Chapter 1
Introduction

1.1 Background

Diabetes mellitus (DM) is a global metabolic disorder in which there are high blood sugars levels over a prolonged period of time [1]. According to International Diabetic Federation, the estimated diabetes prevalence for 2010 has risen to 285 million, which represents 6.4% of the world’s adult population, with a prediction that by 2030 the number of people with diabetes will have risen to 438 million corresponding to 7.8% of the adult population. India has been declared as the “Diabetic capital of world” and currently 40.9 million people in India has been suffering from diabetes and by 2030 it is estimated that it will be the seventh leading cause of death which will account for 3.3 percent of total deaths in the world [2].
Diabetes causes because the pancreatic cells either do not produce enough insulin, produces no insulin or the cells of the body do not respond properly to the insulin the pancreas produces. There are mainly three types of DM:

1) Type 1 diabetes (T1D) is also referred to as "insulin-dependent diabetes mellitus" (IDDM) and results due to the failure of pancreatic β-cells to produce enough insulin.

2) Type 2 diabetes (T2D) is also referred to as "non-insulin dependent diabetes mellitus" (NIDDM) or "adult-onset diabetes", which is a condition where cells fail to respond to insulin properly and ultimately it leads to insulin resistance.

3) Gestational diabetes develops during pregnancy without a previous history of diabetes and it usually disappears after the birth of the baby.

During diabetic condition the prevention and treatment involve a healthy diet, physical exercise, maintaining a normal body weight, controlling of blood pressure and maintaining proper foot care are important for people with this disease. T1D must be managed with insulin injections but T2D may be treated with medications with or without insulin. Insulin and some oral medications generally decrease the levels of blood sugar. For people with obesity, weight loss surgery is the effective measure in those with T2D. Genetically susceptible to T2D people are more vulnerable when these risk factors are present. Physical inactiveness and obesity are basically the main reason for the development of T2D. Obesity is mainly caused due to the imbalance of caloric intake and physical activity that results in insulin resistance. The excess “belly fat” produces hormones and other substances that can cause harmful, chronic effects in the body such as damage to blood vessels. In some people with diabetes, an
abnormal increase in glucose production by the liver also contributes to high blood glucose levels [3].

Normally, the pancreas releases glucagon hormone when there are low levels of blood glucose and insulin levels; it stimulates the liver to produce glucose to release it into the bloodstream. But the glucagon levels drop down when blood glucose and insulin levels are high after a meal and the liver stores excess glucose for later when it is needed. In diabetic people, glucagon levels were found to be higher than needed which causes the liver to produce excess glucose, which contributes to high blood glucose levels.

T2D is characterized by high levels of glucose due to deficiencies in insulin production by pancreatic β cells or insulin resistance in tissues that rely on insulin for glucose uptake [4]. Major complications of diabetes mellitus has been associated with increased oxidative stress, including retinopathy, nephropathy, neuropathy and accelerated coronary artery disease [5]. Insulin act as a master regulator of several key functions in metabolism control and defects in these control points due to inhibition of insulin signalling contribute decisively to the development of insulin resistance and T2D. Oxidative stress induced by reactive oxygen species (ROS) has become apparent as a causative agent for insulin resistance, as it participating in the disease process leading to diabetes and its complications [6]. The impairment of endogenous antioxidant enzymes functioning also induce oxidative insult in cells [7] and prolong exposure to oxidative stress may cause insulin resistance by causing an alteration in the redox balance at the molecular level [8]. During oxidative stress, several reactive products are generated, which include ROS and aldehydes such as 4-Hydroxy-2-nonenal (HNE) through lipid peroxidation process [9, 10]. HNE is a highly reactive
and covalently modifies at cysteine, histidine and lysine residues of proteins. In addition, HNE is known to modify important signalling proteins suggesting a pathogenetic role [11]. The accumulation of HNE in diabetic patients and in diabetic rat’s liver has been reported by Traverso et al., 2002 [12]. Furthermore, exposure of HNE to pancreatic islets decreases insulin secretion [13]. These data reveal HNE as strong oxidative agents and it plays a major role in the development of insulin resistance. However, the specific targets of aldehyde modification which leads to insulin resistance and diabetes still remain unknown.

