1.1 Diabetes Mellitus: An Introduction

Diabetes is emerging as a major global health challenge. The disease is basically due to either insufficient production of a hormone called insulin by the body or due to insensitivity of insulin by the cells in the body that are involved in the generation, transformation and breakdown of sugar. With the progression of the disease, multiple organ systems and metabolic pathways get affected. This results in multiple disease syndromsmaking diabetes one of the most complex metabolic diseases. Diabetes is characterized by high blood glucose levels (hyperglycemia) resulting from either defects in insulin secretion from pancreatic βcells or impaired insulin activity in the peripheral insulin target cells. Depending upon the insulin responsiveness, diabetes mellitus can be classified into two types, type1 and type 2. Type 1 diabetes mellitus (T1DM) or insulin dependent diabetes mellitus (IDDM) is due to autoimmunity mediated destruction of the pancreatic βcells resulting ininsulin deficiency.On the other hand, Type 2 diabetes mellitus (T2DM) or noninsulin dependent diabetes mellitus (NIDDM) is characterized by insulin insensitivity or resistance in the insulin target cells. Skeletal muscle cells, adipocytes and hepatocytes are the major target cells for insulin activity. Both T1DM and T2DM are the responsible factors for hyperglycemia, excessive urine production, compensatory thirst, increased fluid intake, blurred vision, unexplained weight loss, lethargy, and changes in energy metabolism. The International Diabetes Federation (IDF) estimated that diabetes affected 415 million people worldwide in the year 2015, and this number is projected to reach upto to 642 million by the year 2040.In India, 69.1 million people were reported to suffer from diabetesin 2015 which will rise to 123.5 million by the year 2040[1] (Fig. 1.1).

According to the World Health Organization (WHO), T2DMhas turned into epidemic proportion affecting more than 90-95%people of all diagnosed diabetes cases around the world. On the other hand, T1DM is restricted to about 3-5% of the global population[2].

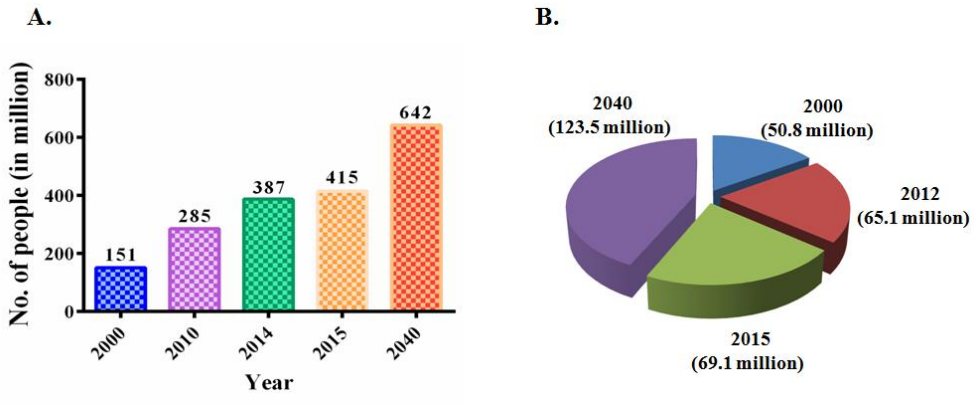

Figure1.1: **Prevalence of diabetes**. Global scenario (A) and the Indian scenario (B)

Genetic susceptibility, environmental factors and lifestyle choices are the most important factors responsible for the development of T2DM.Among these factors lifestyle plays a crucial role as 70-90% of T2DM is attributed to obesity[3,4,5].Obesity induced insulin resistance is a principal risk factor in the development of T2DM. Since this is a common pathological state where the target cells fail to respond to the normal levels of circulating insulin[6,7].

1.2 Insulin and its role in glucose homeostasis

Insulin is a peptide hormone produced in the pancreatic β cells of the islets of Langerhans whichprimarily maintains the normal blood glucose level in the body. It is a dipeptide protein containing A and B chain which are bonded by two disulfide bridges. The chain A of insulin consists of 21 amino acids and the chain B contains 30 amino acids (Fig. 1.2).

