EFFECTS OF AQUEOUS-ETHANOLIC EXTRACTS AND FRACTIONS OF Moringa oleifera Lam. LEAF ON DIABETIC AND METABOLIC SYNDROME RATS

HAFIZ MUHAMMAD IRFAN

UNIVERSITI SAINS MALAYSIA
2017

EFFECTS OF AQUEOUS-ETHANOLIC EXTRACTS AND FRACTIONS OF Moringa oleifera Lam. LEAF ON DIABETIC AND METABOLIC SYNDROME RATS

by

HAFIZ MUHAMMAD IRFAN

Thesis submitted in fulfilment of the requirements
for the Degree of
Doctor of Philosophy

August 2017

ACKNOWLEDGEMENT

I would like to start with Allah Subhana-O-Wataallah for the strength, wisdom and health He bestowed on me, which has been the guiding, force that has helped me to accomplish this phase in my journey of knowledge seeking.

The period of my studying for this Ph.D degree has given me infinite experiences and information, which I believe, will be valuable in my future endeavors and will help me significantly to achieve greater heights. A number of individuals have been instrumental in my study.

My enormous gratitude goes to my supervisor Prof. Mohd. Zaini Bin Asmawi for his innumerable and countless support in guiding me through this learning process of research. Prof. Mohd. Zaini's influence stretch beyond the confines of academic field; the warmness and calm level he develops between him and his students, urging to achieve excellence while understanding our limits and leniency beyond our expectations are attributes that endears both his students and others towards him. His contribution towards my study cannot be overemphasized.

I am also indebted to my co-supervisors, Dr. Nurzalina Abdul Karim Khan, Prof. Amirin Sadikun and Prof. Mohd. Nizam Mordi for their persistent interest in the progress of my research and constant encouragement and invaluable advices they have offered me. Their readiness to offer any help they can is overwhelming and I am grateful for that.

My thanks go to Dr. Yam Mun Fei for his guidance in the initial stages of my candidature. Thank you for the advices and kindness. My appreciation also goes to all my lab colleagues; Idris Bello, Anis, Nasiba Usman Salisu, Adlin Yussof, , Abdulmunem Bakkouri, Majed Al-mansoubb, Bassel Al-Hindi, Raghdaa Al-zouhri and Faramarz for their support during my candidature.

Last but not the least are the most influential people in my life, my parents (Mr. Ghulam Rasul and Mrs. Naziran Begum), for the trouble, patience, encouragement, and moral lessons they have attempted an impact on my life cannot be expressed in words. Thank you for your guidance throughout my life and may Allah reward you with Jannatul Firdaus. I would like to extend my thanks to my wife, Sumaira Sharif, My children, Muhammad Abdullah Irfan, Amina, Abdul Rehman and Saira for their support and encouragement. This would not have been possible without their strength and passion for education and their willingness to make the selfless sacrifices needed to complete this degree. I will forever be grateful to them.

I wish to extend my thank to Prof. Dr. Sajid Bashir, Dean and Chairman, Faculty of Pharmacy, University of Sargodha and Prof. Dr. Muhammad Shoaib Akhtar who helped a lot with valuable inputs and encouragement in making this Ph.D a tenure smooth.

I would like to acknowledge University of Sargodha, Sargodha, Pakistan for providing me faculty development scholarship for Ph.D studies. Many thanks to Mr. Mohammad Razak, Science officer, Centre for Drug Research, USM for HPLC assistance and Mr. Rosli, School of Pharmaceutical Sciences, USM for his technical assistance in experimental work.

Hafiz Muhammad Irfan

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADA	American diabetes association
AF	Aqueous fraction
AMPK	Adenosine monophosphate activated protein kinase
ANS	3-amino-5-nitro-salicylic acid
ANOVA	Analysis of variance
ARASC	Animal research and service centre
ATP	Adenosine triphosphate
BF	Butanol fraction
BG	Blood glucose
BGC	Blood glucose concentration
BGL	Blood glucose level
BW	Body weight
CE	Capillary electrophoresis
CF	Chloroform fraction
CHD	Coronary heart disease
CTLC	Centrifugally accelerated thin layer chromatography
DCCC	Droplet counters currant chromatography
DM	Diabetes mellitus
DMSO	Dimethyl sulfoxide
DNS	3,5-dinitro salicylic acid
DPP-IV	Dipeptidyl peptidase-IV
EE	Ethanol extract
EAF	Ethyl acetate fraction

FBG	Fasting blood glucose
FCR	Folin ciocalteu reagent
GC	Gas chromatography
GIT	Gastrointestinal tract
GLP-1	Glucagon like peptide-1
GLUT 2	Glucose transporter protein 2
GLUT 4	Glucose transporter protein 4
GOD	Glucose oxidase
G6Pase	Glucose 6-phosphatase
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase-3
GTG	Gold thioglucose
HDL	High-density lipoprotein
HF	Hexane fraction
HFD	High fat diet
HMG-CoA	3-Hydroxy-3-methyl-glutaryl-coenzyme A
HMP	Hexose monophosphate
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IDDM	Insulin dependent diabetes mellitus
IDF	International diabetes federation
IDFA	International diabetes federation atlas
IDL	Intermediate density lipoprotein
IGT	Impaired glucose tolerance
IPGTT	Intraperitoneal glucose tolerance test

IRS-1	Insulin receptor substrate-1
KRB	Kreb’s ringer bicarbonate
LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
mAU	Milli absorbance unit
MBC	Minimum bactericidal concentration
MCP-1	Monocyte chemoattractant protein-1
MIC	Minimum inhibitory concentration
NCE	Novel chemicals entities
NDC	Negative diabetic control
NDDG	National diabetes data group
NIDDM	Non-insulin dependent diabetes mellitus
PDC	Positive diabetic control
PEPCK	Phosphoenol pyruvate carboxykinase
POD	Peroxidase
PPAR-g	Peroxisome proliferator activated receptor-gamma
ROS	Reactive oxygen specie
RSD	Relative standard deviation
SD	Sprague dawley
TC	Total cholesterol
VLDL	Very low-density lipoprotein
WHO	World Health Organization

KESAN EKSTRAK AKUEUS-ETANOL DAN FRAKSI DAUN Moringa oleifera Lam. TERHADAP TIKUS DIABETIK DAN BERSINDROM METABOLIK

ABSTRAK

Kemajuan terkini dalam pemencilan dan pengenal pastian sebatian aktif yang ada dalam tumbuhan ubatan telah membawa kapada pendekatan bersasar yang berasaskan pemecahan, dalam pencarian bioaktif. Oleh itu, penyelidikan ini telah direka bentuk untuk menjalankan kajian berpandukan-aktiviti terhadap tikus diabetik bagi menentukan ekstrak paling aktif, pecahan yang aktif dan pengenal pastian bahan utama aktif menggunakan ekstrak akueus-etanolik daun *Moringa oleifera* yang berlainan. Ekstrak ini juga telah dinilai pada tikus bersindrom metabolik. Lima ekstrak berlainan (95, 75, 50 dan 25% [v/v] etanol dan 100% air) telah diuji secara oral dalam tikus Sprague Dawley normoglisemik untuk aktiviti anti-hiperglisemik dan tolerans glukosa manakala tikus diabetik teraruh-STZ telah digunakan untuk aktiviti anti-hiperglisemik akut dan sub-kronik. Ekstrak paling aktif (95% etanol) telah dipecahkan lagi kepada pecahan heksana, kloroform, etil asetat, butanol dan akueus, dan disaring untuk aktiviti anti-diabetik dalam tikus diabetik. Ekstrak dan pecahan aktif kemudiannya di analisis fitokimia secara kualitatif dan kuantitatif, termasuk menggunakan kromatografi lapisan nipis (TLC) dan kromatografi cecair berprestasi tinggi (HPLC) bagi mengenal pasti sebatian penanda. Antara semua ekstrak dan pecahan, ekstrak etanol 95% pada dos 1,000 mg/kg dan pecahan butanol pada dos 500 mg/kg telah didapati paling aktif, menunjukkan penurunan glukosa darah yang signifikan selepas satu pemberian tunggal dalam tikus diabetik. Tiada aktiviti anti-hiperglisemik yang signifikan, dan tolerans glukosa telah tercerap pada