The reactive aldehydes are controlled by enzymatic antioxidants as well as non-enzymatic antioxidants in cells. The most efficient primary scavenger antioxidant enzymes involved in detoxifying ROS are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione s-transferase (GST) [14] while, non-enzymatic antioxidants include non-protein thiol, vitamin C and vitamin E in mammalian systems [15,16,17]. These antioxidant defenses are extremely important because they provide maximum protection to biological sites by directly removing free radicals. Similarly, Fatty aldehyde dehydrogenase (FALDH) is capable of metabolizing HNE which is a microsomal NAD/NADP-dependent enzyme that belongs to the aldehyde dehydrogenase family and catalyzes the oxidation of HNE to the nontoxic 4-hydroxy-2- nonenoic acid [18]. The mRNA of FALDH encodes a protein of 485 amino acids [19]. This protein has a hydrophobic carboxyl-terminal segment that is necessary for microsomal membrane anchoring. A study report of Demozay et al., 2004 has shown that FALDH decreases ROS production induced by HNE and its expression was decreased in the white adipose tissue of diabetic mice [20].
Many studies have provided evidence and suggested the association of oxidative stress and pathophysiology of insulin resistance via insulin signals inhibition [21, 22, 23, 24, 25]. The mechanisms by which this occurs are often multifactorial and quite complex, involving many cell signalling pathways. Even though β cell failure is the essential condition for T2D development, skeletal muscle insulin resistance is considered to be the primary defect which is evident even decades before β cell failure. Oxidative stress activates multiple serine kinase cascades [26, 27], among them insulin signalling occurs through activation of a specific insulin receptor, which belongs to a subfamily of receptor tyrosine kinases [28]. Insulin binding to its receptor results in the tyrosine phosphorylation of insulin receptor substrates (IRS) by the insulin receptor tyrosine kinase (INSR) [29] and allows IRS association with the regulatory subunit of phosphoinositide 3-kinase (PI3K). Further, PI3K activates 3-phosphoinositide-dependent protein kinase 1 (PDK1), which activates a serine kinase Akt. In turn, Akt deactivates glycogen synthase kinase 3 (GSK-3) that leads to activation of glycogen synthase (GYS) and thus glycogen synthesis. Activation of Akt also results in the translocation of GLUT4 vesicles from their intracellular pool to the plasma membrane, where they allow uptake of glucose into the cell [30]. Other signal transduction proteins interact with IRS is PPAR signalling pathway which regulates the lipid metabolism, mitochondrial biogenesis. Peroxisome proliferator activating receptor gamma (PPARγ) is abundant in adipose tissue which is act as a transcription factors in regulating whole-body insulin sensitivity and improved glucose utilization [31].

On a parallel pathway, activated IRS1/2 recruit Grb2, which associates to SOS and activates the Erk1/2 MAPK pathway [4]. The p38 and JNK stress-activated kinases,
whose activation is mainly dependent on stress signals and inflammatory cytokines [32, 33] have also been shown to be activated in response to insulin [34]. The use of antioxidants may be very important in preventing activation of these signalling pathways and owing to the side effects of currently available drugs, the focus has been shifted towards naturally occurring compounds, suggesting the need for new and more efficient antioxidants for the management of diabetes and its complications [35]. The past decade has witnessed abundant scientific studies [36] on phytochemicals which are widely distributed in fruits, vegetables, beverages have been reported to be effective in the cure and management of diabetes [37]. The multifaceted character of antioxidants in countering ROS and diabetes in animal models provides the impetus for identifying new and more efficient antioxidants, for treating diabetes and its complications [38, 39]. Antioxidants of plant origin (widely distributed in fruits, vegetables, beverages, and herbal remedies) that are part of our regular diet can be important molecules to explore their therapeutic potential for countering issues related to ROS generated damages in diabetes. Of the different antioxidants of plant origin Resveratrol, Carvacrol, Curcumin, Baicalein are some of the prominent ones.

Curcumin is the principal curcuminoid found in turmeric with many beneficial effects such as antioxidant, scavenging free radicals, anti-inflammatory and so on [40,41]. Curcumin has been reported to inhibit insulin signalling pathway and glucose transport in 3T3-L1 adipocytes under normal culture condition [42]. Resveratrol is a natural polyphenol who’s hypoglycemic and hypolipidemic effects have been innumerate in streptozotocin-induced diabetic rats [43]. In Swiss 3T3 fibroblasts, resveratrol showed the protective effect against 4-hydroxynonenal (4-HNE) induced oxidative stress and apoptotic death [44]. Carvacrol possesses several biological
actions, including anti-inflammatory, antioxidative, and anti-apoptotic properties [45]. It was revealed that carvacrol has anti-proliferative properties on human non-small cell lung cancer cells, A549 [46]. Carvacrol was found to improve the cognitive function of diabetic rats, which was linked with its hypoglycemic, antioxidant, and anti-inflammatory properties [47]. Flavanoid, Baicalein is another potent antioxidant obtained from Scutellariae radix possesses anti-inflammatory and antioxidant activities [48]. In HMEC-1 cells, Changjiang et al., (2017) reported that baicalein abolishes oxidative stress [49]. A phenolic monoterpene, Carvacrol, is another plant-based antioxidant produced by aromatic plants and spices, present in the genera Origanum and Thymus [50]. Although, Resveratrol, Carvacrol, Curcumin, and Baicalein have been explored individually to establish their hypoglycemic, antioxidant, and anti-inflammatory properties etc., a comparative analysis of their potential in the oxidative setup is still not available. In view of this, it would be interesting to explore the most potent antioxidant molecule possessing ROS quenching properties to bridge this lacking information.

