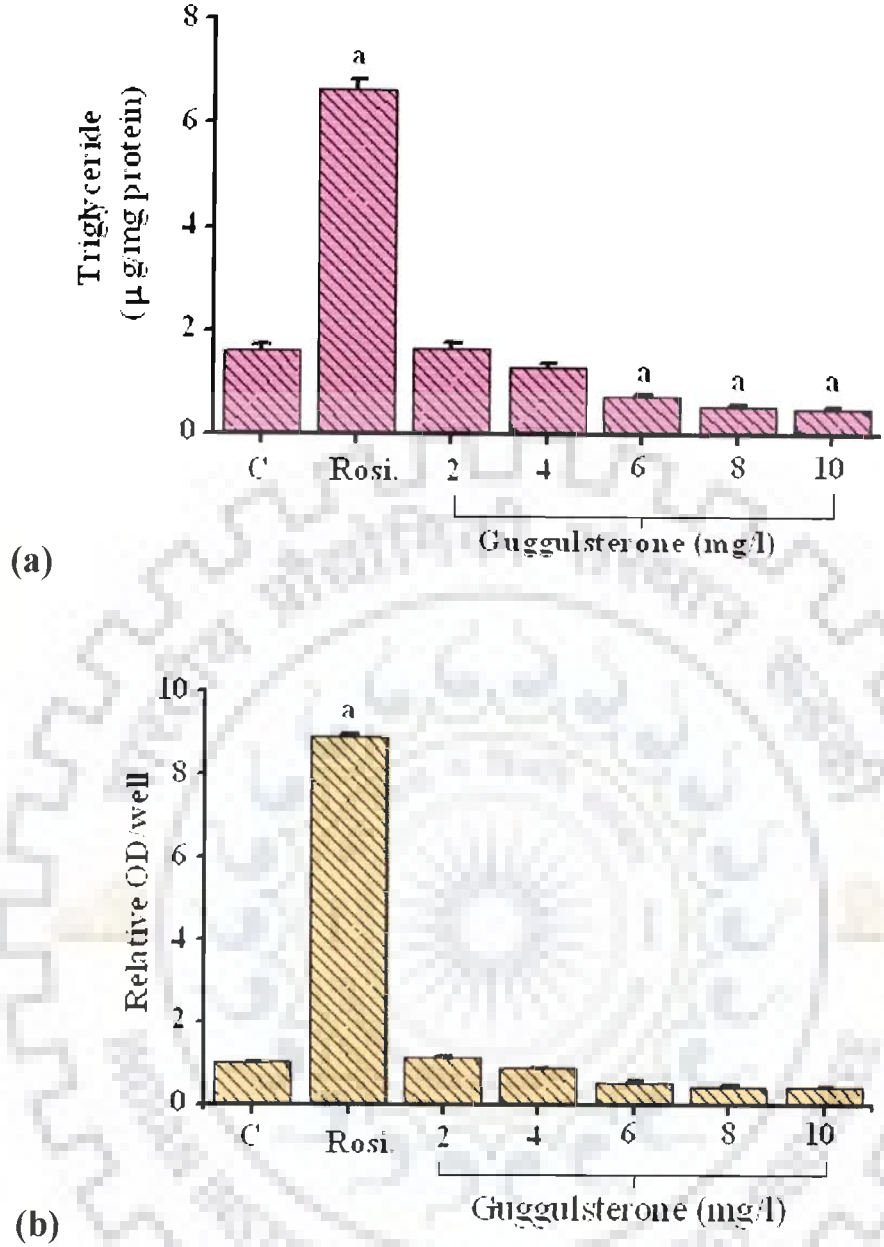


Figure 4.4.13. Dose dependent effect of *guggulsterone* on 3T3-L1 preadipocyte differentiation. (a) The cellular TG content expressed as µg/mg of protein and (b) relative absorption of Oil Red O dye. Data are mean ± S.E.M. of four independent experiments each performed in quadruplicates. a, represents the significant level of differentiation as compared to vehicle treated control incubation ($p<0.05$).

Table 4.4.4. Effect of *guggulsterone* on glucose uptake from medium by psoas muscle isolated from rats. The data is represented as increase in the cellular level of glucose with time, implying the equivalent reduction in glucose level in media by cellular uptake.

Treatment	Glucose uptake (mg/dl)				
group	30 min	60 min	90 min	120 min	150 min
MT	16±1.0	25±1.6	31±1.6	39±1.3	42±1.7
MT + E	24^{a} ±1.7(50)§	32^{a} ±0.8(28)§	38^{a} ±1.6(23)§	52^{a} ±2.5 (33)§	65^{a} ±2.0 (31)§
MT + I	18±1.8	23±3.1	31±2.7	37±2.3	45±2.2
MT + I + E	25±3.1(4)$^{\gamma}$	34±3.5 (6)$^{\gamma}$	41±2.5(8)$^{\gamma}$	53±5.0 (2)$^{\gamma}$	68±.7(5)$^{\gamma}$

Values are mean ± S.E.M. of three independent experiments each performed in quadruplicates. MT: muscle tissue; E: extract; I: insulin.

Extract and insulin in the incubation were added at concentrations of 10 mg/l and 25 mg/l respectively.

§ Values in the bracket indicate percent increase when compared with muscle tissue alone at that particular time point.

$^{\gamma}$ Values in the bracket indicate percent increase when compared with muscle tissue with extract at that particular time point.

a, $p<0.05$ when compared to control (only MT).

4.4.3.2.6 Adipocytes Glucose uptake

Effect of *guggulsterone* on glucose uptake in differentiated adipocytes was analyzed in the presence and absence of 10 µg/ml insulin. *Guggulsterone* did not show any significant increase in glucose uptake in the presence of insulin (Fig. 4.4.14). *Guggulsterone* at a dose of 100 mg/dl could not increase uptake, in the absence of insulin also (data not shown).

4.4.3.3 HPLC analysis

Purified fraction was chromatographed on HPLC. *Guggulsterone* E and Z isomers were identified on the basis of their retention time in comparison with reference standard. Chromatograph showed the presence of both the isomers with the retention times of 8.15 min and 11.22 min for *Guggulsterone E* and *Z* isoforms respectively. Other than E and Z isoforms other unidentified peakes were also present as the compounds were not completely purified as mentioned earlier (Fig. 4.4.15).

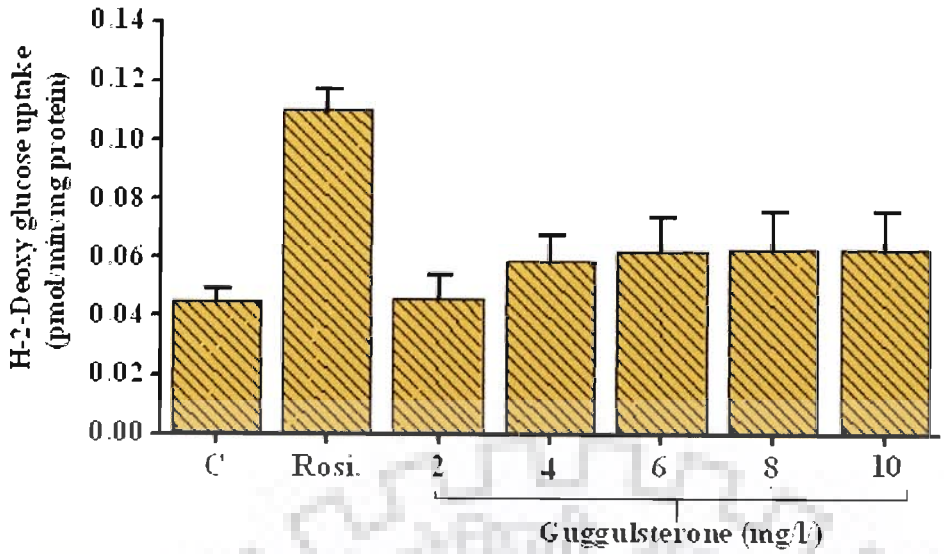


Figure 4.4.14. Effect of *guggulsterone* on glucose uptake in differentiated cells. Data is expressed as mean ± S.E.M. of four independent experiments each performed in quadruplicates. C, vehical control; Rosi., rosiglitazone treated.

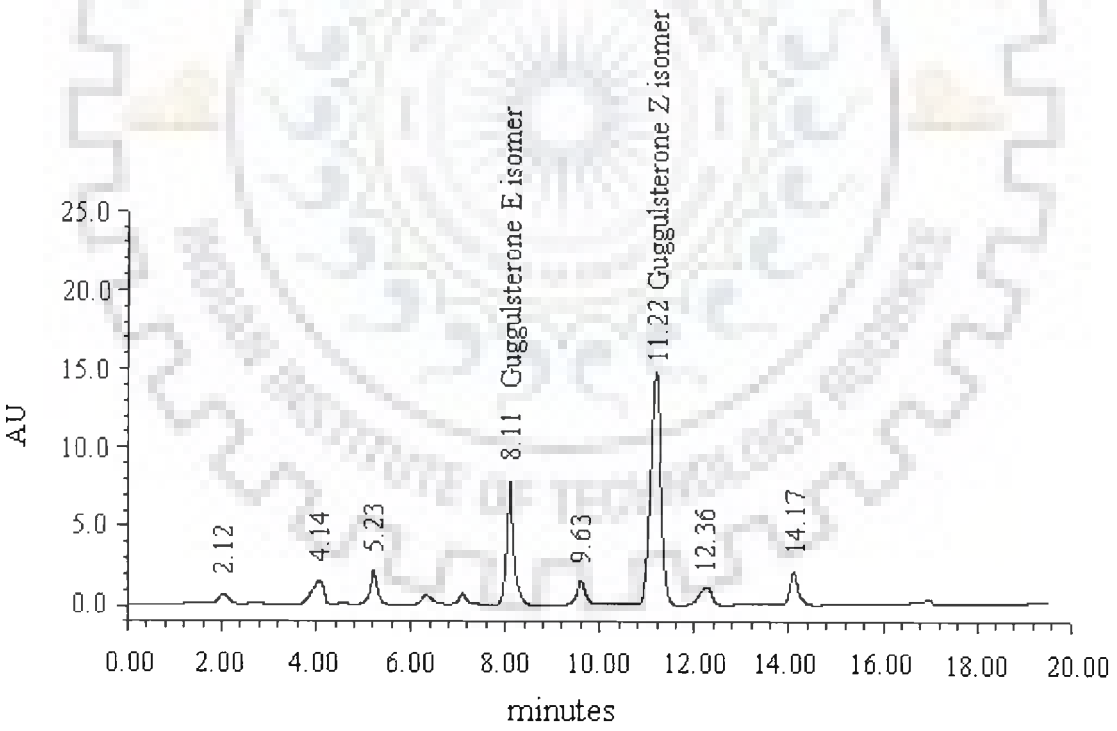


Figure 4.4.15. High performance liquid chromatograms of *Guggulsterone* isolated from *Commiphora mukul*. The peaks were identified on the basis of similarity of retention time in comparison with their reference standards.

4.4.4 Discussion

Diabetes mellitus is a chronic metabolic disorder considered to be among five most life threatening diseases. The so called western life style resulting in obesity, hyperlipidemia and hypertension is supposed to be the root cause for this disorder. Obesity is the outcome of a positive energy balance resulting in an excess of triglyceride storage in the liver and skeletal muscle, subsequently followed by insulin resistance, glucose intolerance and diabetes. The etiological link between obesity and type II diabetes lies in a multitude of factors which includes changes in adipose tissue distribution and metabolism, muscle metabolism, as well as alterations in levels of carbohydrates, fatty acids and adipocyte derived factors including leptin, TNF-α, adiponectin and resistin (Bloomgarden, 2006; Ahren et al., 2008).

