1. INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases in which a person has high blood glucose, either because the pancreas does not produce enough insulin, or because cells do not respond to the insulin that is produced (Shoback et al., 2011). This high blood glucose produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger) (Shoback et al., 2011).

DM is characterized by hyperglycemia arising as a consequence of a relative or absolute deficiency of insulin secretion, resistance to insulin action or both (American Diabetes Association, 2010). This is a major and growing public health problem throughout the world, with an estimated worldwide prevalence of 171 million people in 2000, expected to increase to 366 million people by 2030. In particular, the number of people with diabetes in India, currently around 40.9 million, is expected to rise to 69.9 million by 2030 (Wild et al., 2004). India leads the world with largest number of diabetic subjects thus earning the dubious distinction of being termed the ”Diabetes Capital of the World“ (Mohan et al., 2007).

1.1 Classification of DM

There are three types of DM.

Type 1 DM

Type 2 DM

Gestational diabetes

Other types

Prediabetes and Latent autoimmune diabetes of adults (LADA)
1.1.1. Type 1 DM

Type 1 DM results from the body's failure to produce insulin, and currently requires the person to inject insulin or wear an insulin pump. This form was previously referred to as "insulin dependent diabetes mellitus" (IDDM) or "juvenile diabetes". Type 1 DM is characterized by a deficiency in endogenous insulin production mediated by an autoimmune process destroying the insulin-producing beta cells of the endocrine pancreas resulting in a dependency of exogenous insulin injections (Leslie and Pozzilli, 2002). In general, conventional insulin treatment does not normalise glucose metabolism. Evidence from various clinical observations indicates an association between hyperglycaemia and the development of late diabetic vascular complications (Leslie and Pozzilli, 2002). The diabetes control and complications Trial (DCCT) has emphasised on a strict glycaemic control in patients with type 1 DM in order to delay the onset and slow the progression of diabetic retinopathy, neuropathy and nephropathy achieved by a proper injection technique, frequent self-monitoring of blood glucose and by intensive insulin regimes. Improved control of blood glucose reduced the risk of major diabetic eye diseases by 25%, serious deterioration of vision by nearly 50% and early kidney damage by 33% (DCCT, 1993 and 1997).

Insulin treatment has been available for patients with diabetes for more than 80 years and numerous insulin replacement regimens have been used during the years (Home, 1997). Considerations regarding insulin types, frequency of daily injections, continuous subcutaneous insulin infusions and injection site are some of the major issues in creating the optimal insulin regimen in order to simulate physiological insulin secretion.
The increasing use of intensified insulin regimens in the management of patients with type 1 has raised the question as to whether the overall glycaemic control evaluated by satisfactory glycated haemoglobin value may be improved at the expense of an increased risk of hypoglycaemia. The DCCT trial proved a constant relative risk gradient in which proportional reductions in HbA1c were accompanied by proportional reductions in the risk of complications and an increased absolute risk of severe hypoglycaemia by 26% (DCCT, 1995).

In 1970 continuous subcutaneous insulin infusion (CSII), often called insulin pump therapy, was introduced to achieve and maintain strict control of blood glucose in patients with type 1 diabetes. Buffered soluble insulin or rapid-acting insulin analogues are infused subcutaneously from a portable pump together with continuous infusions i.e. a basal rate. Much research carried out during the years in order to evaluate the effects on the metabolic regulation, frequency of hypoglycaemia and quality of life in patients. In metabolic controlled trails glycosylated haemoglobin was significantly increased (Beck-Nielsen et al., 1990; Feldt-Rasmussen et al., 1986). The incidence of severe hypoglycaemia was decreased due to CSII therapy as compared with multiple daily injections therapy (Bak et al., 1987; Beck-Nielsen et al., 1990; Bode et al., 1996; Feldt-Rasmussen et al., 1986; Dahl-Jorgensen et al., 1986).