tikus biasa. Kajian dos-respons sub-kronik telah menunjukkan penurunan glukosa darah yang amat signifikan dengan dos 500 dan 1,000 mg/kg sahaja. Kumpulan yang terawat juga menunjukkan penurunan jumlah kolesterol, trigliserida, lipoprotin ketumpatan-rendah dan plasma MCP-1. Eksperimen *in vitro* bagi perencatan α-amilase dan α-glukosidase mencadangkan perencatan bukan-kompetitif dan glukosa usus mod campuran masing-masing, dan pengambilan glukosa yang lebih baik oleh otot skeletal. Analisis TLC dan HPLC mempamerkan kehadiran asid cryptochlorogenic, quercetin 3-β-D-glucoside dan kaempferol-3-O-glucoside dalam ekstrak dan pecahan aktif. Ekstrak akueus-etanolik (v/v) daun *Moringa oleifera* juga menurunkan secara signifikan glukosa darah berpuase, berat hati, kerintangan insulin, BMI, lemak abdominal, plasma MCP-1 dan penambahan berat badan dalam tikus bersindrom metabolik. Dapatan kajian menunjukkan bahawa ekstrak etanolik 95% daun *Moringa oleifera* mempamerkan aktiviti anti-hiperglisemik melalui perencatan glukosa usus, fosforilasi glukosa, pengambilan glukosa oleh otot skeletal dan kesan pelindungan melalui pengurangan MCP-1. Tambahan pula, hasil daripada kajian sindrom metabolik menunjukkan yang sensitiviti terhadap insulin diperbaiki. Kesan-kesan ini boleh dianggap disebabkan oleh asid cryptochlorogenic, quercetin 3-β-D-glucoside dan kaempferol-3-O-glucoside.

EFFECTS OF AQUEOUS-ETHANOLIC EXTRACTS AND FRACTIONS OF Moringa oleifera Lam. LEAF ON DIABETIC AND METABOLIC SYNDROME RATS

ABSTRACT

Recent advancements in isolation and identification of active compounds present in medicinal plants have culminated in a fractionation-guided targeted approach in search of bioactivities. Thus, the present research was designed to determine the most active extract, active fraction and to identify active principle from *Moringa oleifera* leaf aqueous-ethanol extracts in diabetic rats. In addition, these extracts were also assessed in metabolic syndrome rats. Five different aqueous ethanol extracts (95, 75, 50, 25% [v/v] ethanol and 100% water) were orally tested in normoglycemic Sprague Dawley rats for anti-hyperglycemic activity and glucose tolerance. Rats with streptozotocin-induced diabetes were used to assess acute and sub-chronic anti-hyperglycaemic activities. The most active extract (95% ethanol) was further fractionated into different polarity solvents of hexane, chloroform, ethyl acetate, butanol and aqueous fractions and these fractions were screened for anti-diabetic activity in diabetic rats. The most active extract and fraction were then subjected to qualitative and quantitative phytochemical analyses; thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used in order to identify anti-diabetic compounds. Among all extracts and fractions, 95% ethanol extract at the dose of 1000 mg/kg and butanol fraction at the dose of 500 mg/kg were found to be most active, showing significant reduction in blood glucose concentration upon single administration in diabetic rats. There was no

significant anti-hyperglycemic activity, and glucose tolerance was observed in normal rats. The sub-chronic dose response studies exhibited highly significant fall in blood glucose at 500 and 1,000 mg/kg doses only. The treated groups also exhibited significant reduction in total cholesterol, triglycerides, low-density lipoprotein and plasma MCP-1. The *in vitro* experiments on α-amylase and α-glucosidase inhibition suggested non-competitive and mixed mode intestinal glucose inhibition respectively and significant glucose uptake by skeletal muscle. TLC and HPLC analyses identified constituents of cryptochlorogenic acid, quercetin 3-β-D-glucoside, and kaempferol-3-O-glucoside in the active extract and fraction. *M. oleifera* aqueous-ethanol (v/v) extracts significantly decreased fasting blood glucose, liver weight, insulin resistance, body mass index, abdominal fat, plasma MCP-1 and body weight gain in metabolic syndrome rats as well. This research findings indicated that *M. oleifera* 95% ethanol extract exhibited anti-hyperglycemic activity through intestinal glucose inhibition, phosphorylation of glucose, glucose uptake by skeletal muscle and protective effect through reduction of MCP-1. Moreover, the results of metabolic syndrome studies indicated that the 95% ethanol (v/v) extract also improved insulin sensitivity. Anti-diabetic and metabolic syndrome effects might be attributed to the compounds cryptochlorogenic acid, quercetin 3-β-D-glucoside and kaempferol 3-O-glucoside.

CHAPTER 1

INTRODUCTION

1.1 Drug development

Drug development is a long-term process, which requires huge financial resources. The pharmaceutical industries generally spend up to 15 years for a drug to be developed, tested, approved by the government authorities, and finally marketed (Principe, 1991). Although, they adopt time saving strategy, structurally modifying an existing drug instead of screening a new drug molecule. Drugs of plant origins are still used e.g. morphine, codeine, atropine, digoxin, digitoxin, vincristine, vinblastine, hyoscyamine, hyoscine, pilocarpine, forskolin and codeine. The natural sources are used especially when the cost of synthetic drug is exorbitant. Therefore, plant materials are still essential raw materials for certain medications (Kadir, 1998; Handa, 2005) and for the preparation of traditional medicine extracts.

Natural products and their derivatives represent more than 50% of all the drugs in modern therapeutics. Over the past few decades, researchers have focused on drug discovery from botanical sources; the success rate of developing a new drug from herbs might be higher than that from chemical synthesis (Pan *et al.,* 2013). Currently, the focus has certainly changed to understand the benefits and risks of commonly used local and traditional plants with the aim of contributing to a better and safer uses of such resources (Heinrich, 2006; Heinrich *et al.,* 2009).

1.2 Advancements in phyto-pharmacological studies

The recent advancements in phytochemistry and pharmacological studies in terms of isolation and characterization have contributed a lot to the knowledge about new therapeutically active compounds obtained from natural products (Filho & Yunes, 1998). These compounds can be used directly as leads for the development of new drugs or as pharmacological tools to discover new active compounds that can improve the quality of life in chronic diseases (Kaplan & Gottlieb, 1990; Elisabetsky & Costa-Campos, 1996). However, it is important to identify active compounds with desired biological activity and to determine its correct use regarding dose and route of administration (Secco, 1990).

It is widely accepted that more than 80% of drug substances are either directly derived from natural products (Maridass & de Britto, 2008) or isolated from herbs/plants for determination of mechanism of action (Krief *et al.*, 2004).