Although target specific drugs are available in the market to manage diabetes, a cluster of components and risk factors associated with the metabolic syndrome warrants the prescription of multiple drugs in the majority of cases (Warner, 2005). One way to overcome this problem is to use drugs with synergistic and pleiotropic effects. Based on the present study we propose the multiple target effectiveness of *guggulsterone E* and *Z* isolated from gum resin of *C. mukul*.

Insulin resistance in peripheral tissues like skeletal muscles and adipocytes impairs their ability to clear glucose from blood stream during glucose intolerance. Skeletal muscle is quantitatively the most important tissue involved in systemic glucose homeostasis, because it accounts for ~80% of glucose disposal following glucose infusion or ingestion (Jozsi and Goodyear, 2006). Improvement in glucose disposal in treated group of rats has raised the possibility that *guggulsterone* might have some promising effect on one or more targets in the muscle tissue. This was further confirmed

by performing the muscle glucose uptake assay where addition of *guggulsterone* resulted in significant improvement in the glucose uptake. Also the expression profile of Glut-4 in muscle and adipose tissues as confirmed by the western blot analysis has clearly shown a marked translocation of Glut-4 from cytoplasm to cell periphery, contributing further in increased systemic glucose clearance and antihyperglycemic effect following a glucose load.

Partially purified fraction of gum resin, commonly known as guggul lipid, has been well documented for its lipid lowering effects (Urizar et al., 2002; Urizar and Moore, 2003). Results from our study have further proved the efficacy of the purified *guggulsterone* in lowering TG, LDL and TC also in the improvement of HDL. Histopathological examination of the liver sections from diabetic group showed significant steatosis (also called fatty liver change) i.e. abnormal retention of lipids within the liver cells which can be correlated to abnormal synthesis and elimination of TG, whereas treatment with *guggulsterone* showed improvement in TG accumulation as demonstrated by absence of steatosis in treated group. This can be correlated with various studies where guggul lipids and synthetic derivatives of *guggulsterone* have been intensively studied for their hypolipidemic effect. In these studies synthetic *guggulsterone E* and *Z* isomers are proposed as or antagonist for farnesoid X receptor (FXR) a nuclear receptor that inhibits bile acid production from cholesterol via inhibiting cholesterol 7α-hydroxylase (CYP7A1) gene transcription. This way *guggulsterone* exerts its hypolipidemic effect by enhancing the removal of cholesterol from body by stimulating its conversion to bile acid (Sinala and Gonzalez, 2002; Owsley and Chiang 2003; Deng et al., 2007). So far the various studies which have been carried out related to the medicinal properties of *guggulsterone* and/or it's synthetic isomers, with major

emphasis on lipid metabolism, here for the first time we propose the direct effect of isolated *guggulsterone* on carbohydrate metabolism. Expression profile of various genes related to glucoregulation i.e. GK, PEPCK, and G6Pase from diabetic and treated group have clearly showen the direct effect of *guggulsterone* on glucoregulation. High G6Pase and low hexokinase activity is a characteristic feature of insulin resistance which results in hyperglycemia. Treatment with *guggulsterone* resulted in low G6Pase and high hexokinase activity in the treated group which can be further correlated with a parallel change in the expression profiles of these enzymes when compared to the diabetic group. Our data showed that this isolated phytochemical do not posses α-glucosidase inhibition activity although it had significant activity in its crude form (as shown in chapter 4.1 of this thesis). This contradiction could be attributed to the fact that the *guggulsterone* in its purified form lack certain factors demonstrating this effect. However, it showed marked improvement in post prandial blood glucose level probably by stimulating peripheral glucose clearance as evident from OGTT as reported earlier by many other plant extracts or phytochemicals..

Remarkable changes were observed in the adipose tissue of *guggulsterone* treated group along with a significant improvement in the expression profile of different target genes providing a preliminary idea about the mechanism of action of *guggulsterone.* Increased expression of Glut-4 and at the same time reduced expression of TNF-α explains improvement in insulin resistance. Activity of PPARγ showed significant improvement during different level of analysis. Improvement in PPARγ activity was clearly evident from expression profile and western blot analysis of the target genes. Transactivation assay further proved the role of *guggulsterone* in transactivating exclusively the PPARγ gene in a dose dependent manner, although the effect was

marginal (20%) as compared to rosiglitazone. This compound surprisingly failed to transactivate the PPARα. This conclusively proved that this phytochemical specifically act on PPARγ. Similar patterns of PPARγ specific action by some other phytochemicals has also been reported earlier (Fang et al., 2008). Histological examination of adipose tissue from diabetic and treated groups showed remarkable differences in size of adipocytes from diabetic and treated groups. Adipocytes from treated groups were significantly smaller than its diabetic counterpart which is indicative of an improved type II diabetic condition as larger adipocytes secrete some adipokines which result in insulin resistance whereas smaller adipocytes are considered as healthy ones that secrete some factors which improve insulin resistance (Scherer, 2006). Although, *guggulsterone* showes specific PPARγ activation but at the same time it inhibited the adipocyte differentiation. Results obtained from TG accumulation and Oil red O staining showed a clear inhibition of differentiation upon incubation of 3T3-L1 cells with different concentration of the extract, which was in parallel with the results obtained by other author's earlier (Yang et al. 2008; Nakai et al., 1999; HSU and Yen, 2007).

The present results showed a contradictory action of this phytochemical on adipocytes in *in vitro* and *in vivo* condition. In *in vivo* condition *guggulsterone* prevented the hypertrophy and necrosis of adipocytes and simultaneously stimulated a healthier tissue profile with smaller adipocyts and increased fat depots. On the contradictory in *in vitro* condition the same phytochemical inhibited adipocyte differentiation and fat accumulation. This paradox might be the result of differential behavior of *guggulsterone* under *in vivo* and *in vitro* condition due to associated parameters, like metabolism in liver, presence of other responsive tissues, and receptor or counter regulatory factors of physiological system.

To check if at all, *guggulsterone* has any insulin secretagogues effect, we analyzed the efficacy of *guggulsterones* to release insulin from purified pancreatic islet cells. As shown in the results section, there was no significant difference in insulin secretion from control and treated groups. Although *guggulsterone* was unable to stimulate insulin secretion from pancreatic islets, yet it could significantly improved insulin sensitivity. This could be attributed to its effect on PTP-1B inhibition activity along with PPARγ activation. In addition to *guggulsterone* PTP-1B inhibition activity has also been shown by several other phytochemicals (Zange et al., 2006). However, to the best of our knowledge this is the first ever report on PTP-1B inhibition by *guggulsterone.*

The effects of high fat diets on pancreas have already been reported earlier by several authors (Capito et al., 1992). In this context the high fat diet fed to the rats in our experiments also showed dramatic reduction in pancreatic islets which improved significantly in response to *guggulsterone*. This further confirmed the action of this compound on pancreatic tissues along with other target organs/tissues. Further, examination of the liver sections depicted two significant observations, firstly, there was no sign of toxicological effect of *guggulsterone* in liver tissues, normally observed in the liver sections of animals treated with crude guggul extract (Healthnotes, Inc. 2000; Antarkar et al., 1984), and secondly, absence of any sign of steatosis in treated group in comparison of high fat diet fed diabetic group. These observations conclusively proved the purified compound to be a safe molecule for the treatment of type II diabetes.

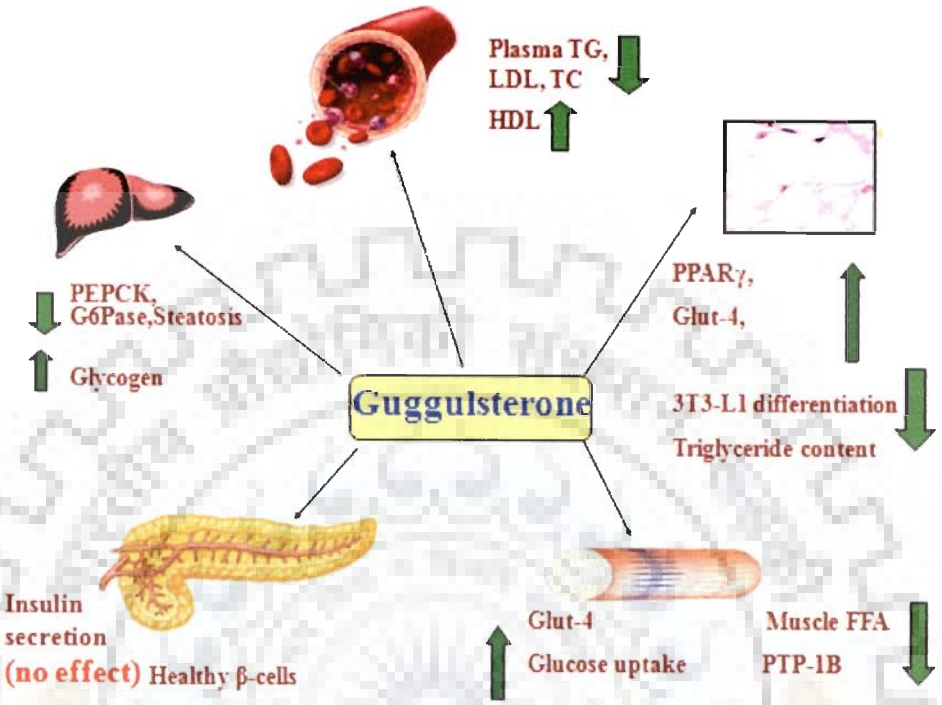


Figure 4.4.16. Effects of *guggulsterone* on various physiological targets for the treatment of diabetes.

Taken together, it is clearly evident from above results and observations that *guggulsterone* has immense potential for antidiabetic treatment not only as a hypolipidemic agent but also because of its ability to act directly on different critical nodes of carbohydrate and lipid metabolic pathways. One of the most interesting features of this molecule is that it inhibited the differentiation of 3T3-L1 preadipocytes unlike any potent PPARγ agonist. This unique feature of *guggulsterone* as PPARγ ligand suggested that the mechanisms underlying the increased insulin sensitivity and adipogenicity may be separate. One possible explanation for the inhibition of adipocyte differentiation by *guggulsterone* could be the absence of coordination of PPAR and C/EBP family of protein in these cells, which are the prerequisite for the differentiation of adipocyte (Young et al., 2008).

Inhibition of PTP-1B, reduced blood cholesterol and TG levels can also provide a possible explanation for the increased insulin sensitivity and improved peripheral glucose clearance during OGTT. Further significant inhibition of 3T3-L1 adipocyte supports the anti-obesity potential of *guggulsterone*. Figure 4.4.16 summarizes the mode of action of *guggulsterone* at various target sites as tested by us. In brief, the present chapter gives an idea that, this could be utilized for the development of novel drugs having multiple potentialities for cure and management of type II diabetes.

4.5 EFFECTS OF *PTEROSTILBENE* ISOLATED FROM *Pterocarpus marsupium* IN HIGH FAT DIET INDUCED DIABETIC RATS

4.5.1 Introduction

Defects in carbohydrate metabolism and consistent efforts of the physiological systems to correct this imbalance place an overexertion on the endocrine system, which leads to the deterioration of endocrine control and later to diabetes. Despite the great efforts that have been made in understanding and management of diabetes, serious problems like diabetic retinopathy, nephropathy and lower extremity amputation continue to confront patients and physicians (Krolewski and Warram, 2004; Stirban et al., 2008). Biomedical science has unraveled complex and nonlinear physiological and pathobiological processes involved in causing/fostering diabetes, and therefore warrants the use of single drug or a combination of drugs targeting multiple sites. Towards this end, the present chapter of this thesis discusses the isolation and biological characterization of a potent bioactive molecule from a common medicinal plant called *Pterocarpus marsupium* Roxb. towards the treatment of diabetes.