The nullification of oxidants by increased antioxidant availability may alleviate the effects associated with oxidative stress in T2D. Antioxidants have been reported to have a protective effect against the development of diabetes by inhibiting peroxidation chain reactions and through several possible mechanisms, such as directly quenching free radicals, chelating transition metals, reducing peroxides, and stimulating the antioxidative defense enzyme system [51]. However, little epidemiological evidence is available on the role of antioxidant intake in the prevention of T2D and only a few studies exist on dietary antioxidants and the risk of T2D. As Indian diet invariably consists of many components of which turmeric
(curcumin), capsicum (capsaicin), tomato (lycopene), fruits (resveratrol) that are well
known antioxidants consumed at daily basis. These widely used as ingredients in
dietary supplements can be exploited to maintain health and prevent oxidative stress-
mediated diseases such as diabetes, inflammation, and aging. Additionally,
compounds Resveratrol, Carvacrol, Curcumin, and Baicalein have been also indicated
to be important in reducing oxidative stress. However, how different genes which are
involved in the mechanism interact and respond to the supplementation with one or
more antioxidants is yet not well characterized. Therefore, it is essential to identify
the role of the antioxidant molecules which are used as part of medicine or dietary
habits at the molecular level using modern molecular biology tools \textit{in vitro} and \textit{in vivo}
models.

T2D is a multifactorial trait in which multiple genetic and environmental
factors interact in complex, non-linear ways to produce the common phenotype of
hyperglycemia. Until recently, research efforts to identify the genetic variants that
contribute to individual differences in predisposition to T2D were met with slow
progress and limited success. Over the past three years, the advent of genome-wide
association (GWA) scan has ushered in a new era regarding the capacity of
identifying common genetic variants that contribute to predisposition to complex
multifactorial phenotypes such as T2D. The identification of the variants, genes, and
pathways implicated in T2D pathogenesis might facilitate its diagnosis and prevention
and offer a route to new therapies [52]. Numerous SNPs have each been associated
with increased risk for T2D. Identification of specific genetic variations in a particular
ethnic group is the other most important area of diabetes research because elucidation
of the diabetes genes (biomarkers) have aided in the mechanistic understanding of the
disease, its complications, cure, and prevention. This makes SNPs valuable for biomedical research and for developing medical diagnostic products. Genome-wide association studies (GWASs) have revolutionized the complex disease genomics research worldwide and have illuminated polymorphisms in several genes including rs5219 in the \textit{KCNJ11} gene, rs13266634 in the \textit{SLC30A8} gene, rs8050136 in the \textit{FTO} gene, rs1801282, in the \textit{PPARG} gene, rs775840, in \textit{CDKAL1} gene, etc.

The present study was carried out hypothesizing that some of the well-known dietary antioxidants may be effective in reducing the oxidative stress-induced impairments’ in the skeletal muscle cell, impressed upon by HNE. The study aims to investigate the \textit{in vitro} ameliorative potential of these four antioxidant compounds in L6 myotubes in combating oxidative stress conditions and also to comprehend the gene expression patterns of oxidative stress genes, insulin signalling genes, MAPK signalling genes and PPAR signalling gene upon supplementation of different antioxidant in induced stress condition. As Insulin is a positive modulator of ROS, I was interested to study the effects of antioxidants treatment conditions in by keeping the Insulin treatment as a comparative reference. I also wanted to study if the antioxidants were able to combat ROS individually or in combination with Insulin. Additionally, we were interested in identifying the most potent antioxidant molecule having the potential to be used as an additive supplement to reduce the risk of oxidative stress-based diseases. Additionally, the role of certain SNP’s on genetic predisposition to T2D in diabetic population was explored.
1.2 Objectives of the study

The objectives taken up for study in the present thesis are:

1. To assess the effect of antioxidants in scavenging ROS by treating cells with the four antioxidants namely Resveratrol, Carvacrol, Curcumin, and Baicalein in *in vitro* system and to perform assays to track down the down-regulation of ROS in *in vitro*.

2. To study upregulation or downregulation of certain genes related to oxidative stress and insulin signalling upon amelioration of ROS by antioxidants.

3. To study the potency of anti-oxidants in scavenging ROS by treating *in vivo* rat models with fruit derived antioxidants.

4. To investigate the distribution of rs9939609 and rs9926289 in *FTO* gene, rs5219 SNP in *KCNJ11* gene and rs13266634 SNP and rs11558471 SNP in *SLC30A8* gene, amongst the diabetic population of North Eastern India and to study their association in T2D occurrence.