Insulin maintains the normal blood glucose level by inducing the uptake of glucose molecules into the glucose storage cells. It also plays an important role in regulating the carbohydrate, protein and lipid metabolism.

There are 3 different ways in which insulin controls the postprandial glucose level in the blood. Firstly, insulin enhances glucose uptake from the blood in the muscle tissue. Secondly, this hormone promotes glycogenesis in the liver. Thirdly, insulin inhibits glucagon secretion from the pancreatic α cells and thereby stops generation of glucose through glucogenolysis and gluconeolysis.

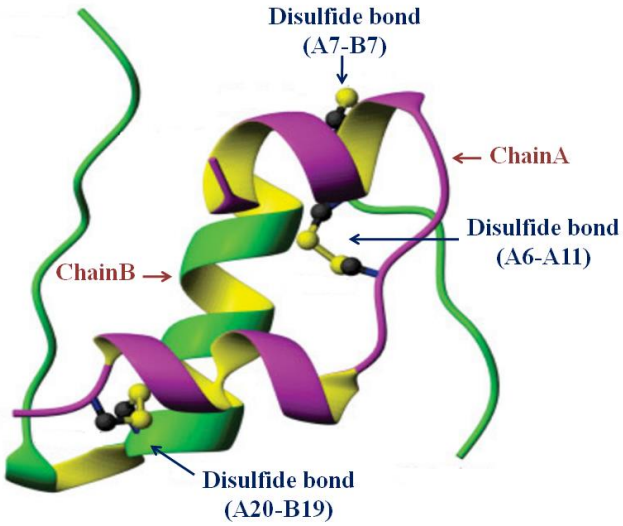

Figure 1.2: 3D structure of insulin monomer. Chain A is represented by green coloured chain and Chain B represented by purple coloured chain. The inter and intra chain disulfide bonds are shown by ball and stick representation[8].

Regulation of insulin action depends primarily on the circulatory glucose concentration. Insulin secretion occurs only when the blood glucose level exceeds more than 3.3 mM/lit. In the postprandial state, glucose stimulates synthesis and release of insulin in and from the β cells of the pancreas in two phases. Apart from glucoseplasma concentration of some amino acids like arginine and leucine are also known to stimulate insulin secretion from the β cells[9, 10,11].

1.3 Actions of Insulin

1.3.1 In carbohydrate metabolism

Insulin plays a major role in carbohydrate metabolism. Primarily, it modulates the GLUT4 translocation for the translocation of glucose molecule into the muscle and fat cells. It induces dephosphorylation of glycogen synthase and glycogen phosphorylase kinase resulting in upregulation of glycogen synthesis and downregulation of glycogen breakdown. It also enhances glycolysis and inhibits gluconeogenesis by dephosphorylating pyruvate kinase and 2, 6 bisphosphate kinase[12].

1.3.2 In lipid metabolism

Insulin plays an important role in stimulating fatty acid synthesisin the adipose tissues, liver and in the lactating mammary glands.In liver and the adipose tissue, it helps in the formation and storage of triglycerides and phospholipid metabolism[12].

1.3.3 In protein synthesis

In some tissue, insulin induces transcription of some specific mRNA as well as translation in ribosomes during protein synthesis. Enhancement of mRNA transcription of the enzyme glucokinase, fatty acid synthase by insulin has been reported. It also downregulates some of the liver enzymes.This hormone influences translation directly or through the mediation various growth factors[12].