1.3 Prevalence of diabetes mellitus

Diabetes mellitus has now become an epidemic, having worldwide incidence of 5% in general population (Sangeeta *et al.*, 2010). According to Shaw *et al.* (2010), there is a 69% increase in numbers of adults with diabetes in developing countries and a 20% increase in developed countries. Not recent any more, the International Diabetes Federation (IDF) estimated that in 2011 there were 366 million people with diabetes and this was expected to rise to 552 million by 2030 (Whiting *et al.*, 2011). According to World Health Organization (WHO), the prevalence of diabetes is now likely to increase by 35% and it is expected to be 592 million in the year 2035, with the greatest increase expected in low and middle-income developing countries [International

diabetes federation atlas (IDFA), 2013]. According to the Malaysian National morbidity survey III, the overall prevalence of diabetes mellitus in Malaysia was 11.6% in 2006 (Letchuman *et al.,* 2010), but has now increased by two fold among Malaysians aged ≥ 30 years to 22.6%. The prevalence amongst Indians and Malays were 37.9 % and 23.8% respectively (Nazaimoon *et al.,* 2013). According to the International Diabetes Institute, Malaysia has the 4th highest prevalence of diabetics and the most over-weight and obese people in Asia. 54% of the adult population is either obese or overweight. As a result, 7 out of 10 Malaysian adults suffer from chronic diseases (Ujang, 2013).

1.4 Metabolic syndrome

Metabolic syndrome is associated with obesity, insulin resistance, hyperinsulinemia, hyperlipidaemia, hyperglycemia, hypertension, fatty liver and impaired energy metabolism. Progression may provoke diabetic and cardiovascular complications such as congestive heart failure, ventricular hypertrophy. Recently, it has acquired more attention worldwide by the physicians and researchers due to its morbidity and mortality factors. Metabolic syndrome can be developed in rodents through high-fat diet and carbohydrate consumption (Angelova & Boyadjiev, 2013; Mamikutty *et al.,* 2014; Grundy *et al.,* 2004). Among carbohydrate, usually D-fructose is preferred as its high plasma level is unable to stimulate the release of insulin from the β-cells of pancreas (Bray *et al.,* 2004). Now-a-days, traditional medicines have gained wide acceptability among general population due to its ease of excess and cost effectivenss (Gurrola-Diaz *et al.,* 2010).

1.5 Diabetes mellitus

Diabetes mellitus is a heterogeneous assembly of syndromes, with persistent high blood glucose level due to change in various metabolic processes like glycolysis, Krebs cycle, gluconeogenesis, hexose monophosphate shunt pathway, glycogenesis and glycogenolysis, cholesterol synthesis, synthesis and release of insulin (Prabhakar & Doble, 2008).

According to WHO, it is a chronic metabolic disorder characterized by common features of chronic hyperglycemia with alteration in carbohydrate, fat and protein metabolism (Mohan, 2005).

1.5.1 Classification of diabetes mellitus

The first real attempt to classify diabetes into (i) asymptomatic diabetes and (ii) clinical diabetes was done in 1964 by World Health Organization Expert Committee. Later, (i) juvenile diabetes, (ii) brittle diabetes, (iii) insulin resistant diabetes, (iv) gestational diabetes, (v) pancreatic diabetes and (vi) endocrine diabetes were introduced. The big breakthrough came, when The National Diabetes Data Group (NDDG) in the USA and second report of the WHO Expert Committee on diabetes mellitus presented a classification that was accepted worldwide (NDDG, 1979; WHO, 1980). Two main classifications were suggested: Type-I, Insulin dependent diabetes mellitus (IDDM) and type-II, non-insulin dependent diabetes mellitus (NIDDM). The specific types for type-II include genetic defects of β-cell function, genetic defects of insulin action, diseases of exocrine pancreas, endocrinopathies, drug/chemical induced and gestational hyperglycemia (WHO, 1985).

1.5.2 Type I diabetes mellitus

Insulin-dependent diabetes mellitus (IDDM) is characterized by the absence or loss of β-cells from the pancreas and demands daily injections of insulin to prevent diabetic ketoacidosis, coma, and death. It may be due to exogenous chemicals from the environment or diet and viral infections (Ilarde & Tuck, 1994).

This can also be due to autoimmune destruction of the β-cells that leads to insulin deficiency and results in resistance to insulin action. Deficiency of insulin on target tissues is responsible for abnormalities in carbohydrate, fat and protein metabolism (Genuth *et al.,* 2003; American Diabetes Association [ADA], 2009).

1.5.3 Type II Diabetes Mellitus

Non-insulin-dependent diabetes mellitus (NIDDM) or maturity-onset diabetes mellitus occurs largely in older people, e.g. 16.8 % of persons over 65 years of age in the United States have NIDDM, and it is often associated with obesity (Ilarde & Tuck, 1994). It represents a diabetic state in which the β-cells are usually low in number relative to α-cells and insulin secretion is usually insufficient to prevent hyperglycemia. The basal rate of hepatic glucose production is elevated and glucagon secretion is increased. Moreover, the efficiency of glucose uptake by the peripheral tissues is also impaired (Porte & Kahn, 1991). The impairment of pancreatic β-cell functions are the dependent risk factors while aging, obesity, insufficient energy consumption, alcohol drinking, smoking, etc are independent risk factors of type-II diabetes mellitus (Ozougwu *et al.,* 2013).

In the early stages, type-II diabetes mellitus is associated with chronic hyperglycemia, hyperinsulinemia and insulin resistance. Insulin resistance is due to loss of tissue sensitivity to insulin and compensatory secretion of the hormone by the β cells. There are the causes of insulin resistance:

1. Obesity
2. Excess glucocorticoids e.g Cushing's syndrome
3. Excess growth hormone e.g acromegaly
4. Pregnancy (gestational diabetes)
5. Polycystic ovary
6. Lipodystrophy (lipid accumulation in liver)
7. Autoantibodies to the insulin receptor
8. Mutations of insulin receptor
9. Mutations of the peroxisome proliferators' activator receptor γ (PPAR γ)
10. Mutations responsible for genetic obesity
11. Hemochromatosis (iron accumulation) (Guyton & Hall, 2006)

Glucotoxicity is also the commonly involved mechanisms for insulin resistance (Robertson *et al.*, 2004). It arises from excessive glucose uptake by the β cells and production of reactive oxygen species (ROS) in the mitochondrial electron transport chain (Kaneto *et al.*, 2007). This results in the stimulation of binding to insulin promoter and insulin gene transcription (Robertson & Harmon, 2006).

1.6 Glucose homeostasis

Glucose homeostasis reflects a balance between glucose production and its utilization. Physiologically, this balance is maintained by the level of circulating insulin and tissue responsiveness to it (Gerich, 2000). This is also the result of a balance between glucose production by the liver, its storage as glycogen and its uptake by peripheral tissues like skeletal muscle and adipose tissue. Insulin is a hormone secreted by the β-cells of pancreas (Fig. 1.1).

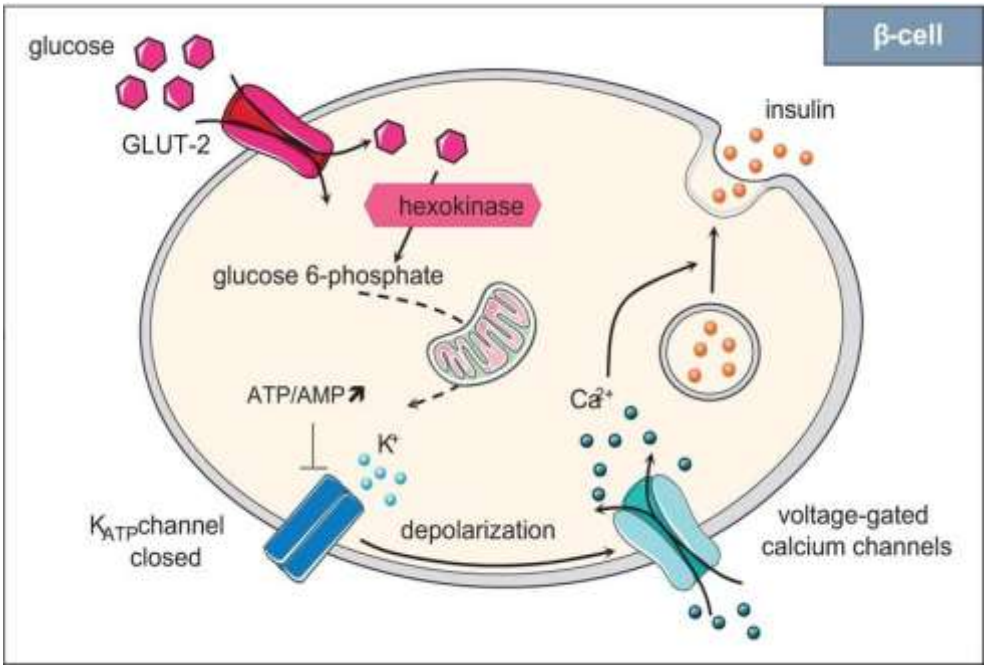

Fig. 1.1 Schematic diagram of insulin release from pancreatic β-cell (adapted from Fournel et al., 2016)