Pterocarpus marsupium Roxb (Sanskrit: Pitasala) (Leguminosae), (Fig. 4.5.1) also known as Indian kino or Bijasar, is a large tree common to the mixed deciduous forests of central and Peninsular India. Traditionally various parts of this plant are used in Indian folklore medicine for the treatment of diseases like diarrhoea, toothache, fever, urinary tract infections, skin infections and diabetes (Manickam et al. 1997; Maurya et al., 2004). The wooden glasses made up of heartwood of *Pterocarpus marsupium* (*PM*) has been reported to be used in drinking water to control blood sugar in Ayurvedic system of medicine (Dhanabal et al., 2006; Grover et al., 2002).

Figure. 4.5.1. *Pterocarpus marsupium* and inset shows the heartwood of this plant.

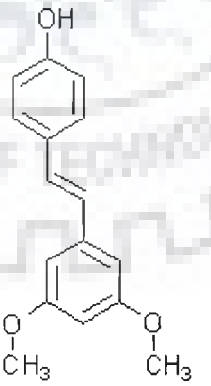


Figure 4.5.2. Structure of *Pterostilbene*

In a multi-centric study *PM* treatment resulted in significant improvement in glucose profile in human subjects with a mean decrease of 32 mg/dl for fasting and 45 mg/dl for post prandial conditions (ICMR Study Group, 2005). A number of phytochemicals have been isolated and characterized from *PM* aqueous extract like pteroside, pteroisoauroside, marsuposide, marsupin, protosupin and pterostilbene flavon C-glucoside, vijayosin, C-b-d-glucopyranosyl-2,6-dihydroxyl benzene, sesquiterpene (Manickam et al. 1997; Maurya et al., 2004).

Pterostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene) $C_{16}H_{16}O_3$, MW 256.3 g/mol, is a stilbene isolated from *PM* stem heart wood (Fig 4.5.2), which has been reported to significantly lower the blood glucose level (Suh et al., 2007). In another study, treatment with *pterostilbene* resulted in a significant reduction of glycosylated hemoglobin and an increase in total hemoglobin level and significant changes in the glucose homeostatic enzymes like hexokinase, G6Pase and fructose-1,6-bisphosphatase (Grover et al., 2002; Mukhtar et al., 2005). *Pterostilbene* is also an established agonist for PPAR with special emphasis on PPARα, a transcription factor involved in lowering cholesterol and other blood fats (Rimandoet al., 2005; Mizuno et al., 2008).

On the basis of available previous reports and results obtained from preliminary studies from our laboratory, we carried out a stepwise activity based fractionation of aqueous extract using high fat diet diabetic rat models. Finally *pterostilbene* was purified and identified as the most active constituent in an active fraction of the extract of *PM* showing potent antidiabetic, antihyperlipidemic and antihyperglycemic activities. This was validated using commercially available standard of *pterostilbene*. Efficacy of *pterostilbene* against insulin resistance and type II diabetic conditions was evaluated on

the basis of different experimental evidences. On the basis of results obtained from a broad range of experiments it is evident that *pterostilbene* has the ability to combat various targets of insulin resistance and type II diabetic condition.

4.5.2 Animal groups and treatment

The animals were randomly divided into four groups as given below:

Group I - Normal palate diet fed (control)

Group II - High fat diet fed rats (diabetic)

Group III - High fat diet fed rats treated with 100 mg/kg bw *pterostilbene* (treated)

Group IV - High fat diet fed rats treated with 20 mg/kg bw Metformin (metformin)

Rats from group II, III and IV were fed with modified high fat diet (Table 3.2) for 16 weeks and rats from group I were fed normal palate diet. On completion of 16 weeks, once the type II diabetic condition was confirmed rats from group III an IV were treated with isolated *pterostilbene* and metformin respectively for another 8 weeks. Rats from group I and II were given 0.01 % ethanol as control.

4.5.3 Results

4.5.3.1 Effects of pterostilbene on diet induced diabetic rat models

4.5.3.1.1 Fasting blood glucose level and oral glucose tolerance test

Fasting blood glucose level of high fat diet fed diabetic group showed significant elevation in comparison to their control and treated counterparts. However this level reduced upon treatment with 100 mg/kg bw of the *pterostilbene* (FBG data, Fig. 4.5.3). An acute elevation of blood glucose level followed by a sharp decline (25%) was seen in treated rats with glucose load during OGTT.

Further decline in blood glucose level was parallel with that of the control group. Although blood glucose profile could not achieve the normalcy, yet improvement was significant in comparison to it's diabetic counterpart (Fig. 4.5.3) ($p < 0.05$).

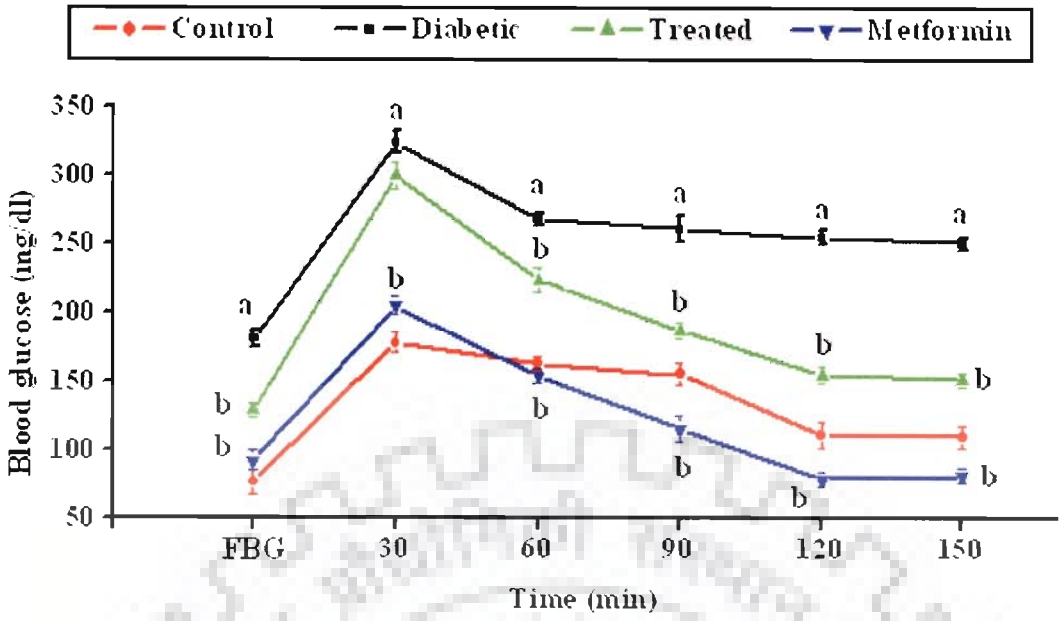


Figure 4.5.3. Oral glucose tolerance test (OGTT) in high fat diet induced diabetic rats in response to *pterostilbene*. Results are mean ± S.E.M. of n = 10. FBG, Fasting Blood Glucose. a and b, represent significant level of difference with respect to control and diabetic group respectively at each corresponding time period ($p<0.05$).

Table 4.5.1. Plasma level of various lipids after eight weeks of treatment with *pterostilbene* in high fat diet induced diabetic rats

Treatment Group	Plasma Lipid level (mg/dl)				
	TC	HDL	LDL	VLDL	TG
Control	154±9.3	44.1±2.4	76.6±8.8	33.1±2.1	165±10
Diabetic	220±8.2[a]	28.5±.3.2[a]	111.8±10.7[a]	79.7±1.4[a]	398.3±6.8[a]
Treated	182±6.4	32.5±2.6	109±6.2	40.5±1.9[b]	206±9.2 [b]
Metformin	73.2±5.9[b]	45.7±3.3[b]	89.5±6.7[b]	38.0±1.5[b]	190.1±7.3[b]

Values are presented as mean ± S.E.M., n = 10;

a and b, represent statistically significant ($p<0.05$) as compared to normal control and diabetic groups respectively among each class of lipids.

4.5.3.1.2 Effects on plasma lipid profile

High fat diet induced diabetes caused hyperlipidaemia i.e. high TG levels accompanied by a raised concentration of LDL and diminished HDL. However treatment with *pterostilbene* showed significant improvement in lipid profile as also reported by other authors (Rimando et al., 2005; Zhiqiang et al., 2008). About 12 and 48% reduction in TC and TG levels, respectively were found in diabetic rats treated with *pterostilbene* ($p<0.05$). In addition, HDL levels were found to be increased by 14% (as compared to diabetic control) in *pterostilbene* treated rats (Table 4.5.1).

4.5.3.1.3 Effects on hepatic and muscle glycogen content

High fat diet resulted in a severe depletion of liver and muscle glycogen content which was also evident from histological analysis of liver tissues where marked steatosis was seen. Further treatment with *pterostilbene* caused improvement in glycogen content of liver by 75% and muscle by 17% which further validated the effectiveness of *pterostilbene* for treatment of diabetes which was almost comparable of metformin treated group (Fig.4.5.4).

4.5.3.1.4 Enzyme activity in liver

G6Pase is an important target for antidiabetic agents as its activity increases during diabetic conditions. Induction of diabetes with high fat diet resulted in an increased G6Pase in the diabetic group compared to the control group. However, treatment with *pterostilbene* resulted in a significant reduction (40%) in its activity.

Hexokinase, another important glucose homeostatic enzyme showed just the opposite pattern of that of G6Pase. In our assay hexokinase enzyme showed almost 25% increases in its activity on treatment with *pterostilbene* as compared to diabetic counterpart (Fig. 4.5.5).

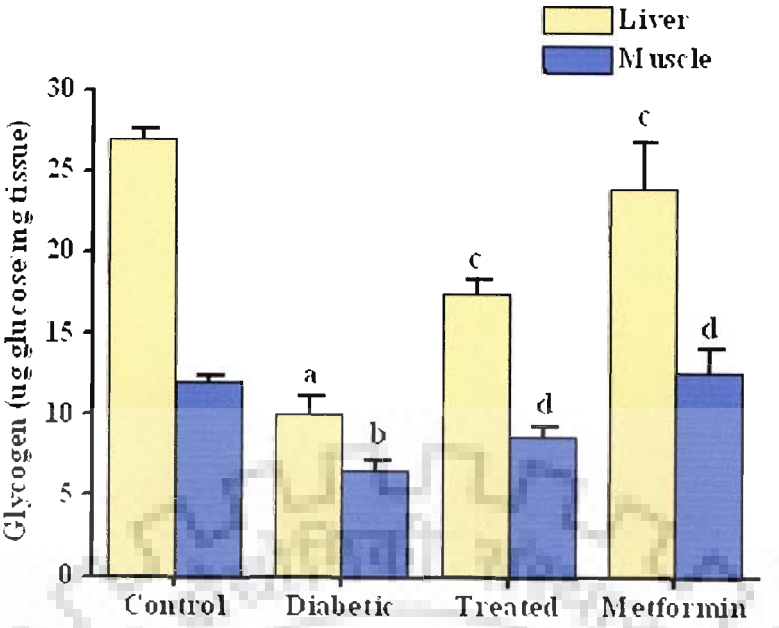


Figure 4.5.4. Effect of *pterostilbene* on liver and muscle glycogen contents in high fat diet induced diabetic rats. Data is expressed as mean ± S.E.M; n = 10. a and c, indicate the significant level of differences in liver glycogen, b and d, indicates the significant level of differences in muscle glycogen levels, as compared to control and diabetic groups respectively ($p<0.05$).