1.4 Insulin resistance and role of free fatty acid (FFA)

Free fatty acid (FFA) is found to be one of the major players in obesity induced insulin resistance and T2DM. Several reports demonstrate that the elevation of the circulatory FFA due to the oversupply of lipid leading to the development of T2DM. The excess of lipid leads to the generation of hyperlipidemia resulting in acute insulin resistance. The signs of abnormal lipid metabolism, increased circulatory lipid concentration and elevated deposition of lipid in the skeletal muscle are seen in patients suffering from insulin resistance[13,14,15]. Though it is clear that FFA plays a key role inthe development of insulin resistance and T2DM, not much is reported on to how FFA does this. Elevated levels of FFA impairs phosphorylation of IRS1 and reduces the activation of PI3 kinase[16,17,18].This in turn downregulates the phosphorylation of AKT and thereby inhibiting glucose uptake through the glucose transporters. It is inferred therefore that insulin resistance is associated with the defects in the insulin signalling pathway. Moreover, the decrease in lipid content improves the action of insulin in skeletal muscle cells, adipocytes as well as in the liver. The chronic elevation of plasma FFA level also leads to the destruction of the β cells thereby contributing towards the development of T2DM[19].

1.4.1 Molecular mechanisms of FFA induced insulin resistance: an overview

Several pathways are involved directly or indirectly in regulating insulin signalling pathway.Impairment of any of these pathways due to the elevated level of FFA leads to insulin resistance in the insulin target cells.The molecular mechanism underlying muscle insulin resistance depends on the activity of the protein kinase Cs (PKC).The isoforms of nPKCs e.g., PKCε, PKCθ and PKCδ are found to be the major PKCs associated with FFA induced insulin resistance. FFA activatedPKCε leads to the suppressionof the insulin receptor (IR) gene expression leads to the development of insulin resistance in the muscle cells[20,21,22]. On the other hand, activated PKCθ directly induces the phosphorylation of the Ser residue of IRS1 instead of the Tyr residue and impairs the insulin signaling pathway[23]. FFA induced low grade inflammation has also been reported as one of the responsible factors for inducing severe inflammation and leads to the development of insulin resistant state in the muscle cells[24].

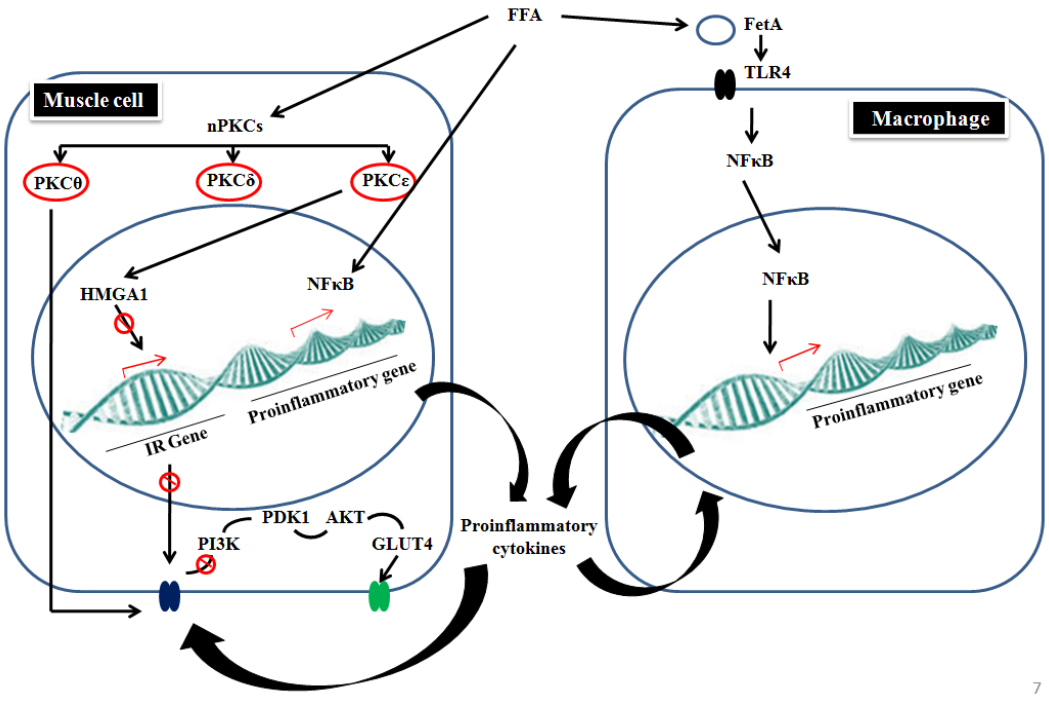

Figure 1.3: Schematic diagram represents the molecular mechanisms of FFA induced insulin resistance in muscle cells