In normal state, it decreases glucose production in the liver, stimulates its uptake by skeletal muscle and peripheral tissues, enhances its storage as glycogen in the liver and muscles as shown in Fig. 1.2 (Saltiel, 1996) while in case of obesity, the extra glucose is converted to triglycerides and stored as lipid droplets in adipocytes.

Therefore, insulin deficiency combined with insulin resistance; contribute to a state of hyperglycemia (Gonz´ alez *et al.*, 2006; Dashty, 2013). As the blood glucose level rises, the osmotic pressure increases the blood volume and urine output. The glucose starts appearing in the urine when the level exceeds its renal threshold (180 mg/dl). This result in loss of water from the body and patient feels thirsty (International Diabetes Federation, 2007). Other signs and symptoms include polydipsia, weight loss, polyphagia, blurred vision, tachycardia and hypotension (Rang *et al.*, 2012). Uncontrolled diabetes causes ketoacidosis and nonketotic hyperglycemic coma (Tripathi, 2008).

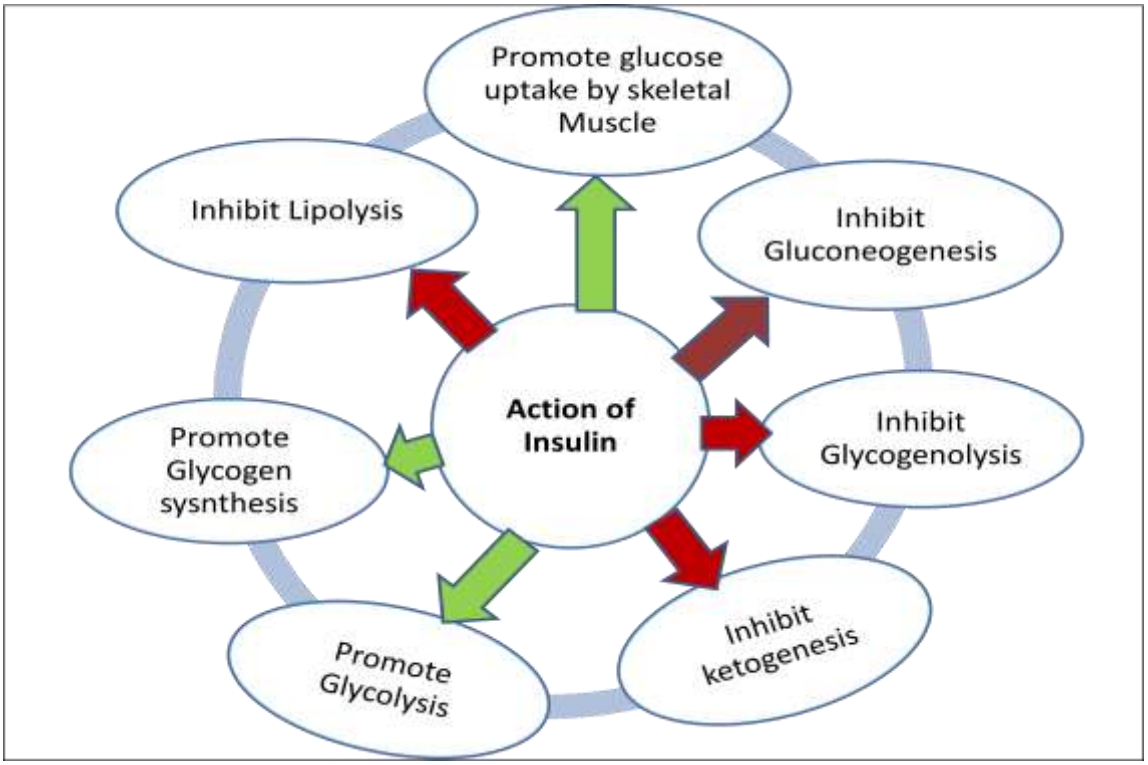

Fig. 1.2 Effects of insulin on carbohydrate metabolism

The chronic hyperglycemia is also linked with long-term damage or failure of various organs like the eyes, kidneys, nerves, heart, and blood vessels. During this

asymptomatic period, it is possible to measure plasma glucose in the fasting state or after oral glucose load. (ADA, 2009).

- 2-h postload glucose <140 mg/dL (7.8 mmol/L) = normal glucose tolerance
- 2-h postload glucose 140–199 mg/dL (7.8–11.1 mmol/L) = IGT (impaired glucose tolerance)
- 2-h postload glucose ≥200 mg/dL (11.1 mmol/L) = provisional diagnosis of diabetes

1.6.1 Carbohydrate metabolism by liver

Gluconeogenesis is the major metabolic pathway through which the liver synthesizes glucose from lactate, glycerol, amino acids and pyruvate. It is mainly activated after a fast in normal individuals and in diabetic patients (Watford, 2005), and is controlled by three major enzymes namely, phosphoenol pyruvate carboxykinase (PEPCK), fructose-1,6-biphosphatase, and glucose-6-phosphatase (G6Pase). Insulin normally reduces the activity of these enzymes to help in normalizing blood glucose (Puigserver *et al.,* 2003).

Glucocorticoid excess also promotes stimulation of gluconeogenesis in the liver as well as an inhibition of insulin sensitivity both in the liver and in the skeletal muscles. This is probably responsible from an impairment of glucose tolerance to an overt diabetes mellitus in susceptible patients with Cushing's syndrome (Pivonello *et al.,* 2010).

Glycogenesis is the storage of glycogen in both the liver and skeletal muscle that is catalyzed by glycogen synthase (GS). This enzyme is regulated by several

transcriptional factors and kinases, the most important one is the glycogen synthase kinase-3 (GSK-3). It is expressed in many diseases such as diabetes, cancer, inflammation, Alzheimer (Martinez *et al.,* 2002) and phosphorylates/inhibits GS, thus decreases glycogen synthesis in liver and muscles (Wang & Roach, 1993).

1.6.2 Carbohydrate metabolism in intestine

Dietary carbohydrates are composed of hexoses such as sucrose, lactose, galactose, maltose and pentoses such as xylose and arabinose (Rosensweig & Herman, 1968). The salivary α-amylase starts digestion of food in the mouth, hydrolyses α-1, 4 linkage of starch, and converts it to maltose. The pancreatic amylase digests 60% of starches. Moreover, intestinal epithelial cellular enzymes (α-glucosidases) cause degradation of lactose, sucrose (Mac *et al.,* 2008) and maltose to two glucose molecules (Franco *et al.,* 2002; Notkins, 2002). The neuronal signals, enterohormones like incretins, meal composition and the intestinal normal flora are also involved in glucose absorption. The 5C-carbohydrates such as xylulose, arabinose, ribose and ribulose easily diffuse into the intestinal absorptive cells and do not need degradation. Absorption of the 6C-carbohydrates from intestinal epithelium occurs through passive and active transport systems. In the passive diffusion, phosphorylation of carbohydrate results in facilitated transfer to the circulation while in the active diffusion, it uses sodium/potassium (Na^+/K^+) pump and against the gradient enter into enterocytes together with Na^+ ion (Stevens *et al.,* 1984).