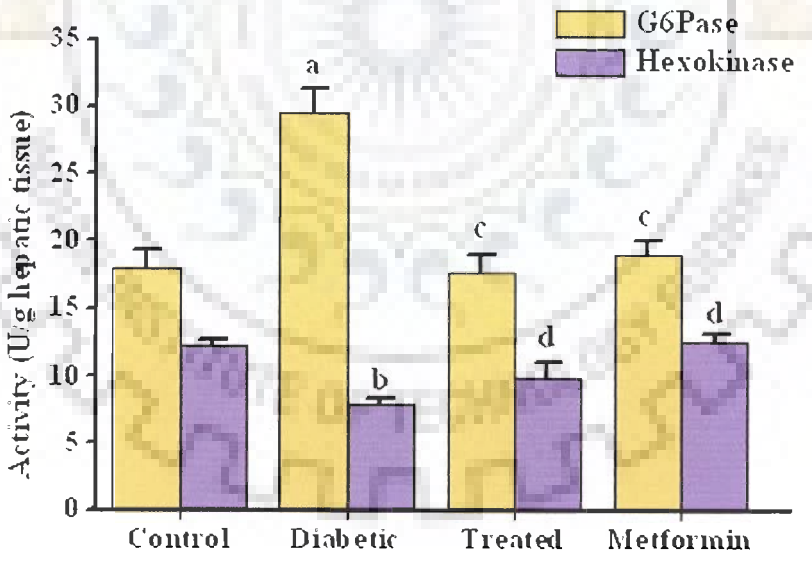


Figure 4.5.5. Effect of *pterostilbene* on hepatic G6Pase and hexokinase activity in high fat diet induced diabetic rats. Data is expressed as mean ± S.E.M; n = 10. a and c, indicate the significant level of differences in G6Pase. b and d, indicate the significant level of differences in hexokinase activity levels, as compared to control and diabetic groups respectively ($p<0.05$).

4.5.3.1.5 Gene expression profile

Expression profiles of different target genes in liver, muscle, adipose tissue, and kidney were studied in control, diabetic and treated groups. The expression of GK increased in treated rat, while at the same time there was a clear reduction in the expression of gluconeogenic enzymes G6Pase and PEPCK. This indicates the direct effect of this phytochemical on enzyme activity, as discussed in last section, and is compatible with activity analysis of these enzymes (Fig.4.5.6a).

Expression of Glut-4 was significantly low in diabetic group when compared to control group and treatment with *pterostilbene* resulted in a profound increase in expression of these gene (Fig. 4.5.7). Under similar circumstances as discussed in earlier chapters, the expression of TNF-α was upregulated in diabetic rats and treatment with *pterostilbene* led to a significant decrease, which further proved the efficacy of *pterostilbene* for the treatment of diabetes mellitus (Fig. 4.5.6b). In way it was marginally more efficient than metformin in decreasing the expression of TNF-α gene.

Effect of *pterostilbene* on PPARα and γ activity was prominent, which was confirmed on the basis of different experimental evidences. PPARγ and PPARα which regulate carbohydrate and lipid metabolism and control the adipocyte differentiation showed increased expression after the treatment with *pterostilbene* in adipose tissues (Fig. 4.5.6b). For their expression also *pterostilbene* was found to be more efficient then metformin. Like TNF-α *pterostilbene* was again slightly more efficient in regulating PPARγ gene expression.

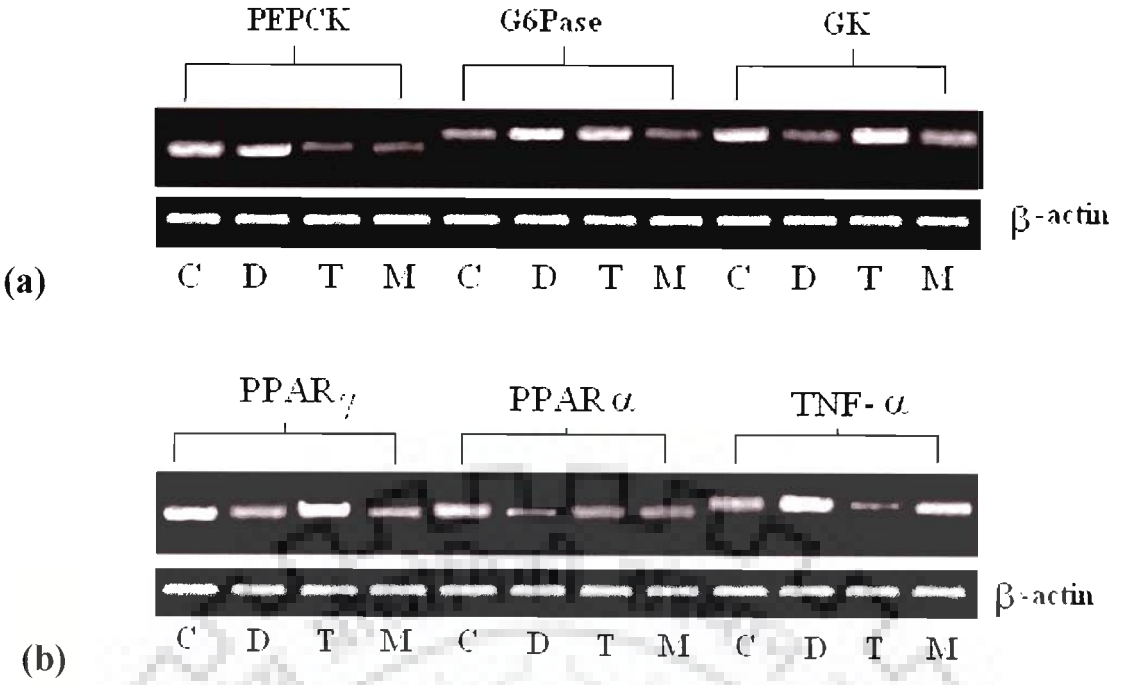


Figure 4.5.6. RT-PCR analysis of (a) liver mRNA expression of GK, G6Pase and PEPCK (b) adipose tissue mRNA expression of PPARγ, PPARα and TNF-α genes in control, diabetic, *pterostilbene* treated and metformin treated groups. GK, Glucokinase; G6Pase, Glucose 6 Phosphatase; PEPCK, Phosphoenolpyruvate carboxykinase; PPARγ, Peroxysome prolifirator activated receptor γ; PPARα, Peroxysome prolifirator activated receptor α; TNF-α, tumor necrosis factor-α.; C, control; D, diabetic; T, *pterostilbene* treated; M, metformin treated.

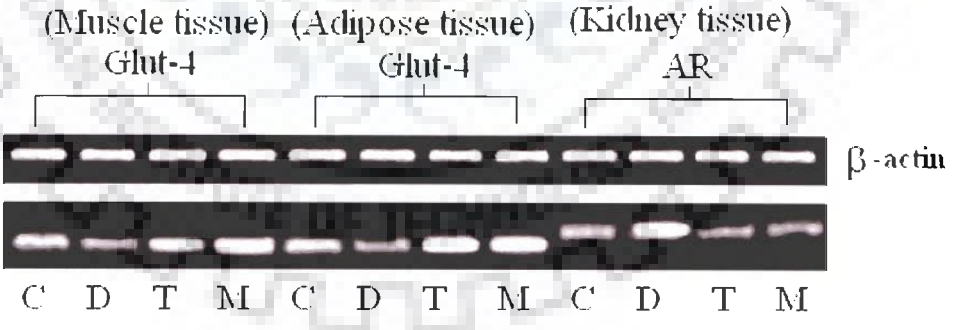


Figure 4.5.7. RT-PCR analysis of Glut-4 and AR, mRNA in muscle, adipose and kidney tissue from control, diabetic, *pterostilbene* treated and metformin treated groups. Glut-4, glucose transporter-4 and AR, aldose reductase; C, control; D, diabetic; T, *pterostilbene* treated; M, metformin treated.

4.5.3.1.6 Western blot analysis

Changes in the activities and expression profile of PPARγ and PPARα were further confirmed by western blot analysis. Western blot clearly showed a significant improvement in expression of both PPARγ and PPARα in *pterostilbene* treated groups as compared to its diabetic counterpart (Fig. 4.5.8a).

Further treatment with *pterostilbene* resulted in an increased expression as well as translocation of Glut-4 from cytoplasmic fraction to plasma membrane, as evident from the western blot analysis of the LDM and PM fractions of adipose tissue from diabetic and treated rats when equal amount of protein content was loaded (Fig. 4.5.8b).

4.5.3.1.7 PTP-1B inhibition activity

Although the crude extract showed a prominent PTP-1B inhibition activity (IC_{50} value 7.2 mg/ml), the purified *pteostilbene* did not show any inhibitory effect on the enzyme even at the highest concentration of 20 mg/ml as tested by us.

4.5.3.1.8 α-Glucosidase inhibition activity

Pterostilbene was tested for its inhibition effect on α-glucosidase activity in a dose dependent manner against acarbose as positive reference. *Pterostilbene* showed a significant inhibition potential with an IC_{50} value of 0.5 mg/ml. Another interesting feature of this part is the improvement in the inhibitory activity by about 6 folds in the purified form as compared to the crude ethanolic extract. This supports the notion that at certain instances purified phytochemicals show better result as compared to crude complex chemicals.

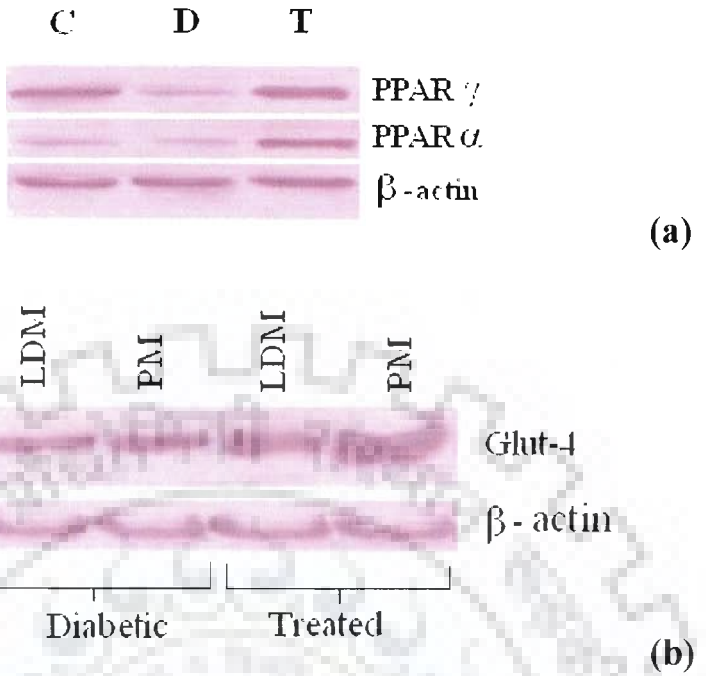


Figure 4.5.8. Immunoblot showing the expression of (a) PPARα and PPARγ protein in adipose tissues from control, diabetic and treated animals (b) Glut-4 expression in adipose tissue from diabetic and treated groups, in response to *pterostilbene* treatment. The protein was extracted from adipose tissues and immunoblot analysis was performed as described in chapter 3. C, control group; D, diabetic group; T, treated group, LDM, low density microsome; PM, plasma membrane.

4.5.3.1.9 Histopathological studies

Treatment with *pterostilbene* for eight weeks resulted in a clear protective effect on pancreas, liver and adipose tissue from treated group in comparison of diabetic group. High fat diet caused a marked reduction in size of pancreatic islets due to excessive oxidative stress and β-cell exertion (Fig.4.5.9b). However this condition improved upon eight weeks treatment with *pterostilbene* almost close to control (Fig. 4.5.9a and c). Other than pancreas, *pterostilbene* showed a direct effect on adipose tissue, where it stimulated differentiation of new smaller adipocytes (Fig. 4.5.9i) along with increased visible adipose tissue depot in comparison with diabetic group (Fig. 4.5.9h).

In order to check if *pterostilbene* has any toxic effect in physiological system, liver tissue from treated group was compared with control group, where it showed no sign of toxic effect. Another major observation in diabetic rats was the sever steatosis (Fig. 4.5.9e) i.e. accumulation of lipid droplets. On the other hand, upon treatment with *pterostilbene* there was no sign of steatosis (Fig. 4.5.9f). This may be the result of lowered lipid level and/or decreased uptake of lipid in hepatocytes followed by effective removal of triglycerides.