1.1.2. Type 2 DM

Type 2 DM results from insulin resistance, a condition in which cells fail to use insulin properly, sometimes combined with an absolute insulin deficiency. This form was previously referred to as non insulin-dependent diabetes mellitus (NIDDM) or "adult-onset diabetes". The last 30 years, type 2 diabetes has changed from being considered a relatively mild ailment associated with ageing, to one of the major
contemporary causes of premature mortality and morbidity worldwide (Roglic et al., 2005). Diabetes is ranked among the leading causes of blindness, renal failure and lower limb amputation, and is one of the major causes of cardiovascular death (American Diabetes Association, 2007).

Type 2 DM is the most common endocrine disorder worldwide covering 90-95% of all diabetes cases (Goldstein, 2007). The classification and pathogenesis of Type 2 DM involves abnormalities in glucose and lipid metabolism, inadequate insulin secretion from pancreatic beta-cells and resistance to insulin activity (Goldstein, 2007). Insulin resistance and impaired glucose tolerance, both conditions preceding the development of Type 2 DM are closely related to obesity (Sharma et al., 2006). The contribution of excess visceral fat to the development of insulin resistance, due to pronounced lipolysis and the subsequent release of free fatty acids that can directly block insulin signaling pathways is well established (Mlinar et al., 2007 and Reimann et al., 2009). Increasing environmental pressure may widen the susceptibility profile of Type 2 DM (Reimann et al., 2009). Increased oxidative stress has been proposed to be one of the major causes of the hyperglycemia-induced trigger of diabetic complications (Valko et al., 2007).

1.1.3 Gestational diabetes

Pregnancies complicated by gestational diabetes mellitus (GDM) are at increased risk for caesarean delivery as well as adverse neonatal outcomes, with hypoglycaemia being the most frequent metabolic complication (Blank et al., 1995). Neonatal hypoglycaemia is common in the first hours of life of newborns due to immaturity of gluconeogenesis and ketogenesis (Levitt-Katz et al., 1996; Stanley and Baker, 1999). It is a common gestational complication, affecting 1-14% of pregnant
women each year (American Diabetes Association, 2003). In 30-70% of GDM patients, Type 2 DM may develop at a later age (Kim et al., 2002; O’Sullivan, 1991), making GDM a clinical condition that deserves to be taken very seriously. Although impaired insulin secretion from the pancreas and increased insulin resistance, features also shared by Type 2 DM, have been implicated in the pathogenesis of GDM, the underlying metabolic mechanisms have yet to be clearly elucidated (Carpenter and Cousta, 1982; Falavigna et al., 2013).

Infants of diabetic mothers are at increased risk for hypoglycaemia as a secondary complication of foetal hyperinsulinism due to maternal hyperglycaemia and thus the risk for neonatal hypoglycaemia is extended by several hours. On the other hand, –desirable values‖ for glycaemia in newborns have yet to be determined since insufficient evidence exists regarding the level and duration of hypoglycaemia that can cause neurologic damage (Cornblath et al., 2000; Ozuguz et al., 2011; Stanley and Baker, 1999). Few studies have characterized glucose concentrations in infants of GDM mothers in the first day of life (Balsells et al., 2000; Chertok et al., 2009; Maayan-Metzger et al., 2009). Most studies evaluating risk factors for hypoglycaemia in infants of women with GDM took only gestational and maternal characteristics into account whereas peripartum factors, such as intrapartum glycaemic control known to increase the risk of neonatal hypoglycaemia in pregestational diabetes, have not been fully assessed in women with GDM (Esakoff et al., 2011; Maayan-Metzger et al., 2009; Sarkar et al., 2003; Silva et al., 2006).

GDM has been defined as glucose intolerance of variable severity with onset or first recognition during pregnancy (Metzger and Coustan, 1998). The incidence of GDM has increased markedly in recent years in large part due to
the obesity epidemic (Ferrara et al., 2004) and will increase further with the adoption of the diagnostic criteria proposed by the International Association of Diabetes in Pregnancy Study Groups (IADPSG) (Metzger et al., 2010), recently adopted by the American Diabetes Association (American Diabetes Association, 2011).