The management of diabetes can be achieved by reducing post-prandial hyperglycemia by delaying the activities of α-amylase and α-glucosidase, respectively (Ali *et al.,* 2006; Bhandari *et al.,* 2008).

1.6.3 Glucose transportation in skeletal muscle

Skeletal muscle is the principal site for postprandial glucose utilization and disposal and has a prominent role in energy balance (Zaid *et al.*, 2008). Insulin plays an essential role in glucose regulation by promoting the disposal of postprandial glucose into the skeletal muscle that constitutes the major portion of peripheral tissue (Goodman & Gilman's, 2006). Glucose uptake in the skeletal muscle is regulated through translocation of glucose transporter 4 (GLUT4) from the endoplasmic reticulum to the plasma membrane (Klip & Ishiki, 2005). Thus a defect in glucose transportation by transporters results in insulin resistance (Reaven, 1995). Upon binding of insulin, the insulin receptors become activated, and this leads to the phosphorylation of the insulin receptor substrates (IRS-1) and/or (IRS-2) on tyrosine residues. In skeletal muscle, IRS-1 is primarily responsible for inducing glucose uptake. It activates Phosphatidylinositol-3-kinase (PI-3-kinase), which in turn promotes the activation of downstream targets and translocation of GLUT4 from intracellular to cell surface where it facilitates the entry of glucose into the cell (Fig. 1.3). In case of type 2 diabetes mellitus, there is impairment in the insulin-stimulated translocation of GLUT4 to cell surface and thus leading to defect in insulin-stimulated glucose uptake in peripheral tissues like skeletal muscle and fat (Roach, 2005; Goodman & Gilman's, 2006; Harvey *et al.*, 2012; Tripathi, 2008).

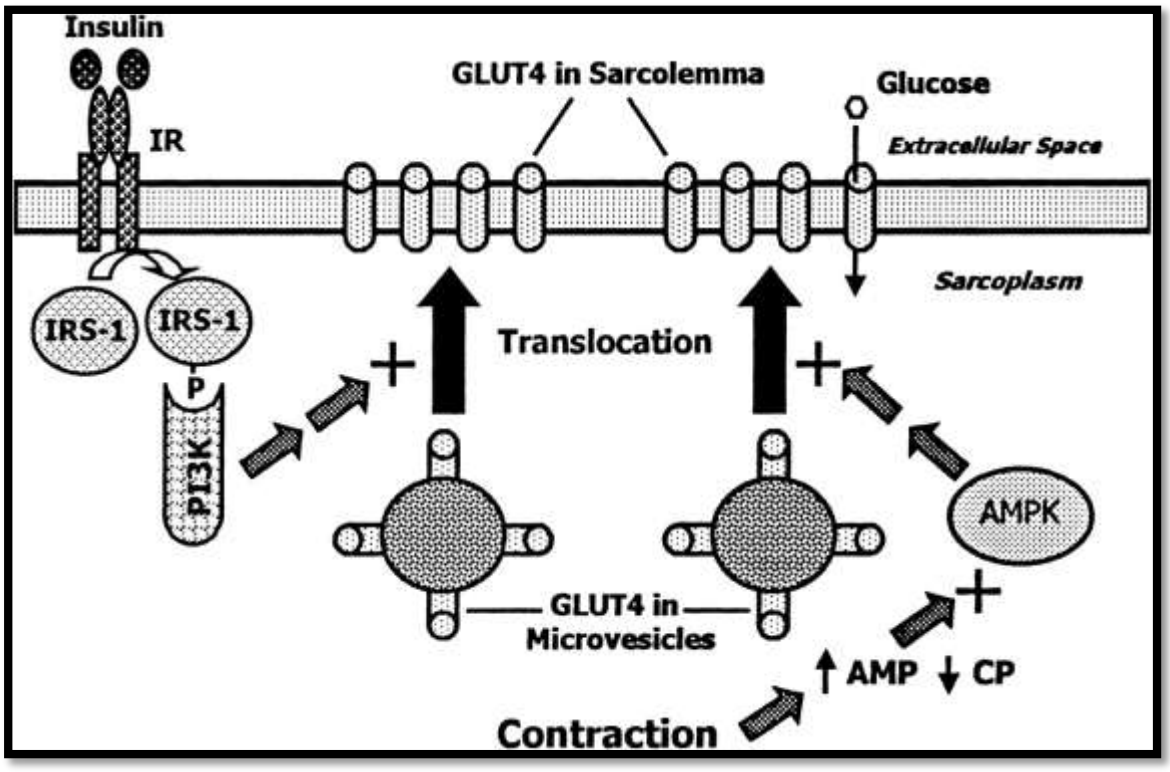

Fig. 1.3 Glucose transportation in skeletal muscle (adapted from Winder, 2001)

1.7 Hyperlipidemia and diabetes mellitus

The main forms of lipids in the body include cholesterol, phospholipids and triglycerides. They are involved in a variety of biological processes such as membrane formation, intracellular and intercellular signaling, as well as energy storage and production. The body derives its lipids from cellular biosynthesis and from nutrition. Cellular biosynthesis of lipids is regulated at the transcriptional level by sterol-regulated element-binding proteins-1 & 2 that promote the biosynthesis of fatty acids, triglycerides and cholesterol (Horton, 2002).

Intestinal and plasma lipids are transported by lipoproteins particles. Lipoproteins vary in density and, depending on their relative contents are identified as chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL),

intermediate density lipoprotein (IDL) and high-density lipoprotein (HDL) (Havel & Kane, 2001). Excess circulating free fatty acids deposited in the muscle and liver tissues of obese individuals lead to an increase in the level of intracellular triglycerides. These triglycerides and products of fatty acid metabolism are potent inhibitors of insulin signaling and result in an acquired insulin resistance state. A decrease in the activity of key insulin signaling mediates the lipotoxic effects of free fatty acids (Shulman, 2000). Overall, 30-40% of patients with diabetes have triglyceride levels > 200 mg/dL, and 10% have triglycerides > 400 mg/dL (Cowie & Harris, 1995). Anti-diabetic drug (metformin) are known to reduce VLDL through activation of adenosine monophosphate protein kinase (AMPK) and reduction of sterol regulatory element binding protein as shown in Fig. 1.4 (Phielix *et al.*, 2011).

1.8 Geriatric patients and diabetes mellitus

The prevalence of diabetes increases with age and up to 25-30% of elderly people suffer from this condition and 10-25% have impaired glucose tolerance (Valle *et al.*, 1997). Older adult's diabetic patients vary in duration of diabetes, degree of hyperglycemia, frequency of diabetes-related complications and disease burden (Blaum *et al.*, 2010). They are typically at greater risk for development of microvascular complications e.g. diabetic retinopathy, nephropathy, and neuropathy (Booth *et al.*, 2006), premature death, functional disability, and co-existing illnesses such as hypertension, coronary heart disease (CHD), and stroke (Schwartz *et al.*, 2002; Songer, 1995). It has been estimated that the number of people over 65 with diabetes are expected to increase by 4.5-fold between 2005 and 2050 (Narayan *et al.*, 2006). The elevated rate of impaired glucose metabolism may be due to a complex group of risk

factors that contribute to increasing insulin resistance with aging and impaired β-cells function (Halter, 2011). Modification in lifestyle can slow down age-related hyperglycemia by reducing the demand on functional β-cells. However, loss of β-cells function shifts the patient towards insulin therapy (Halter & Merritt-Hackel, 2011). Beside this, the use of oral hypoglycemic agents can compensate for worsening pancreatic islet function (Lee *et al.*, 2012).