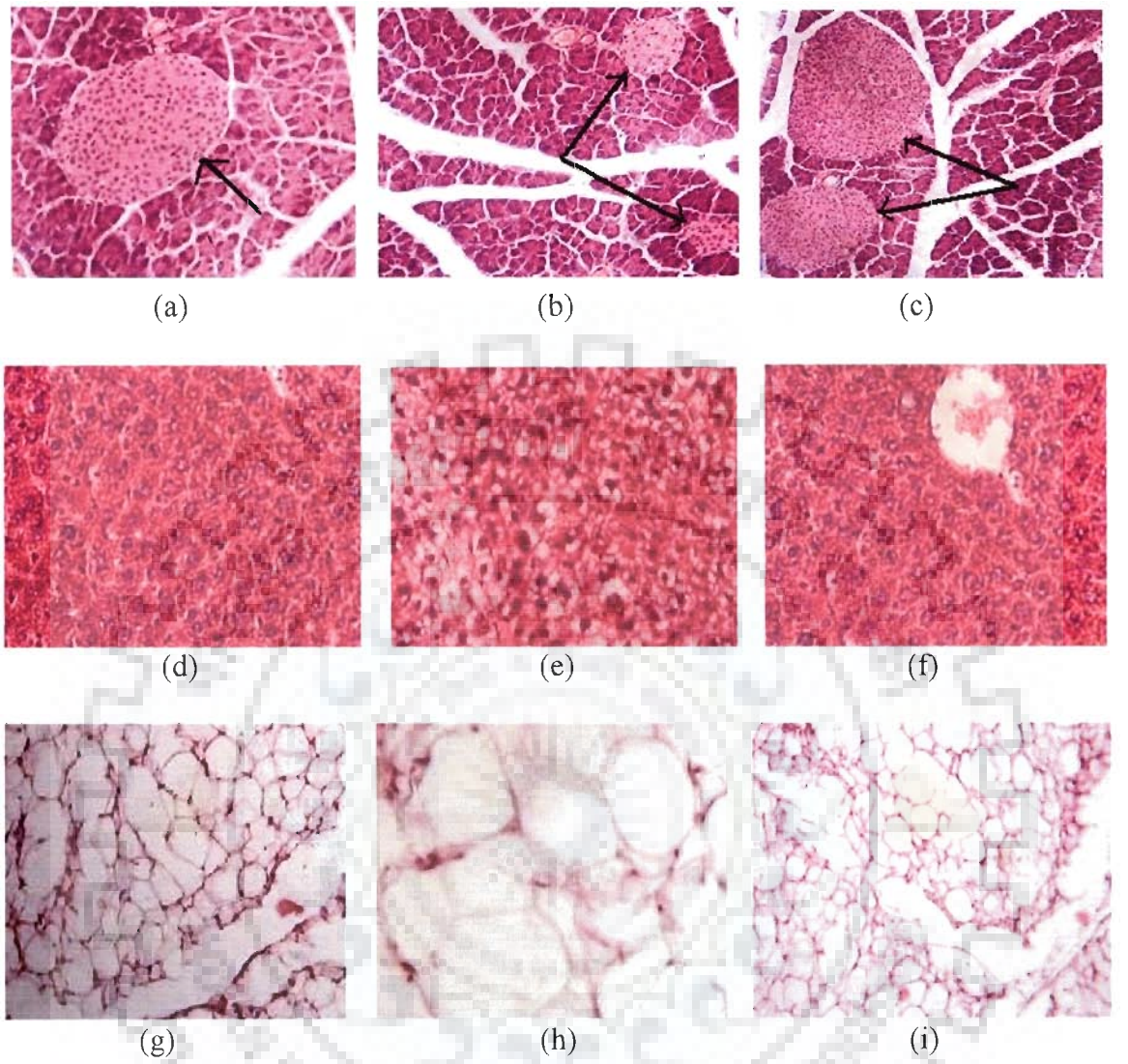


Figure 4.5.9. Photomicrograph of the sections from the pancreas (a-c), liver (d-f) and adipose tissues (g-i) of various groups of animal. (a), (d), and (g) represent the respective tissues obtained from control group of animals. (b), (e), and (h) represent the respective tissues obtained from diabetic group of animals. (c), (f), and (i) represent the respective tissues obtained from *pterostilbene* treated group of animals. The arrow indicates the islet region of the section. Haematoxylin and Eosin, 20X objective.

4.5.3.2 In vitro analysis of antidiabetic potential of pterostilbene

4.5.3.2.1 Effect of pterostilbene on cell viability

Effect of *Pterostilbene*'s effect on cell viability was tested using MTT assay. *Pterostilbene* did not show any significant toxic effect on cell viability even at the highest dose of 100 mg/l tested by us (Fig. 4.5.10) ($p<0.05$). This was further confirmed by observing the cells microscopically which did not show any alterations in cellular morphology even at the highest concentration tested by us. Thus the doses used in the following set of experiments were below toxic level. However, at a further higher dose of 500 mg/l there was a marginal decrease in cell viability (although not significant).

4.5.3.2.2 Identification of peroxisomal prolifirator activity by the pterostilbene

To test if *pterostilbene* could induce the PPAR activity, the phytochemicals was tested in a transactivation assay using Hep G2 cells. As shown in fig 4.5.11, *pterostilbene* induced the activation of both PPARα and PPARγ in a dose dependent manner albeit at a marginally high efficiency for PPARα. *Pterostilbene* treatment resulted in significant up regulation for both PPARα and γ for concentrations ranging from 10 mg/l to 50 mg/l (42% and 30% transactivation of PPARα and γ respectevely) after which it leveled off (Fig. 4.5.11). This further conclusively proved that although, *pterostilbene* has the capacity of dual transactivation of both PPARα and PPARγ but it is more efficient as an legend for PPARα.

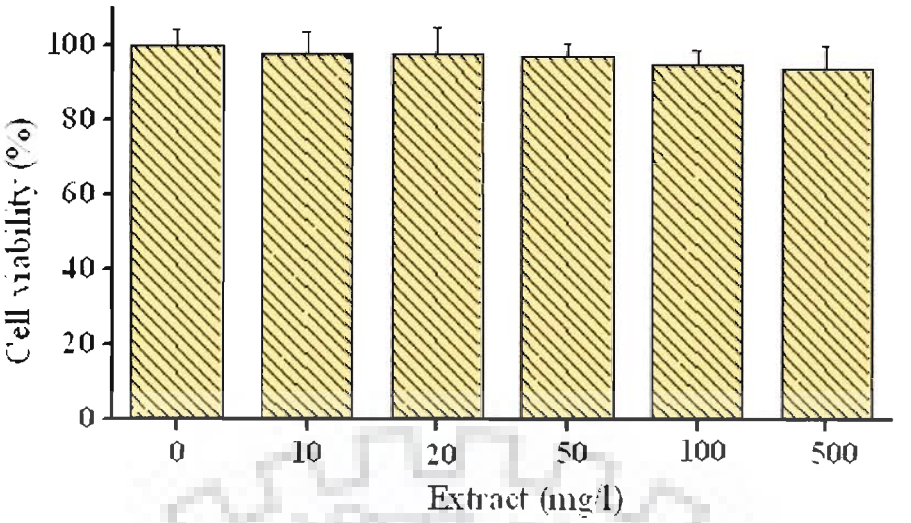


Figure 4.5.10. Dose dependent effect of *pterostilbene* on cell viability. Cells were incubated for 1 h with various concentrations of *pterostilbene*. Cell viability was expressed in comparison to vehicle treated control cells which was given a value of 100%. Values represent means ± S.E.M. of three independent experiments each performed in triplicates.

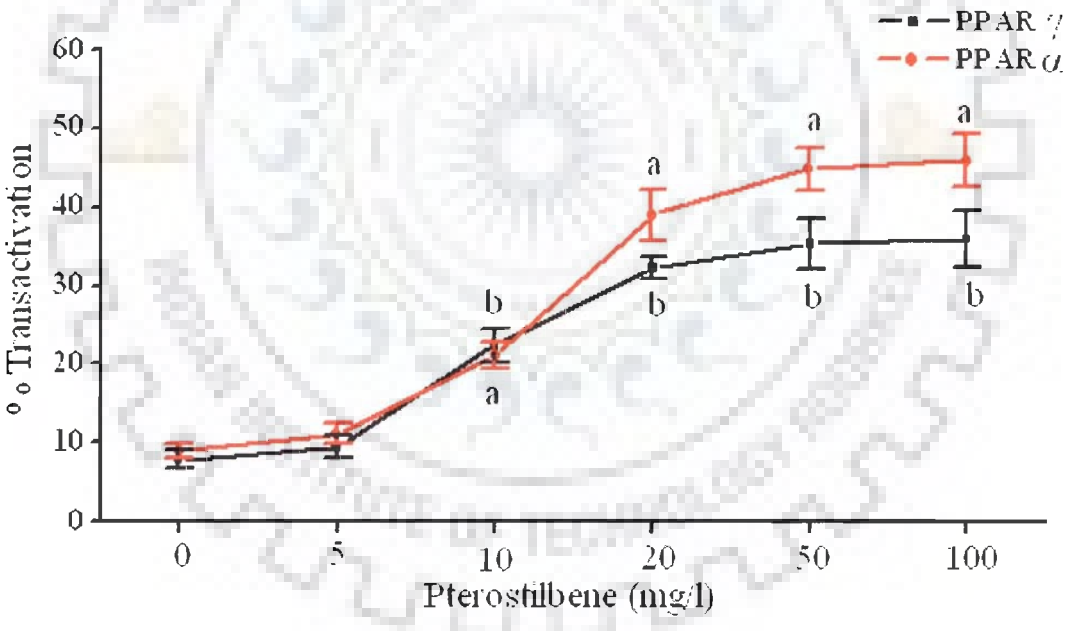


Figure 4.5.11. Transactivation assay to determine the PPAR activation. Values are mean ± S.E.M. of five independent experiments each performed in quadruplicates and are expressed as percentage of the positive controls for PPARα (Wy 14643, 16.2 mg/l) and PPARγ (Rosiglitazone 1.8 mg/l) which were given a value of 100% respectively. a and b, indicates the significant level of difference in values as compared to vehicle treated control incubation for PPARγ and PPARα respectively($p<0.05$).

4.5.3.2.3 Effect on adipocytes differentiation

Incubation of 3T3-L1 preadipocytes with *pterostilbene* resulted in an increased adipocytes differentiation (Fig. 4.5.12a). Up to 10 mg/l dose *pterostilbene* showed only marginal (not significant) TG accumulation. Further increase in the concentration of *pterostilbene* caused a significant differentiation which was evident from the increased triglyceride accumulation in the adipocytes (about 2 fold with respect to control). Similar pattern of differentiation was demonstrated by Oil red O accumulation in adipocytes (Fig 4.5.12b). At 50 mg/l dose, the accumulation was almost 4 times that of vehicle treated control. This further confirmed the role of this phytochemical in differentiation of adipocytes.

4.5.3.2.4 Adipocytes Glucose uptake

Effect of *pterostilbene* on glucose uptake in differentiated adipocytes was analyzed in the presence and absence of 10 µg/ml insulin to check the efficacy of *pterostilbene* in glucose uptake. *Pterostilbene* showed a significant improvement in glucose uptake in presence of insulin (Fig. 4.5.13). *Pterostilbene* showed a gradual increase in glucose uptake with maximum effect at a dose of 50 mg/l afterwards there was no further increase in glucose uptake even at a dose of 100 mg/l. However *pterostilbene* was not able to stimulate glucose uptake in absence of insulin.