Adipocytes and macrophages are the well known sources of inflammatory cytokines where FFA plays a crucial role resulting insulin resistance[25,26]. FFA induces the secretion of proinflammatory cytokines by activating the TLR4 pathway[27]. It has been reported, fetuin A, a liver secretory protein helps in binding of FFA to TLR4 to induce the expression of the proinflammatory genes. This results the development of severe load of proinflammatory cytokines[28]. Similar to the muscle insulin resistance, these cytokines released from adipocyte develops adipocyte insulin resistance by adipose tissue macrophage activation.

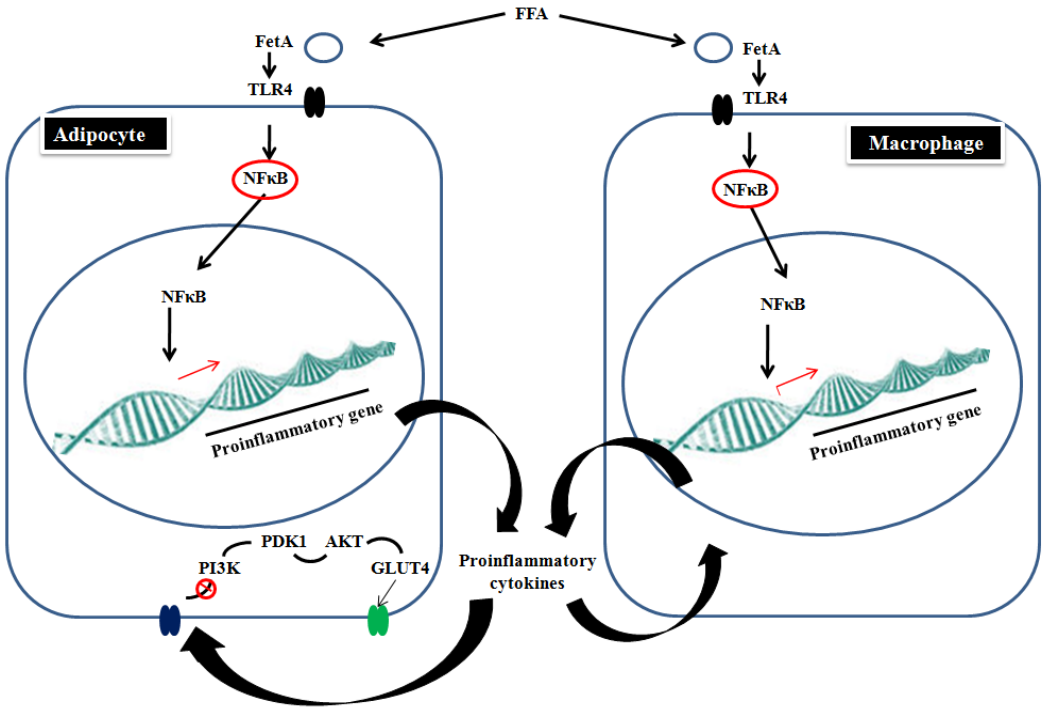

Figure 1.4: Schematic diagram represents the molecular mechanisms of FFA induced insulin resistance in adipose cells