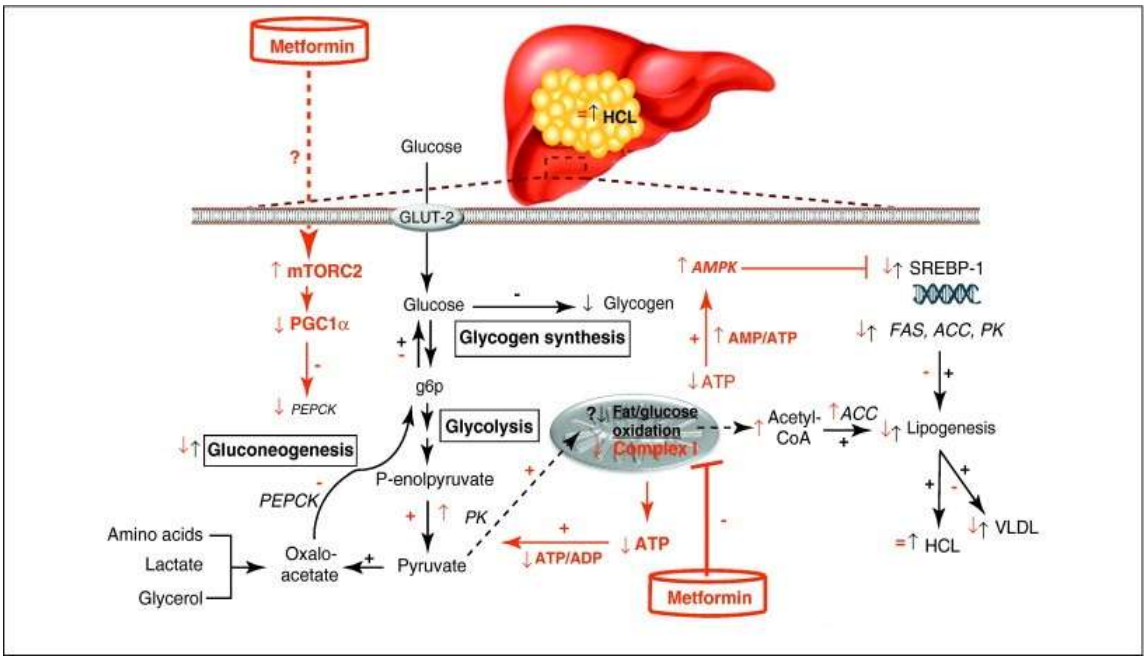

Fig. 1.4 Scheme diagram showing effects of metformin in liver (Adopted from Phielix *et al.*, 2011)

Key: G6P=Glucose-6-phosphate, FAS=Fatty acyl-CoA synthetase, ACC=Acetyl-CoA carboxylase, mTORC2=Mammalian target of rapamycin2, SREBP=Sterol regulatory element binding protein, PEPCK=Phosphoenol pyruvate carboxykinase, PK=Pyruvate kinase.

The management of diabetes can also be achieved by reducing post-prandial hyperglycemia by delaying the activities of α-amylase and α-glucosidase enzymes which are responsible for the digestion of carbohydrates and absorption of glucose in the digestive tract, respectively (Ali *et al.,* 2006; Bhandari *et al.,* 2008).

1.9 Monocyte chemoattractant protein-1 and diabetes mellitus

Chemokines constitute a family of chemoattractant cytokines that play a major role in migrating monocytes, neutrophils, and lymphocytes, as well as in inducing chemotaxis through the activation of G-protein-coupled receptors towards site of injury. However, chemokines transmit cell signals that generate multiple responses. Specifically, alteration of plasma monocyte chemoattractant protein-1 (MCP-1) concentration in metabolic disorders and the presence of circulating chemokines reservoirs. MCP-1 is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages (Rull *et al.*, 2010; Satish *et al.***,** 2009). MCP-1 has been extensively studied in the etiologies of obesity and diabetes-related diseases. (Panee, 2012). Pancreatic β-cells are selectively destroyed during the course of type 1 diabetes (Fig. 1.5). In the early stages of the disease, inflammatory infiltrates of mononuclear cells, containing predominantly monocytes and T-cells, are present in the islets (insulitis). Chemokines, such as MCP-1, play a key role in the recruitment and activation of these immunocytes (Kutlu *et al.,* 2003).

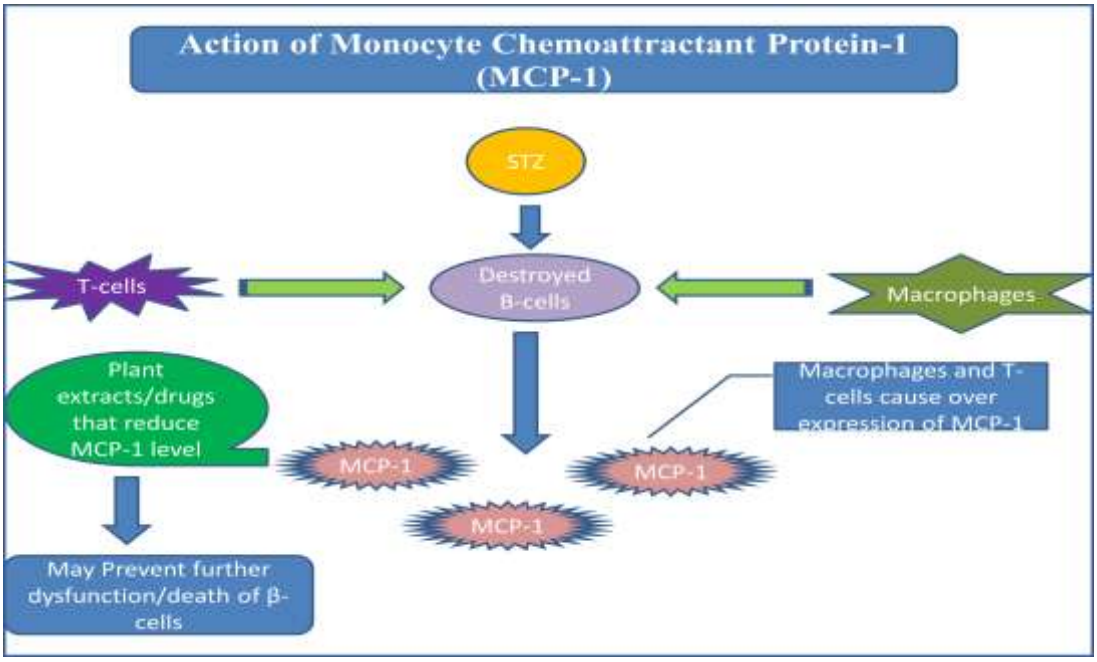

Fig. 1.5 Monocyte chemoattractant protein-1 and its role in diabetes mellitus

1.10 Chemical induction of diabetes mellitus

Gold thioglucose (GTG), alloxan/ streptozotocin are the chemical compounds used to induce diabetes in laboratory animals, which can be either type-I or type-II depends upon animal species and route of administration (Etuk, & Muhammed, 2010). GTG is not recommended, as 16-20 weeks are required to induce hyperglycemia, hyperphagia, obesity and insulin resistance (Le Marchand Brustel, 1999). The alloxan exhibits severe hyperglycaemia, glucosuria, hyperlipidaemia, polyphagia, polydypsia and diabetic complications. The major disadvantages of alloxan are the fluctuation of the glucose values among different doses, incidence of ketosis and reversal of hyperglycaemic state due to pancreatic regeneration. For those reasons, it is now replaced with streptozotocin for induction of diabetes (Battell *et al.*, 1999; Srinivasan & Ramarao, 2007).