GDM is generally asymptomatic, usually being detected through systematic screening after the 24th week of pregnancy. Evidence to support screening for GDM is indirect and strongly based on the potential adverse effects of hyperglycemia on pregnancy outcomes (Black et al., 2010; Metzger et al., 2008; Wendland et al., 2011; Wendland et al., 2012), and on the effectiveness of GDM treatment in preventing these outcomes (Crowther et al., 2005; Landon et al., 2009). Two systematic reviews have summarized the evidence available for the effectiveness of GDM treatment (Alwan et al., 2009; Horvath et al., 2010). The first, performed by Alwan et al., was conducted prior to the publication of the Landon et al. study, a large and well designed randomized trial (Landon et al., 2009). The second, conducted by Horvath et al., did not analyze preeclampsia, a common and clinically important complication of pregnancy, found to be reduced by in recent GDM trials (Crowther et al., 2005; Falavigna et al., 2012; Landon et al., 2009).

1.1.4 Other types of DM

1.1.4.1 Prediabetes

Prediabetes indicates a condition that occurs when a person's blood glucose levels are higher than normal but not high enough for a diagnosis of type 2 DM. Many people destined to develop type 2 DM spend many years in a state of prediabetes which has been termed America's largest healthcare epidemic (Handelsman, 1999; Tanaka et al., 2013).
Prediabetes describes individuals who have impaired fasting glucose and/or impaired glucose tolerance (American diabetes association, 2009). Among adults aged 45 years and older, the prevalence of prediabetes is approximately 25% (Benjamin et al., 2003; Dunstan et al., 2002; Glumer et al., 2003). Evidence suggests moderate PA of at least 150 minutes per week (min/wk), in combination with a 5-10% weight loss, is associated with reducing the risk of developing type 2 diabetes and helps to maintain weight loss in those with prediabetes (Diabetes Prevention Program Research Group, 2002; Hamman et al., 2006; Li et al., 2013; Pan et al., 1997; Tuomilehto et al., 2001).

1.1.4.2 Latent autoimmune diabetes of adults (LADA)

LADA is a subgroup of type 1 diabetes that showed preserved β-cell function initially misclassified as type 2 DM (Littorin et al., 1999; Pozzilli and Mario, 2001). The development of symptoms is insidious and the clinical diabetes becomes overt at the age of 30s or older, and often is maintained in good metabolic control on diet or oral hypoglycemic therapy for up to several years before insulin dependency (Kobayashi et al., 1993; Stenstrom et al., 2005). So it has been suggested that LADA deviates from classic type 1 diabetes (Palmer et al., 2005). Until now, LADA remains poorly understood at both a clinical and research level (Fourlanos et al., 2005).

LADA defines initially non-insulin requiring adult-onset diabetic patients with the islet-related autoantibody that, in a number of cases, eventually require insulin treatment. The prevalence of LADA has been reported to be about 10% of adult-onset type 2 diabetic patients in Caucasians (Li et al., 2011; Turner et al., 1997; Tuomi et al., 1999). Residual insulin secretion is important in maintaining good glycemic control and preventing diabetic complications in type 1 DM (Nakanishi et al., 1995) and time course changes for serum C-peptide including young
adult-onset autoimmune (over 15-20 years old) diabetes with type 2 diabetic phenotype has been reported (Borg et al., 2001; Gottsater et al., 1993; Kasuga et al., 1999; Torn et al., 2000), but there have been few reports regarding changes in insulin secretion in LADA patients of onset over 30 years old (Niskanen et al., 1995) which has been proposed as one of the criteria for LADA (Fourlanos et al., 2005; Murao et al., 2008).

1.2 Mechanism of action of DM

1.2.1 Metabolic action of insulin

A basic requirement for all vertebrates is stability of the level of blood glucose. This is essential for brain function. Regardless of large fluctuations in physical activity and food intake, blood sugar levels are held within very narrow limits. The key to this is insulin, the secretion of which is closely regulated by circulating substrates of energy metabolism. Insulin signals food abundance and initiates uptake and storage of carbohydrates, fats and amino acids. Energy supply and stability of blood sugar levels postprandial is usually accorded to glucagon and the catecholamines, but the reduction in insulin signalling postprandial is almost certainly just as important.