1.5 Oxidative stress in insulin resistance and T2DM

Several studies proposed that oxidative stress plays a primary role in the development of obesity induced insulin resistance and T2DM by inhibiting the insulin signaling process[29,30,31,32,33].Oxidative stress is a physical state with higher level of the reactive species like ROS, RNS. Mitochondria being the reaction site of the electron transport chain (ETC), is thought to be the main source of ROS. In presence of molecular oxygen, leakage of electrons from the ETC leads to the formation of superoxide radical. The superoxide anions act as the mediator and

precursor of many other ROS[34]. Some ROS are beneficial for normal cellular functions regulating intracellular signalling but excessive ROS leads to the oxidative damage to the cells[35,36,37]. The detailed mechanism of how oxidative stress leads to insulin resistance is not clear. The general concept is that oxidative stress stimulates MAPKs (Mitogen-activated protein kinases) and JNK (c-Jun N-terminal kinases) and inhibits insulin signaling. In this process ROS play dual role by activating JNK through oxidation or by deactivating JNK, thereby inhibiting MAPK phosphatase. Deactivation of JNK through inhibition of Glutathione-S-transferase was also reported[38]. Oxidative stress induces the expression of several proinflammatory cytokine genes including TNF-α, IL6 leading to insulin resistance.

1.5.1 Role of antioxidant enzymes during oxidative stress

There are several enzymes involved in the body to regulate oxidative stress. Mainly, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidises (GPx) and glutathione reductase (GR) are involved in this ROS management. Briefly, SOD reduces the superoxide level in the cell by converting it intomolecular oxygen and H_2O_2. SOD is the first line of defense against superoxide radicals producedby the mitochondria during cellular respiration and by other cellular sources such as NADPH oxidases. The function of catalase is to convert H_2O_2 to molecular oxygen and water. Catalase is ubiquitously expressed in the mammaliantissues. GPx reduces peroxides (e.g. hydrogen peroxide and lipid hydroperoxides) to water and alcohol. Depending upon the tissue localization and substrate specificity, GPx can be classified into 8 different categories. Among these, GPx1 is the most abundant isoform in the mammalian tissues.Glutathione reductase (GR) is a critical antioxidant enzyme in the cellular defence system. It reduces the oxidised form of glutathione (GSSG) to sulfhydril form GSH, which is also a cellular antioxidant[39].

1.5.2 Role of free fatty acid in oxidative stress

Several studies reveal that fat accumulation (i.e., obesity) is related to the state ofoxidativestress. The plasma of the obese patients showed elevated levels of 8-OHdG and of thiobarbituric acid reactive substances (TBARS) which arethe

indication of lipid peroxidation[40,41]. In the mammalian system, consumption of fatty diet and the accumulation in adipose tissues increase the level of oxidative stress[40,41,42,43,44]. It is also reported that palmitate induced insulin resistance occurs due to stimulation of ROS as a result of increased lipid accumulation[45].

1.6 Network pharmacology in drug discovery

Multi targeting of efficient drugs has fascinated scientists and a novel concept of Network Pharmacologyis being evolved[46,47]. This concept integrates multidisciplinary concepts of biology, *viz.* Systems Biology, Biochemistry and Bioinformatics to create a molecular network[48]. These molecular networks help in unravelling the complex disease with involvement of multiple genes as well as functional proteins [49] . Thus, network pharmacology assists in systematic characterization of drug targets to reduce potential challenges of drug discovery[50]. This new approach of drug discovery helps in better understanding of disease pathogenesis using system level approach without the limitations of single target and its side effects. These advances in the development of lead molecules from the traditionally used medicinal plants and the understanding their modes of actions have proved to be very effective in drug target identification and safety assessment[51].