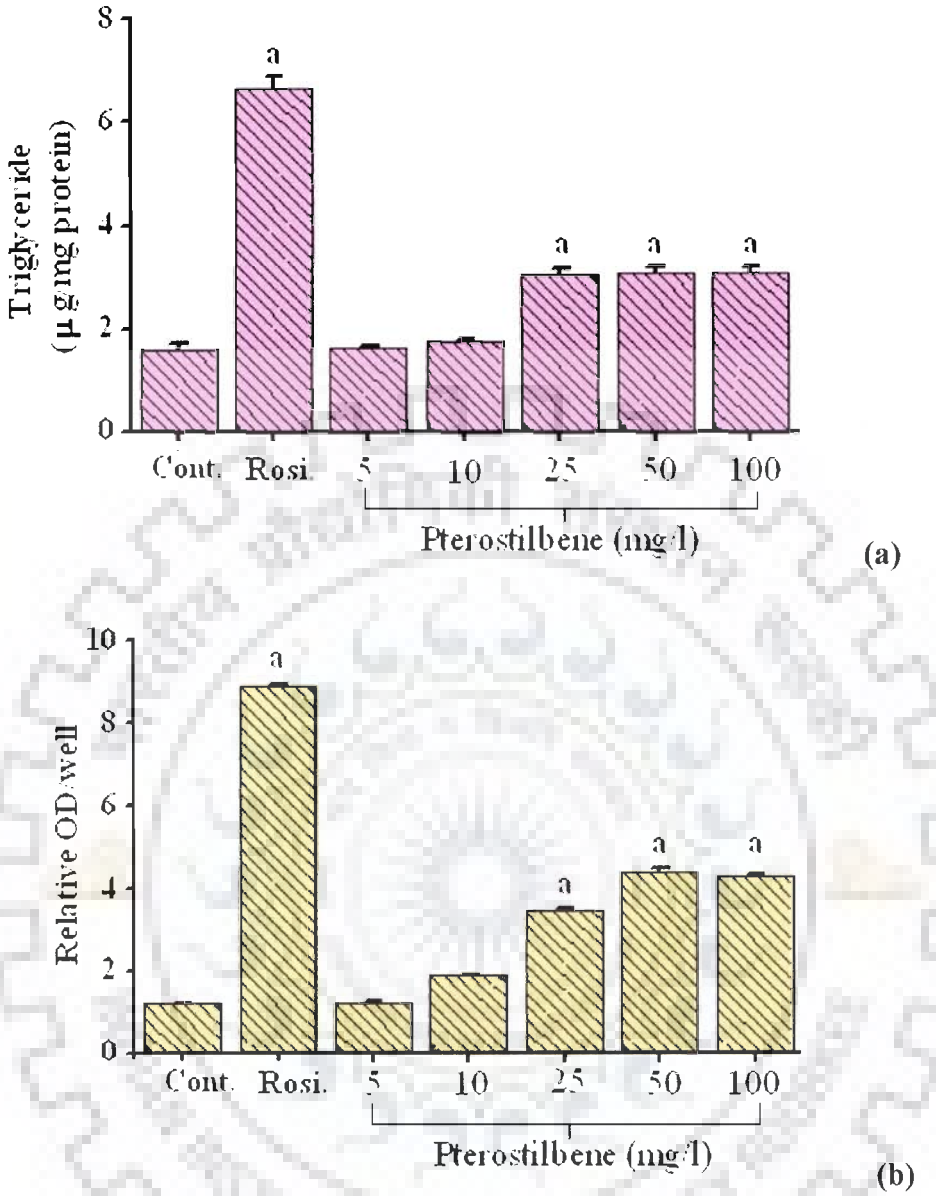


Figure 4.5.12. Dose dependent effect of *pterostilbene* on 3T3-L1 preadipocyte differentiation. (a) The cellular TG content expressed as µg/mg of protein and (b) relative absorption of Oil Red O dye. Data are mean ± S.E.M. of four independent experiments each performed in quadruplicates. a, represents the significant level of effect as compared to vehicle treated control groups ($p<0.05$). Cont., vehicle treated control; Rosi., rosiglitazone treated.

4.5.3.3 HPLC analysis

HPLC analysis of purified fraction showed the presence of *pterostilbene* at the retention time of 20.37 min (Fig. 4.5.14). Although some other minor peaks were also present but they were not significant in comparison of *pterostilbene*, which was the most prominent component of the isolated fraction.

4.5.4 Discussion

Diabetes is a chronic metabolic disorder affecting a major part of population worldwide. A sudden switch from basic life style to a so called western life style along with other genetic and environmental factors resulting in obesity, hyperlipidemia and hypertension is the reason behind its deadly growing speed (ACP, 2007). Diets containing high fat contents result in an elevated plasma free fatty acid level which further cause a systemic insulin resistance. Insulin resistance in peripheral tissues like skeletal muscles and adipose tissue ameliorates the ability to clear glucose from systemic blood flow (Antti et al., 2005; Kraegen et al., 1991 and Bozkurt et al., 2007). Accumulation of lipid in liver due to high plasma free fatty acid level inhibits glucose uptake and glycogen storage, at the same time this results in an enhanced gluconeogenesis and increased glucose output from liver (Ahren, et al., 2008; Aronoff et al., 2004). All these factors collectively increase blood glucose level i.e. hyperglycemia, a root cause of multiple complications associated with diabetes.

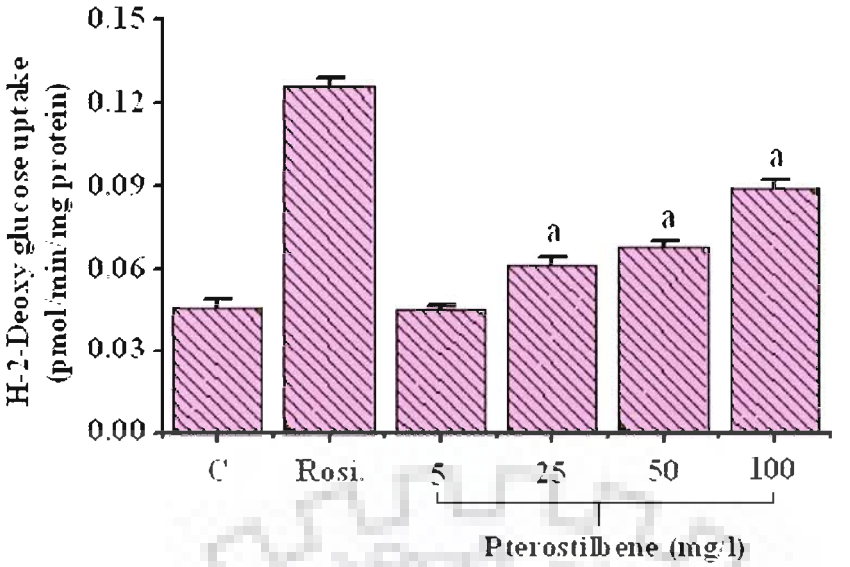


Figure 4.5.13. Effect of various doses of *pterostilbene* on glucose uptake in differentiated adipocytes. Data is expressed as mean ± S.E.M. of three independent experiments each performed in quadruplicates. a indicates the significant levels of difference in glucose uptake as compared to activity without extract ($p<0.05$).

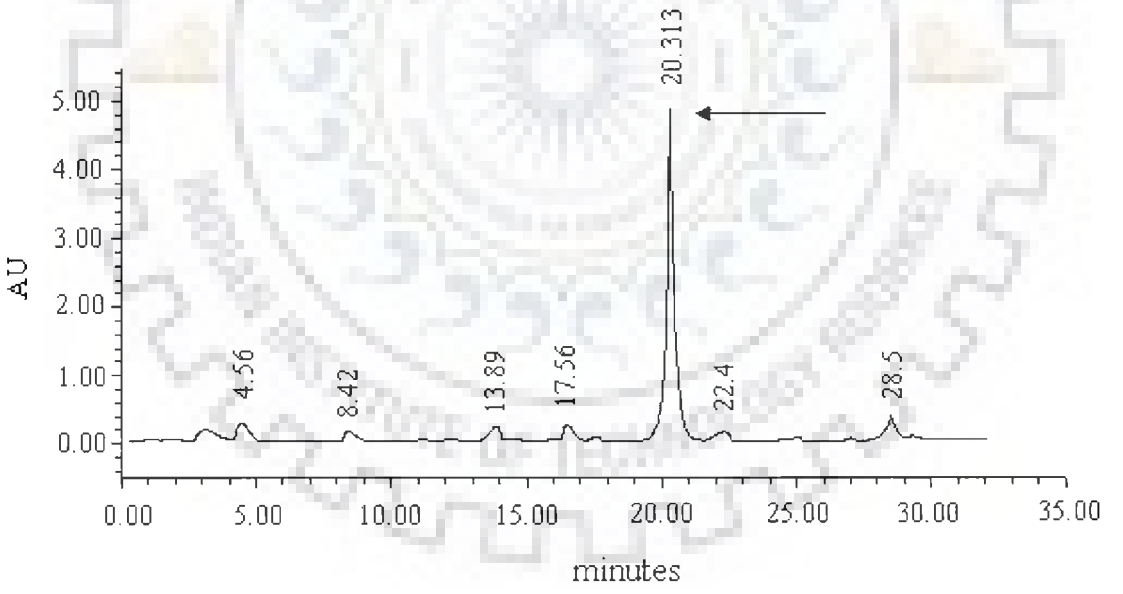


Figure 4.5.14. High performance liquid chromatograms of *pterostilbene* isolated from *Pterocarpus marsupium*. The arrow indicates *pterostilbene* identified on the basis of similarity of retention time in comparison of is reference standard.

Present study gives us an idea that how *pterostilbene* can be utilized for targeting multiple sites associated with diabetes in order to treat high fat diet induced type II diabetic condition. Skeletal muscle, adipose tissue, liver and pancreas are the four basic organs related with overall metabolism and in the present study *pterostilbene* has shown it's affects in all these four organ directly or indirectly. Skeletal muscle is quantitatively the most important tissue involved in systemic glucose clearance and incubation of muscle tissue in presence of *pterostilbene* isolated from *P. marsupium* further improved its efficiency of glucose uptake. Improvement in glucose disposal could be attributed to the enhanced expression profile of Glut-4 in muscle tissue in comparison of diabetic counterpart.

Pterocarpus marsupium or *PM* is a traditional medicinal plant known for its lipid lowering effects (Bose and Sephaha 1956, Dhanabal, 200625, Grover et al., 2002; Amarnath and Pari, 2006). Results from our study further confirmed it by showing it's efficacy in the lowering of TG and TC in type II diabetic animals. This was further confirmed by adipocytes differentiation assay where incubation with *pterostilbene* enhanced adipocytes differentiation and triglyceride accumulation in 3T3-L1 cells. A similar pattern of results were also shown by some other hypolipidemic plant extracts (Wu et al., 2007; Martineau et al., 2006).

Following mucles, liver is the second most important tissue for glucose homeostasis. A fat enriched diet causes an increased accumulation of triglycerides in liver due to abnormal synthesis and elimination of triglycerides (also called fatty changes) (Arion et al., 1998). Treatment with *pterostilbene* for eight weeks resulted in reduced hepatic lipid accumulation perhaps due to improved lipid metabolism in liver

along with reduced plasma triglyceride content. Triglyceride accumulation in liver tissue results in increased gluconeogenesis, glycogenolysis, reduced glycogen synthesis and contributes further in a hyperglycemic condition. High G6Pase and low hexokinase activity is a characteristic feature of insulin resistance which results in hyperglycemia (Bonoro, 2000). However, treatment with *pterostilbene* caused improvement in glucoregulation, as evident from activity and expression analysis of GK, PEPCK ad G6Pase enzymes. Improved glucoregulation can be attributed to increased expression of glucokinase, a key regulator for glycolysis and increases glucose utilization as energy source. Further decreased activity of G6Pase, a key regulator in gluconeogenesis, reduces the glucose production from liver and counteracts the hyperglycemic condition of diabetes. In addition this phytochemical proved to be a potent inhibitor of α-glucosidase enzyme, which is responsible for ingested carbohydrates. Further experiments are needed to suggest the exact mode of interaction of this chemical with this enzyme as has been shown earlier by some authors (Li et al., 2005; Onal et al., 2005). Reduced expression of aldose reductase (AR) focuses on an additional role of *pterostilbene* in the treatment of type II diabetes (Nishimura, 1998).