Control of the key enzymes of metabolism can be divided into two classes:

1. Covalent modification of enzymes, usually by phosphorylation or dephosphorylation of serine, threonine or tyrosine residues.

2. Allosteric feedback and feed-forward regulation by metabolic intermediates.

Enzymes involved in metabolism can be either activated or inactivated by phosphorylation. Glycogen phosphorylase and hormone-sensitive lipase which are activated when phosphorylated and glycogen synthetase and pyruvate dehydrogenase
are inactivated through phosphorylation. The protein kinases that catalyze phosphorylation of these enzymes are subject to control through cyclic nucleotides (PKA and cyclic AMP), Ca\(^{++}\) and diacylglycerol (PKC) and PI (3,4,5)P3 (PKB).

Hormone-sensitive lipase activity in fat cells is regulated largely through cAMP activation of protein kinase A (PKA). The cyclic nucleotide levels are controlled through the balance between hormone-regulated G-protein control of adenylate cyclase and breakdown of cAMP catalyzed by phosphodiesterase. Insulin regulates cAMP levels through its stimulatory effect on the esterase and reduction of cAMP levels. Fig 1.2.1 shows mechanism of insulin action.

![Fig. 1.2.1 Mechanism of insulin action (Petersen et al., 2007).](image)

Insulin regulates glucose homeostasis by promoting glucose disposal in skeletal muscle and adipose tissue (Petersen et al., 2007). In addition to its direct actions on the skeletal muscle, insulin regulates nutrient delivery to target tissues by actions on microvasculature (Barrett et al., 2011). These vasodilator actions of insulin are nitric oxide (NO)-dependent and lead to increased skeletal muscle microvascular perfusion that further enhances glucose uptake in skeletal muscle.
(Muniyappa et al., 2007). These actions of insulin on skeletal muscle microvasculature appear to be a rate limiting step for insulin-mediated glucose disposal. At the cellular level, balance between phosphatidylinositol 3-kinase-(PI3K)-dependent insulin signaling pathways that regulate endothelial NO production and mitogen activated protein kinase (MAPK)-dependent insulin-signaling pathways regulating the secretion of the vasoconstrictor endothelin-1 (ET-1) determines the microvascular response to insulin. Insulin resistance is frequently present in obesity, hypertension, coronary artery disease, dyslipidemias, and metabolic syndrome (Petersen et al., 2007). Insulin resistance is typically defined as decreased sensitivity or responsiveness to metabolic actions of insulin such as insulin-mediated glucose disposal. However, diminished sensitivity to the vascular actions of insulin also plays an important role in the pathophysiology of insulin-resistant states (Hua et al., 2009; Muniyappa et al., 2013). Endothelial insulin resistance is typically accompanied by reduced PI3K-NO pathway and an intact or heightened MAPK-ET1 pathway (Muniyappa et al., 2007).

1.2.2 Autoimmune disorder

Type 1 DM is an autoimmune disorder resulting from loss of immunologic self tolerance and characterized by Th1-mediated cellular destruction of insulin-producing pancreatic β-cells (Huang et al., 2009). Sub-clinical insulitis/inflammation, infiltration of pancreatic islets by mononuclear cells, typically characterizes the pathological presentation of Type 1 DM and subsequently leads to insulin deficiency and clinical symptoms of hyperglycemia when most b cells have been destroyed (Bluestone et al., 2010). The development of Type 1 DM involves a complex interaction between pancreatic β-cells and immune cells including autoreactive T cells, regulatory T cells (Treg) and dendritic cells (DC). Autoreactive T cells, both CD4+Th1 and CD8+ cells, have been implicated as active players in β-cell
destruction (Knip and Siljander, 2008; Perone et al., 2009), and deletion of these cells can prevent diabetes in non-obese diabetic (NOD) mice (Han et al., 2005). It is generally accepted that pathogenic T cells are normally held in check by the forkhead box transcription factor (Foxp3)-expressing CD4+CD25+ Tregs (Xue et al., 2012). Tregs suppress the differentiation of islet-reactive CD8+T cells to cytotoxic T cells (Green et al., 2003). In both mice and humans a progressive loss of Treg suppressive capacities correlates with diabetes development (Gregg et al., 2004; You et al., 2005; Lindley et al., 2005; Pop et al., 2005; Sgouroudis and Piccirillo, 2009). The transfer of Tregs in NOD mouse model or the potential of strategies to promote Treg cells was shown to protect pancreatic β-cells from autoimmune destruction and against disease progression, suggesting that Treg dysfunction contributes to the pathogenesis of diabetes and Tregs are crucial for controlling Type 1 DM development (Petzold et al., 2010; Battaglia and Roncarolo, 2011).