1.7 Traditional Knowledge in drug discovery

The medicinal uses of plants have been known to mankind since the beginning of civilization. The oldest evidences of such knowledge are found in the *Rigveda,* dating back to 4500-1600 BCE[52]. The use of plants for their medicinal properties has also been seen in the different ethnic populations around the world for a long time. The use of medicinal plants has two fold advantages: to conserve the biodiversity by maintaining the position of plants in the ecosystem; and to initiate the discovery of new drugs from the plant sources. In addition to these, it also enables an easier access to different kinds of medicines and drugs to the rural population who donot have easy access to modern medicine[53]. It has been seen that more than two third of the population in the developing countries are dependent on medicinal plants for treatment of various diseases. Easy availability of the plants, belief and acceptance among the local people sustain the use of these traditional medicines through many generations. Medicinal plants don’t just act as healing

alternatives, but also serve as a repertoire for the ecological, cultural and traditional values of the society, building an affective relationship between the society and the ecology[54]. Margaresiet, et al., 2007 also noted how the information on medicinal plants in folklores has been an important tool in the rediscovery of numerous medicinal plants[55]. Two third of the total population in the developing countries believe and use traditional medicines and other herbal plant based drugs for the initial primary treatments of several diseases. Due to the easy availability of the plant based traditional medicines and because of their acceptance among the local people, plant based traditional medicineshave been in use for generations[54]. Information on the use of medicinal plants through folklore has been an important tool in revealing numerous plants with medicinal properties[55].

1.7.1 Natural products in drug discovery

For thousands of years, natural products have been the basis of treatment and prevention of human diseases. Many of the clinically used therapeutic agents are used in their naturally occurring forms or as derivatives or analogues after structural optimization. The influence of natural products on drug discovery is well recognized and documented[56]. The use of these natural products comprising of plant and animal sources and various minerals as potent drugs are known since time immemorial[57]. Twenty-five percent of the drugs used worldwide have their origins in plants and 121 such active compounds are still in use today. Eleven percent of the 252 basic and essential drugs, as claimed by the World Health Organization (WHO), originate from plants and other natural sources[56]. Instances of such chemical compounds having plant origins are digoxin from *Digitalis* sp., quinine and quinidine from *Cinchona* spp., vincristrine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum*[58]. It has been estimated that 60% of anti-tumor and anti-infectious drugs which are being sold or clinically tested have natural origins[59]. But it must also be noted that the use of plants as an alternative source for new drugs has not been exhaustively explored yet. From the estimated 250,000–500,000 plant species, only a small percentage has been investigated phytochemically and pharmacologically. Only 5000 plant species have been studied for medical use[60]. In recent times natural products have taken the forefront of drug discovery[61]. In the

last decade itself natural product derived molecules contributed to nearly half of all small molecules that are approved as drugs. Ethnobotanical approach and the traditional herbal medicines provide the much required platform for discovery and development of new drugs[62].

Medicinal plants have many industrial uses ranging from traditional medicines, herbal teasand health foods such as nutraceuticals to galenicals, phytopharmaceuticals and industrially produced pharmaceuticals. Furthermore, these constitute a source of potential chemotherapeutic agents since these are a ready source of drugs such as quinine and reserpine, galenicals like tinctures and of intermediates (e.g. diosgenin from *Discorea* sp.)[58]. Of the 974 small chemical entities reported as drugs during 1981-2006, 331 were of natural origin, either extracted or semi-synthetic[56]. Over 350 natural products have been evaluated for their anti-mycobacterial activities[63]. Herbal remedies play an essential role and they are extremely popular in the rural societies of South Africa. The preparation of these traditional medicines often depends on their cultural contexts[64].

In the mid 1900s, natural product sources such as fungi, plants, and actinomycetes were the major antibiotic producers[65]. Numerous novel drugs, which were discovered from natural sources, could be included among the most effective drugs available now for the treatment of human diseases. These include quinine and artemisin for malaria treatment, paclitaxel for cancer treatment, lovastatin as a lipid control agent, and morphine as an analgesic[65]. In addition, more than two-thirds of the current antibiotics used in the clinic, including the β-lactam class originated from natural sources[66]. However, the relatively low profit margin compared to high development costs and the rapid development of drug resistance has restricted natural antibiotic discovery efforts in most pharmaceutical companies. In addition, researchers have repeatedly isolated the same compounds from natural sources because of a lack of efficient dereplication technology. As a result, the relatively low cost synthetic drug is in a favorable position in comparison to the labour intensive production of naturally occuring drugs. However, the interest in development of drugs of medicinal plant origin is still high. The reasons are, conventional medicine can be inefficient (e.g. side effects, ineffective therapy and high expense). Secondly, a large percentage of the world's population does not have access to conventional pharmacological treatment.