Improvement in peripheral glucose tolerance is the key feature of *pterostilbene* treatment, which is evident from sharp decline of blood glucose level during OGTT. Improvement in peripheral glucose uptake can be attributed to the improved insulin sensitivity due to lower plasma lipid profile and reduced free fatty acids induced insulin resistance in peripheral system. Other plant extracts have also shown similar pattern of results earlier under similar conditions (Soo and Sunmin, 2002; Anwer et al., 2008). Along with this, a clear improvement in Glut-4 both at transcriptional and translational

level, also contributes towards improved glucose uptake from peripheral tissues. Treatment with *pterostilbene* resulted in a clearly enhanced translocation of Glut-4 from cytoplasmic fraction to cell membrane as evident from western blot analysis in diabetic and treated groups. Similar pattern of results were observed from grape seed-derived Procyanidins treatment of 3T3-L1 preadipocytes, where the treatment resulted in enhanced Glut-4 translocation from intracellular reserves to the plasma membrane (Pinent et al., 2004).

Expression profile of different target genes in the adipose tissue provides a preliminary idea about the mechanism of action of *pterostilbene*. Increased expression of Glut-4 with simultaneously reduced expression of TNF-α explains improvement in insulin resistance condition. Activity of PPARα and γ showed significant improvement during different levels of analysis. In addition transactivation assay further proved the role of *pterostilbene* to be acting as agonist for PPARα as well as γ. Dual regulation of PPARα and γ has been reported by several other plant extract as well earlier (Han et al., 2008; Li et al., 2007). However this phytochemical has a higher affinity for PPARα as compared to PPARγ. PPARα is predominantly expressed in tissues catabolizing high amounts of fatty acids, such as liver, heart and brown adipose tissue and controls the expression of various genes involved in intra- and extra cellular lipid metabolism, such as acyl coA oxidase, acyl-coA synthetase and apolipoproteins A-I, A-II and C-III. As the PPARγ specific agonist are associated with the extensive weight gain, coactivation of PPARα using a seprate PPARα agonist or dual activator has been proposed as an effective remedy for this side effect (Chakravarthy et al., 2005; Xu et al., 2002). Although *pterostilbene* stimulates adipocytes differentiation in *in vitro,* but it did not

cause any significant weight gain, this effect could be attributed to the dual nature of *pterostilbene* as PPARα and γ agonist, where PPARα controls the overall lipid metabolism and counteracts the weight gain tendency of PPAR agonist. Absence of any effect on PTP-1B activity as shown by our data conclusively proves the direct effect of this chemical on insulin receptor sensitization via PPAR activation other than any other associated pathways.

Histological examination of adipose tissue from diabetic and treated groups showed remarkable differences in size of adipocytes from diabetic and treated groups. Adipocytes from treated group were significantly smaller than its diabetic counterpart which is a positive sign as larger adipocytes secrete some adipokines which result in insulin resistance, whereas smaller adipocytes are considered as healthy ones that secrete factors which improve this condition (Hotamisligil, 1999).

To interpret if at all *pterostilbene* has some insulin secretagogues effect, we checked the effect of *pterostilbene* on insulin release from purified pancreatic islets. There was no significant difference in insulin secretion from islets of control group or treated with *pterostilbene* (Data not shown). Histological examination of pancreatic tissue from diabetic and treated group showed a clear significant difference, where the pancreatic islets were found to be comparatively smaller in the diabetic group. Toxicological evaluation of liver section did not showed any sign of toxicity in comparison of control group, hence proving the *pterostilbene* safe for diabetes management.

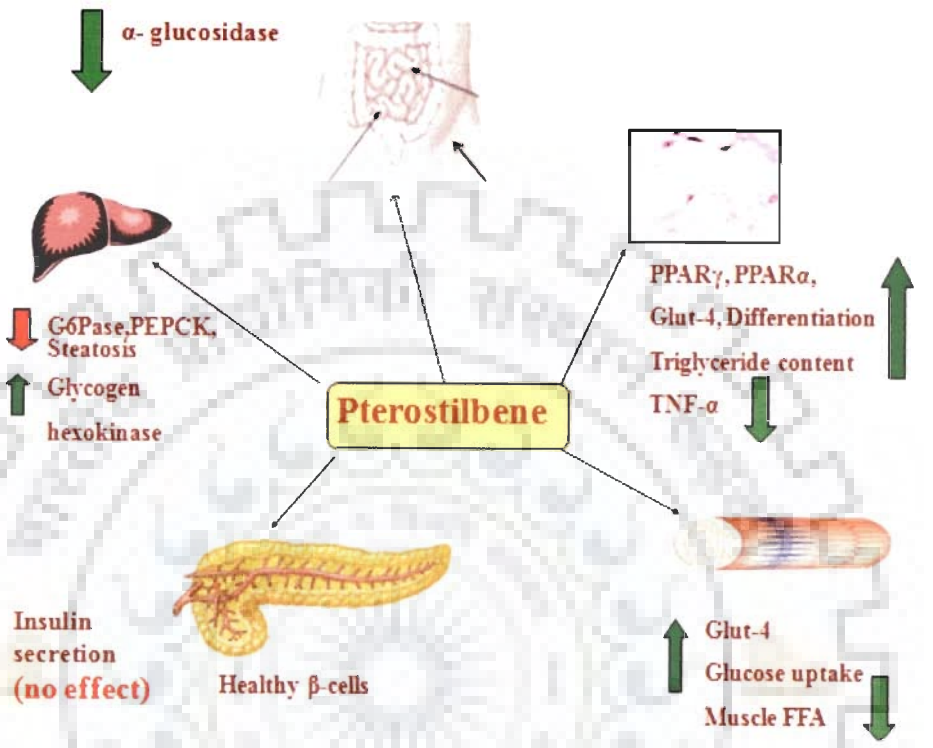


Figure. 4.5.15. Effects of *pterostilbene* on various organs for the treatment of diabetes.

Results obtained from the present study gives a clear idea about multi-target potential of *pterostilbene* in the treatment of diabetes mellitus. *Pterostilbene* treatment resulted in a dual regulation for antidiabetic treatment. Firstly, *pterostilbene* showed its effect via regulation of adipose tissue and stimulated triglyceride accumulation, finally resulting in an improved plasma lipid profile and secondly, it regulated the Glut-4 expression and function further contributing in improvement of systemic glucse tolerance (Fig. 4.5.15).

In summary, it is clearly evident from above results and observations that *pterostilbene* has the ability to act on various critical nodes of carbohydrate and lipid metabolic pathways. To the best of our knowledge this is the first ever report that this phytochemical has been purified from a plant source and analyzed to get a comprehensive idea about its mode of action.

CHAPTER- V

SUMMARY

Diabetes mellitus is complex metabolic disorder resulting from either insulin insufficiency or insulin dysfunction. Type I diabetes (insulin dependent) is caused due to insulin insufficiency due to the lack of functional β-cells. Patients suffering from this type of diabetes are therefore totally dependent on exogenous source of insulin. While the patients suffering from type II diabetes (insulin independent) fails to respond to insulin. These patients can be cured mainly with dietary changes, exercise and medications. Type II diabetes constitutes about 90% of the diabetic patients. Both these conditions results in increased concentration of glucose in the blood, which in turn damage many of the body's systems. The major chronic complications associated with diabetes include retinopathy, neuropathy, nephropathy and artherosclerotic vascular disease (Kaczmar, 1998). In addition to hyperglycemia, diabetes is associated with several other factors like hyperlipidemia and enhanced oxidative stress which makes pathogenesis of this disorder further complicated. Diabetes affects about 5% of the global populations and according to a recent estimation, this disease is approaching the midst of a diabetes pandemic (Dewanjee et al., 2008; Mukherjee et al., 2006). It is expected that by the year 2010 the total number of people worldwide affected with diabetes would be close to 293 million (Rao et al., 2004). In spite of alarming situation with this disorder, management of diabetes without any side effects is still a challenge to the medical system (Kameswara Rao et al., 2003). The principal aim of any antidiabetic drug is the lowering of blood glucose level to normalcy. As diabetes is a multifunctional disease leading to several

complications, it therefore demands a multiple therapeutic approach. As discussed earlier, patients of diabetes either do not make enough insulin or their cells do not respond to insulin. In case of total lack of insulin, the patients are given insulin injections. On the other hand, in case of those patients who do not respond to insulin various kinds of drugs have been developed keeping into consideration the possible disturbances in carbohydrate metabolism. For example, to manage post-prandial hyperglycemia at the digestive level, α-glucosidase inhibitors like acarbose, miglitol and voglibiose are used. These agents inhibit degradation of carbohydrates thereby reducing the glucose absorption by the intestinal cells. To enhance glucose uptake by peripheral cells biguanides such as metformine is used. Similarly, sulphonylureas like glibenclamide acts like an insulinotropic agent and function as secretagogues for pancreatic cells. Further, some molecules of troglitazone group, like rosiglitazone, acts in sensitizing the insulin receptors thus paving ways for the treatment of type II diabetic condition (Modak et al., 2007; Mukherjee et al., 2006). Although all these are in use for the treatment of diabetes, there are certain limitations for their use like high cost, severe side effects such as development of hypoglycemia, weight gain, gastrointestinal disturbances, liver toxicity etc. (Modak et al., 2007; Dey et al., 2002).

Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. According to WHO estimations the populations in developing countries like India (70%), Rwanda (70%), Uganda (60%), Tanzania (60%), Benin ((80%) and Ethiopia (90%) extensively use traditional and alternative systems of medicines for health care. In case of developed countries like Belgium (31%), USA (42%), Australia (48%), France (49%), Canada (70%), a significant percentage of the population has been reported to use traditional and

alternative remedies albeit at a lower rate than the developing countries (WHO, 2002; Mukherjee; Wahile, 2006). In almost all the traditional system of medicines, the medicinal plants play a major role and they are used as basic constituents. Indian Materia Medica includes about 2000 drugs of natural origin almost all of which derived from different traditional systems and folklore practices (Narayana et al., 1998; Mukherjee and Wahile, 2006). Like many other diseases the use of herbs in the management of diabetes mellitus has been prevalent since a long time. Recently, two national surveys were carried out in USA to examine the use of herbal medicines for the cure of diabetes among them. One of the studies reported that 8% of the diabetic populations were completely dependent on herbal remedies (Egede et al., 2002). In another set of surveys on specific diabetic populations, 39% of Navajo, two-thirds of Vietnamese and 49% of a largely Hispanic population in South Texas used herbal therapy (Kim and Kwok, 1998; Mull et al., 2001; Noel et al., 1997). All these studies conclusively proved the popularity of the plant based cure for diabetes even in developed countries like USA. The ethnobotanical information reports about 800 plants that may possess antidiabetic potential (Grover et al., 2002). A wide array of plant derived active principles representing numerous chemical compounds have demonstrated activity consistent with their possible use in the treatment of NIDDM. Majority of these compounds are alkaloids, glycosides, peptidoglycans, hypoglycans, polysaccharides, steroids, flavonoids, terpenoids, amino acids and inorganic ions (Grover et al., 2002). The best example for the plants as source of drugs is demonstrated by hypoglycemic drug, metfromin, which was isolated from the plant *Galega officinalis*.

In spite of the fact that the plants are a potential source of antidiabetic drugs (and others too) this aspect of herbal research has not gained enough momentum or interest in

the scientific community. This could be attributed to several factors like lack of belief among the practitioners of conventional medicines over alternative medicine, alternative forms of medicine are not very well defined and above all these drugs may vary tremendously in content, quality and safety. The biggest controversy in this regards is whether to use the whole extracts obtained from various parts of these plants or their isolated active constituents. In theory, the activity shown by a mixture, such as an extract, is due to the sum of the activities of the individual constituents. Thus fractionation, leading to isolation of individual compounds, should result in aliquots having a higher activity than the original extract (Houghton et al., 2007). However, in some instances the situation is reversed such that the activity shown by the total extract is higher than that of from the activities of the individual major components (Ren et al., 2004). This could be justified by two reasons, firstly, the probable loss of activity by decomposition of the active compounds during fractionation process and secondly, oxidation of some active constituents during isolation procedure also contributing to this process. The best example of latter is the phenolic compounds which get oxidized in presence of silica during fractionation (Houghton et al., 2007). Such situations make the ethnopharmacology research a bit complicated and controversial. In order to prove these facts more logically it is important to validate each phytochemical for their functional activities before they are used for human consumptions.