Streptozotocin (STZ) is produced by *Streptomyces achromogenes* that has antibacterial and diabetogenic properties. Streptozotocin substantially damages predominantly the pancreatic β-cells due to the presence of glucose moiety in its molecule. The glucose transporter protein 2 (GLUT2) in the plasma membrane of β-cells has higher affinity for streptozotocin to transport it into the cell from plasma as compared to other cells (Elsner *et al.,* 2007), causing the β-cells to be more susceptible to damage by the drug. STZ major cytotoxic mechanism is DNA alkylation and strand breakage in both bacterial and mammalian cells (Fig. 1.6) but mammalian β-cells are less affected due to lower expression of GLUT2 transporter proteins (Eleazu *et al.,* 2013). Physico-chemical properties indicate that it is N-acetyl glucosamine derivative and crystalline powder, which is very soluble in water (Ventura-Sobrevilla *et al.,* 2011). It is stable at pH 7.4 at 37°C up to 1 hour. After reconstitution, it should be protected from light and can be stored at room temperature up to 12 h but suggested to be used within 10 min (Povoski *et al.,* 1993; Sharma *et al.,* 2010; Joo *et al.,* 2010).

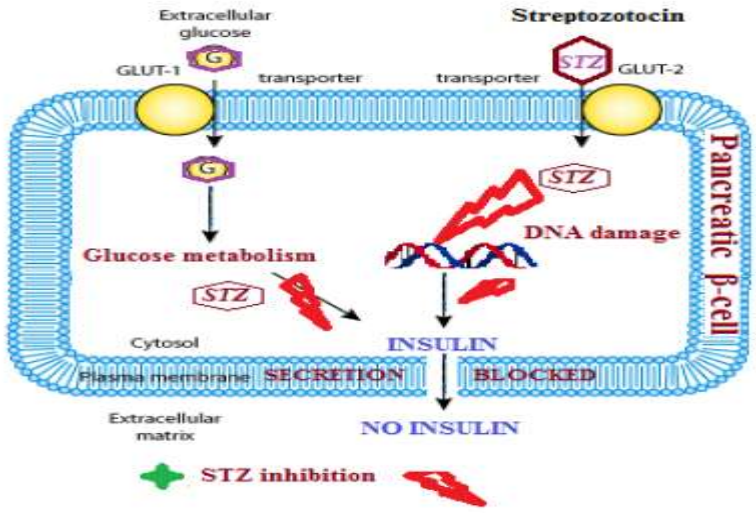

Fig. 1.6 Pancreatic β-cell death by streptozotocin (Adopted from Goud *et al.,* 2015)

After streptozotocin administration, there is a state of transient hyperglycemia due to sudden breakdown of liver glycogen, followed by hypoglycemia that may be lethal, succeeded finally by permanent hyperglycemia that ensures at about 10 to 12 hour after STZ administration (Joo *et al.,* 2010).

1.11 Anti-diabetic drugs and their mechanisms of action

Anti-diabetic drugs (Fig. 1.7) are prescribed to type II diabetic patients that fulfill the following criteria (Timby & Smith, 2013)

1. Fasting blood glucose level less than 200 mg/dL
2. Insulin requirement of less than 40 units/day
3. No ketoacidosis
4. No renal or hepatic disease

1.11.1 Sulfonylureas

Sulfonylureas e.g. glibenclamide act primarily by increasing insulin secretion and decreasing the secretion of glucagon. The release of insulin from pancreatic beta cells takes place through inhibition of ATP sensitive potassium channels in the plasma membrane. When sulfonylurea drug binds to its receptors, it closes the potassium channels that lead to the β-cell depolarization, influx of calcium and release of insulin from the β-cells. The glucagon secretion decreases from alpha cells because of insulin release and pancreatic somatostatin secretion. These also increase insulin sensitivity and gain in body weight. The most common adverse effects are hypoglycemia, leucopenia, thrombocytopenia and skin rashes (Brenner & Stevens, 2012).

1.11.2 Biguanides

Metformin a biguanide exerts its effects primarily by decreasing hepatic glucose output mainly through inhibition of gluconeogenesis. It does not increase plasma lactic acid levels in healthy people or patients with normal renal functions. The most common reported adverse reaction to metformin therapy is gastrointestinal upset e.g. nausea, vomiting, anorexia and diarrhea, metallic taste and weight loss. It is generally started in low doses 500-850 mg and maximum up to 2,550 mg daily with meal (Bailey & Turner, 1996; DeFronzo & Goodman, 1995).

1.11.3 Thiazolidinediones

Peroxisome proliferator activated receptors (PPARs) are located throughout the body in many different tissues, especially in adipose tissues and present mainly as PPARα, PPARδ, and PPARγ forms. The PPARγ is the major target site for thiazolidinediones (Nathan *et al.*, 2006). These drugs bind with PPARs in the cells and result in the transcription of genes, thus stimulate the production of proteins (adiponectin) that promote insulin sensitivity and block transcription of insulin resistance or inflammatory proteins (Yki-Jarvinen, 2004; Willson *et al.*, 2001; Chinetti *et al.*, 2000). These drugs can produce hepatotoxicity. Therefore, patients should undergo hepatic function tests before initiation of these medications and followed by regular monitoring when on this medication. Thiazolidinediones also cause bone weakness (Schwartz *et al.*, 2006).