Thirdly, it has already been stated that folk medicine and "natural" products are harmless. Scientific knowledge about these medicinal plants and bioactive compounds should enable researchers to determine efficacy, stability, best drug delivery systems and quality control processes.

The majority of traditionally used crude drugs are derived from plant extracts. This knowledge has formed a base of information which provides a potential platform for the research for new drug discovery. A different set of metabolites is usually produced in the different parts of the plant (e.g. root, leaves and flower). The botanical knowledge is thus crucial for the correct taxonomical determination of the plants from where the bioactive molecules are sourced[56].

Historically, there have been evidences that medicinal plants have helped to introduce single chemical entities in modern medicine. Current drug discovery from plants has mainly relied on bioactivity-guided isolation methods, e.g., important anticancer agents, paclitaxel from *Taxus brevifolia* and camptothecin from *Camptotheca acuminata*. In addition, combretastatin A4, isolated from the South African medicinal tree, *Combretum caffrum*, was developed to combretastatin A4 phosphate and is now in phase II trials[67,68,69] .

Assam lies in the North-eastern part of India and falls in the Indo-Burman region which has been identified as one of the eight biodiversity hotspots in the world depending on five factors that include Endemic plants[70]. As a result, Assam, with its vast and unique plant resources has huge potential and possibilities in the field of drug discovery. Of the three thousand odd plant species found in the state, some 950 species are known to possess definite medicinal properties[65]. Some of these species have been used in the *Ayurvedic*, *Unani*, and other traditional alternative systems of medicine since time immemorial. This huge traditional knowledge is an invaluable asset for the process of drug discovery. It has also been observed that many of these native plant species in Assam are traditionally found to be antidiabetic. These plants need to be investigated further for validating their antidiabetic properties. Drug discovery efforts can justifiably be focused on these species for identifying the much needed potent molecules having antidiabetic activity.

1.8 Objectives

Although diabetes is the most widespread non-communicable disease in the world, its primary cause still remains unclear. Insulin resistance is one of the common features of T2DM patients. There are several drugs present in the market for the treatment of diabetes *viz.*sulfonylureas, metformin and thiazolidinediones (TZD) which either increases insulin release from the β-cell or decreases glycogen mobilization to glucose and carbohydrate absorption. The best choice for insulin resistance appears to be the TZD class of drugs but has a number of adverse effects and it does not address T2DM directly.

In view of the above background and lacunae, the following questions were raised:

- Do the traditionally used medicinal plants to treat diabetes have any role in reverting FFA induced insulin resistance?
- If yes, what could be the probable active principle(s)?
- How does the active principle(s)/compound(s) ameliorate insulin resistance induced by FFA?
- Does the active principle(s)/compound(s) retain the same activity in *in-vivo* diabetic mice and rat models?
- Do the various medicinal plants used traditionally to treat diabetes have any synergistic effect?

With these research questions, the work embodied in this thesis proceeded with the following objectives which are identified against the broad perspectives of the urgent necessity to forwardnew molecules as potential drugs to cure insulin resistance and T2DM:

- Isolation and purification of active compound(s) from the screened plants with known antidiabetic effects.

- Assessment of the purified active compound(s) on free fatty acid (FFA) induced T2DM.
- *In vivo* study of the potential antidiabetic compound(s).
- Role of saturated fatty acid in alteration of antioxidant enzymes and its amelioration by the plant derived bioactive compound(s).
- Identification of probable antidiabetic compound(s) from plants and target(s) through *in silico* network pharmacology based approach.

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