According to a recent report, out of an estimated 250 000 higher plants, less than 1% have been screened pharmacologically and very few for diabetes mellitus. Therefore it is prudent to search for options in herbal medicines for diabetes as well (Grover et al., 2002). Based on some of the promising results obtained WHO have recommended that traditional medicinal plants are further investigated (Modak et al., 2007). Considering all

these facts in the present thesis we looked at the medicinal plants from two different aspects for the cure and management of diabetes. In the first approach we selected about nine medicinal plants: *Tinospora cordifolia*, *Phyllanthus emblica*, *Murraya koenigii*, *Capparis deciduas*, *Eugenia jambolana*, *Aegle marmelos*, *Pterocarpus marsupium*, *Eucalyptus globules* and *Commiphora mukul*. The plants were selected based on their preliminary data on hypoglycemic activities. During this part of study all these plants were screened for their anti-diabetic activities with special emphasis on type I diabetes using STZ induced diabetic mice models. Our data reveled that out of all those plants at least four plants namely *Eugenia jambolana, Capparis decidua, Commiphora mukul* and *Pterocarpus marsupium* demonstrated overall significant antidiabetic activities. The basic parameters tested here were fasting blood glucose levels, oral glucose tolerance tests, estimation of enzymes responsible for maintaining glucose homeostasis, liver tissue glycogen content. In addition these plant extracts also improved the lipid profiles of the treated animals. As a whole, some of the plants selected by us showed potent antihyperglycemic and hypolipidemic activities. Although some of these plants like *Commiphora mukul*, *Eugenia jambolana*, *Capparis decidua* and *Pterocarpus marsupium* have been reported to show antidiabetic activities (Grover et al., 2002, Modak et al., 2007; Mukherjee et al., 2006), but to the best of our knowledge there was no systematic studies on these plants about their comparative hypoglycemic activities. The results of this part of study showed that the basic extracts (aqueous or alcoholic) of these plant's parts like leaves, seeds, bark could control fasting and post-prandial blood glucose levels, glycogen content etc. Such an ethnomedical approach for diabetes is a practical, cost effective and logical for its treatment (Grover et al., 2002).

Once the extract showed promising bioactivity towards the cure and management of diabetes, those best active plants were taken further to test their efficacy in the isolated fractions rich in a particular group of compounds like flavonoids, alkaloids, steroids and polysaccharides from various plants. Although we tested all these groups of chemicals from various plants, in the present thesis the basic emphasis was given on flavonoids and alkaloids isolated from *Eugenia jambolana* and *Capparis decidua*, respectively. The main reason for this is that, although the other fractions isolated from the plants selected by us showed some response but response were not comparable to the foresaid group's effects. Also taking into consideration all the groups was beyond the scope of this thesis. Hence only flavonoids and alkaloids were considered for this thesis.

As discussed in chapter 4.3 of this thesis, flavonoids represent a beneficial group of naturally occurring compounds with hypoglycemic potentials. These are widely distributed in plant kingdom and exhibit distinctive pharmacological properties. One of the earliest and best studied flavonoid which possesses antidiabetic activity was quercetin. It has been reported to enhance insulin release from isolated rat islet cells partly by changing Ca^{2+} metabolism (Vessal et al., 2003). In our study too, the flavonoids rich fraction isolated from *Eugenia jambolana* seeds showed insulin enhancing activity in addition to other glucose lowering activities (Sharma et al., 2008). Further studies were carried out to test if this enriched fraction could play some role in the cure and management of type II diabetes as has been reported earlier by some authors (Mukherjee et al., 2006). The soy isoflavones genistein were found to improve diabetes complications in male and female zucker rats, a model for type II diabetes (Mezei et al., 2003). Peroxisome-proliferator activated receptor (PPAR) are promiscuous nuclear receptors that regulate the transcription of genes involved in lipid and glucose homeostasis and

lipid metabolism within the cells. The results from several laboratories including ours showed that flavonoids or their derivatives significantly improved lipid and glucose metabolism by acting as a hypoglycemic PPAR agonist (Fan et al., 2006; Fang et al., 2008; Guo et al., 2006; Sharma et al., 2008). In addition to these effects the flavonoids have also been reported to regulate the expression profiles of various genes directly involved in glucose homeostasis like PEPCK, PI3-kinase, G6Pase, hexokinase, Glut-4 (Sharma et al., 2008; Waltner-Law et al., 2002). Some reports also exist on the role of flavonoids as insulinomimetic activity which also stimulated glucose uptake by insulin sensitive cells *in vitro* (Pinent et al., 2004). In the present thesis the flavonoid rich fraction isolated from *Eugenia jambolana* seeds showed many of the activities which have been reported earlier by flavonoids isolated from other plants. Finally when we tested the fraction for its content of various flavonoids by HPLC, it was found to contain rutin, quercetin, myricetin and kaempferol. The responses shown by the flavonoid rich fractions hence could be attributed to these compounds. In conclusion, this part of study showed that the *Eugenia jambolana* seeds are rich in various types of flavonoids which play an important role in the management of diabetes.

In a similar context, the alkaloid rich fraction isolated from *Capparis decidua* was tested for its antidiabetic activities. Earlier various alkaloids have been isolated from several Indian medicinal plants and investigated for their possible hypoglycemic activity in different animal models. The best studied of them is berberine which has been shown to act as antidiabetic agent by inhibiting α-glucosidase activity and/or by decreasing glucose transport through the intestinal epithelium (Pan et al., 2003). On our study, the alkaloid rich fraction also demonstrated prominent hypoglycemic activity. In addition to all other normal parameters controlled by any potent antidiabetic agent, the alkaloids of

this plant also prominently affected the PPAR activity like that of flavonoids, thus confirming its direct involvement in type II diabetic conditions as well. Thus the results of this part of study showed that the alkaloid rich fraction from the *Capparis decidua* has the potentiality to act at multiple target sites for the management of diabetes and its related complications like hyperlipidemia. However, we failed to identify the exact alkaloids present in the extract due to the lack of proper standards.

In the next phase of our study two other plants showing best antidiabetic activities in the preliminary screening were selected for further isolation of single major constitutes almost to homogeneity and to check their action as antidiabetic agent. The plants tested in this section were *Pterocarpus marsupium* and *Commiphora mukul*. Both these plants are found throughout India and have been used commonly for the cure of diabetes and its related complications. *Pterocarpus marsupium* has been used in folk medicines since long as a hypoglycemic agent (Bose and Sepaha, 1956; Gupta 1963; Shah 1967). Various parts of this plant like bark, latex, etc., have been investigated and reported to have hypoglycemic activities (Vats et al., 2002, 2004, Kar et al., 2003; Abesundara et al., 2004). Further some of the phytochemicals isolated from the bark and heartwood of this plant like (-)-epicatechin, marsupin, pteroupin, and *pterostilbene* were found to possess blood sugar lowering activity (Mukherjee et al., 2006). However, all these data did not provide the exact mode of actions of these active constituents. In the present thesis we purified the *pterostilbene* almost to homogeneity from *Pterocarpus marsupium* plant. Although there are several reports on the action of this phytochemical in ameliorating the oxidative stresses and other related complications, there are almost no reports on its effects on diabetes especially in type II diabetes. Our data showed that this phytochemical significantly improved the glucose homeostatic conditions in high fat induced obese and

diabetic rats. The use of diet induced diabetic model is considered to be a better option as compared to transgenic animal models (*db/db* or *ob/ob*) since these transgenic model do not reflect the exact pathophysiology of diabetes due to a single gene mutation (Winzell and Ahren, 2004). On the other hand the high fat diet affects the multiple target sites thus providing a better model close to human type II diabetic condition. There was clear improvement in the lipid profiles in these groups of animals and they were comparable to the known hypolipidemic drug i.e., metformin. Finally, when this phytochemical was tested in PPAR transactivation assay it was found to stimulate both PPARα and PPARγ induced transactivations albeit to a lower level in the latter than the former one. This was in agreement with earlier report by Rimando et al. (2005) where they have reported the PPARα agonistic activity of this chemical. As expected, *pterostilbene* also induced the differentiation of 3T3-L1 adipocytes like any other PPARγ agonist. Further it also inhibited α-glucosidase activity which elucidates it's another mode of action in the prevention of blood glucose level. Based on these data it could be concluded that the *pterostilbene* acts as an antidiabetic agent both by increasing insulin sensitivity as well as stimulating peripheral glucose clearance.

In a similar context we tested for the other phytochemical i.e. *guggulsterone* isolated from *Commiphora mukul*. This is one of the most prominent chemicals in the resin of this plant which exists in cis and trans forms. The most studied effects of *guggulsterone* are the lipid lowering and cholesterol lowering effects. Early *in vivo* studies showed that oral administration of this phytochemical decreased serum cholesterol levels in hypercholesteremic animals (Yang et al., 2008). More recent studies have confirmed the clinical effect and have shown that the activity is at least partly due to antagonism of nuclear farnesioid X receptors (FXRs) (Wu et al., 2002; Urizar et al.,

2002; Yang et al., 2008). It is used in various Ayurvedic formulations for the treatment of obesity and hyperlipidemia and it has been shown to decrease body weight in human and animals (Yang et al., 2008). On the other hand there are several contrary reports on this chemical's role in the cure of obesity and diabetes (Bhatt et al., 1995; Ulbricht et al., 2005). In order to throw some lights on this controversial issue next part of the thesis was taken up. Literature survey showed that majority of the earlier reports used either the synthetic *guggulsterone* or the guggul lipids. In the present thesis we isolated *guggulsterone* from the *Commiphora mukul* which was purified almost to homogeneity consisting of both E and Z isoforms. This particular fraction actually was a compromise between the above two categories which has not yet been tested so far in the cure of diabetes. Our data showed that *guggulsterone* exhibited that potent antidiabetic activity by improving fasting and post-prandial glucose load, improving lipid profiles and altering the glucose homeostatic enzyme activities as expected from any chemical showing antidiabetic activities. The most promising effect shown by this plant was the protection of pancreatic β-cells. This was in accordance to an earlier report by Lv et al. (2008) using synthetic *guggulsterone*. Further when tested for adipocyte differentiation, the compounds failed to differentiate 3T3-L1 cells *in vitro* although it showed marked improvement in the adipocytes of the high fat diet obese animals. At this point it is difficult to predict any reason for this disparity of this compound in *in vitro* and *in vivo*. One possible reason could be its partial agonistic activity for PPARs atleast in the *in vivo* condition. Further PTP-1B assay depicted that this phytochemical could significantly inhibit the activation of this enzyme, thus proposing yet another mode of action for it where the insulin receptor desensitization is prevented. Taken together, *guggulsterone* isolated from *Commiphora mukul* acts at multiple target sites in the cure of diabetes and

obesity. However, further characterization of this phytochemical will elucidate its exact mode of action to be considered as a new drug entity.

In conclusion, diabetes is a disorder of carbohydrate, fat and protein metabolism attributed to diminished production of insulin or mounting resistance to its action. Herbal treatments have been used since long to manage these complications. Scientific validations as depicted in this thesis proved the efficacy and modes of action some of these botanical extracts in the management and cure of diabetes. This thesis thus provides the comprehensive mode of actions of some common Indian medicinal plants. From the data presented here it is conclusively proved that these botanicals has tremendous potentiality against both type I and type II diabetes, which needs further exploration and for necessary development of drugs and neutraceuticals from natural sources.

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