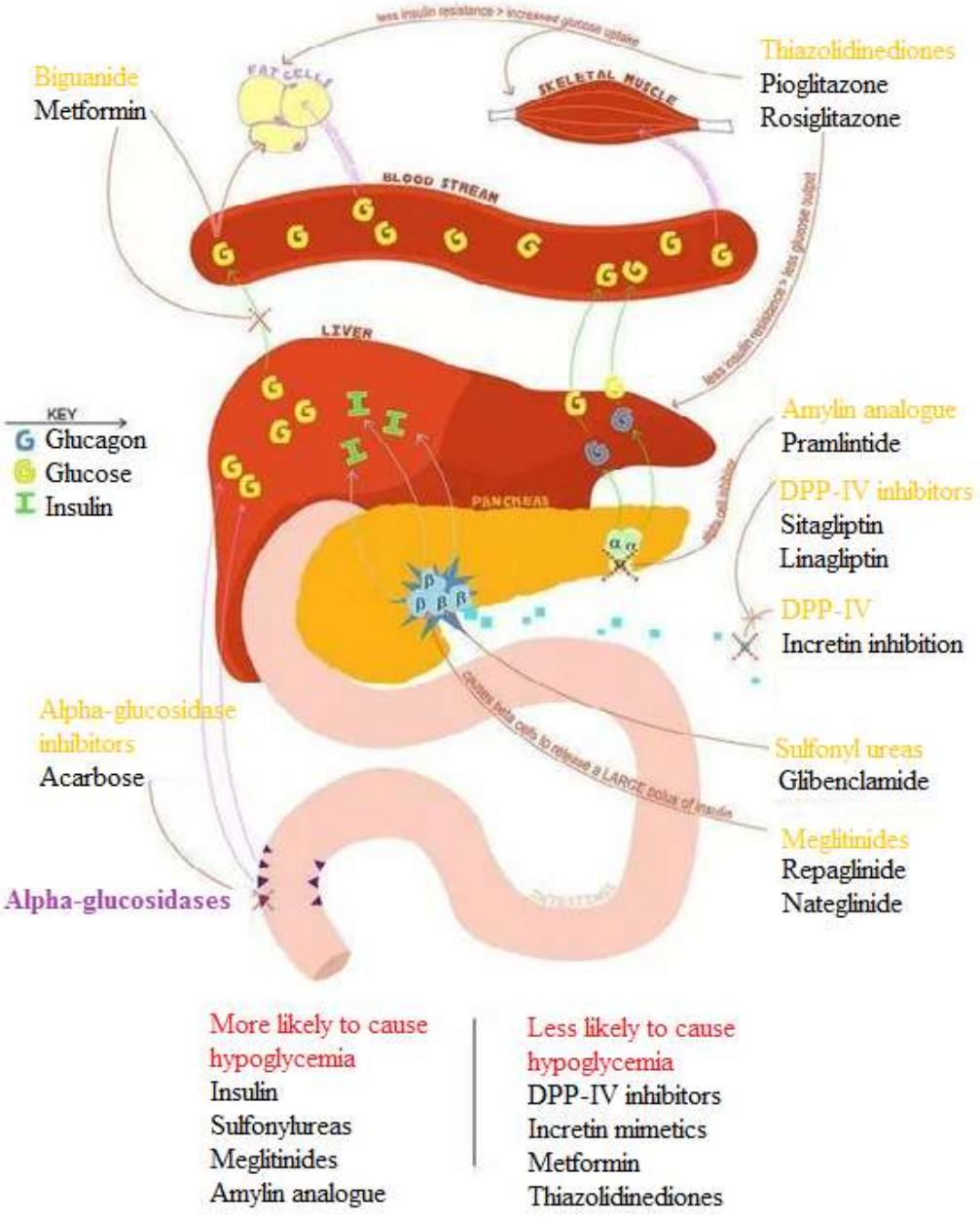

Fig. 1.7 Schematic diagram of mechanisms of action of anti-diabetic drugs (adapted from Pinterest. The World's catalog of ideas. Pharmacology)

1.11.4 α-glucosidase inhibitors

α-glucosidase inhibitors (e.g. acarbose) are competitive inhibitors of intestinal α-glucosidases that hydrolyze oligosaccharides, trisaccharides and disaccharides to glucose and other monosaccharides in the small intestine and thus delay postprandial glucose absorption (Van de Laar, 2008). These agents are available as a first-line treatment in patients with slightly increased fasting blood glucose and marked postprandial hyperglycemia (Krentz & Bailey, 2005). Derosa *et al.* (2009) demonstrated that both repaglinide and acarbose had a similar effect on reducing postprandial glucose levels. Drugs must be taken 15 min before meal and effective in postprandial hyperglycemia. The possible adverse effects include abdominal pain, flatulence, diarrhea and hypoglycemia (Timby & Smith, 2013).

1.11.5 Meglitinides

Meglitinides includes repaglinide and nateglinide that stimulate insulin secretion from β–cells (Black *et al.*, 2007). Repaglinide is the first therapeutically available insulin nonsulfonylureas secretagog that specifically enhances early-phase prandial insulin response by increasing the sensitivity of β-cells under hyperglycemic conditions (Raskin, 2008; Johansen & Birkeland, 2007). The in *vitro* studies indicate that repaglinide is five-times more potent than glibenclamide a sulfonylureas in stimulating insulin secretion. Experimentally, it was observed that repaglinide promotes insulin release from β-cells only in the presence of glucose whereas glibenclamide can stimulate insulin secretion in the absence of glucose also (Fuhlendorff *et al.*, 1998).

1.11.6 Dipeptidyl peptidase inhibitors

Dipeptidyl peptidase-IV (DPP-IV) inhibitors act by inhibiting the enzymatic degradation of glucagon-like peptide 1 (GLP-1). It is an incretin hormone produced by the distal small intestine and released into the bloodstream. This hormone delay gastric emptying, suppresses glucagon release and stimulates insulin release, thus limiting postprandial hyperglycemia (Ahren *et al.*, 2003). Sitagliptin, linagliptin, saxagliptin require only once daily dosing. These drugs are well tolerated and do not cause hypoglycemia and gastrointestinal side effects (Brenner & Stevens, 2012).

Exenatide is a short acting glucagon-like peptide-1 (GLP-1) analogue (Boon *et al.*, 2006) that enhances glucose-dependent insulin secretion (mimic the effects of gut incretin). It has similar efficacy to bedtime insulin when added to failing oral regimen, but considerably more expensive (Irwin & Rippe, 2009; Katzung, 2007). Glucagon-like peptide-2 (GLP-2) is also an intestinal secretion which manages intestinal glucose transport (Baggio & Drucker, 2004).

1.11.7 Amylin analogue (pramlintide)

Amylin (Pramlintide) is a peptide co-secreted with insulin from β-cells. It suppresses postprandial glucagon secretion and slows gastric emptying and thus retards digestion and absorption. The glycosylated haemoglobin level decreases on average by 0.1 or to 0.6 mg/dL. If used in combination with insulin, doses should be decreased by half or more to avoid the risk of hypoglycemia (Irwin & Rippe, 2009; Madison Clinic, 2009).

1.12 Diabetic patient's compliance towards herbal medicines

Many diabetic patients rely on herbal medicines for the management of their diseases particularly in under developed subcontinent (Grover *et al.,* 2002). It is estimated that about 80% of the population in African and Asian countries depend on conventional medicine for treatment (WHO, 2008) and have an ease access to take such medications (WHO, 2013). Today's plant materials have provided the models for 50% of Western drugs (Robbers & Tyler, 1996; Hammer & Carson CF Riley, 1999). The most important ones are the secondary metabolites, which include alkaloids, phenolic compounds, tannins, phytosterols and terpenoids (Bandow *et al.,* 2003).

1.13 Extraction and activity profiling of plant extracts

Extraction is the separation of medicinally active portions from crude source by using standard procedures e.g. maceration and percolation. The purpose of extraction procedures is to obtain the therapeutically desired material and to eliminate unwanted material with a selective solvent (Handa, 2005). Polar solvents are frequently employed for the recovery of polyphenols from a plant matrix. The most suitable of these solvents are hot or cold aqueous mixtures containing ethanol, methanol, acetone and ethyl acetate, to extract compounds from various natural sources (Peschel *et al.*, 2006).

The natural sources (plants, animals and micro-organisms) provide a big source for deriving active compounds. Predominantly, the plant kingdom offers a variety of species act as remedies for several diseases in many parts of the world (Duraipandiyan *et al.,* 2006; Grover *et al.,* 2002). As stated by Newman and Cragg (2010) that natural products continued to play a highly significant role in the drug discovery and development process. These novel chemicals entities (NCE) are

secondary metabolites e.g. alkaloids, terpenoids and phenolic compounds that are present in plant extracts.

An extract that obtained through extraction, may be used as such (as medicinal agent) or it can be further processed to isolate individual chemical compounds. The basic parameters that influence the quality of an extract are the plant parts used as starting material, the solvent used for extraction and the extraction process. However, it is not always an easy, to select extracts from primary screens that are likely to contain novel compounds. By comparing the types and levels of activities of different extracts, it can be possible to predict the best extract that likely contains components with the appropriate pharmacological activity (Littleton *et al.*, 2005; Handa, 2005).

1.14 *Moringa oleifera* Lam. tree

1.14.1 Locality and habitat

Moringa oleifera Lam. belongs to Moringaceae family of perennial angiosperm plants, having 12 other species from the family (Olson, 2002) with a single genus family of shrubs (Jahn, 1984; Ndabigengesere *et al.*, 1995). All Moringa species are native to Asia (India), from where they have been introduced into other warm countries, such as Malaysia and other tropical countries (Fig. 1.8). The tree can tolerate temperatures from 19 to 28 ºC (Karmakar *et al.*, 2010) and a wide range of rainfall annually from 250 mm to over 3000 mm (Palada & Changl, 2003).