1.3 Complications of diabetes

The chronic complications of DM affect many organ systems and are responsible for the majority of morbidity and mortality. Chronic complications can be divided into vascular and nonvascular complications. The vascular complications are further subdivided into microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular complications (coronary artery disease, peripheral vascular disease, and cerebrovascular disease). Nonvascular complications include problems such as gastroparesis, sexual dysfunction, and skin changes. As a consequence of its chronic complications, DM is the most common cause of adult blindness, a variety of debilitating neuropathies, and cardiac and cerebral disorders (Collins et al., 1995; Tripathi and Srivastava, 2006).

1.3.1 Microvascular complication

1.3.1.1 Diabetic retinopathy (DR)
DR is a severe complication of diabetes and the leading cause of blindness among working adults worldwide. According to World health organization, the prevalence of DR is expected to increase, and the number of people at risk of vision loss is predicted to double by the year 2030 with the increasing rate of diabetes epidemic (Crosby-Nwaobi et al., 2012; Wild et al., 2004). DR is a multifactorial progressive disease of the retina where the pathogenesis of the disease is extremely complex involving many different cells, molecules, and factors. Diabetes manifests in damaging all of the major retinal cells including endothelial, ganglion, and pigment epithelial cells. Diabetes induces dysregulated levels of metabolites such as glucose, lipids, amino acids, hormones, and nutrients, and several factors have been found to activate those retinal cells before the damage. A number of studies suggest a high plasma level of homocysteine in diabetic patients (Coral et al., 2009; Ganapathy et al., 2009; Gowda et al., 2011). DR is a common and specific microvascular complication of diabetes, and its significance is likely to increase with increasing frequency of diabetes (Cheung et al., 2010; Jeganathan et al., 2008; Kempen et al., 2004). A recent epidemiological study has revealed that DR is the leading cause of preventable blindness in working-aged people (Mohamed et al., 2007). However, the underlying mechanism of how these diabetic microvascular complications occur remains largely unknown (Varma, 2006).

In addition, dysregulated level of taurine and its transporter in diabetic retina has been shown to be implicated in pathophysiology of DR (Chen et al., 2009; Heller-Stilb et al., 2002). Increased level of adenosine in the retina may also be implicated in the pathophysiology of DR since the non proliferative to proliferative selective adenosine receptor antagonist has been found to inhibit endothelial cell proliferation, cell migration, and tube formation and also inhibit neovascularization (Grant et al., 2001). Moreover, the role of nutrients such as alpha lipoic acid, folic
acid, vitamin C, vitamin E and minerals is gaining interest in diabetes, where dysregulated levels play an important role in the pathophysiology of DR (Bartlett and Eperjesi, 2008; Ola et al., 2012). The major emphasis is given on diabetic-induced metabolic changes in the retina which induce a range of molecules and pathways involved early in the pathophysiology of DR which are briefly discussed, and those major cascades of events are shown in the schematic diagram as depicted in Fig. 1.3.1.1 (Ola et al., 2012).

**Fig. 1.3.1.1** General features for diabetes induced neurovascular damage in DR. Diabetes induces a number of mediators including growth factors, hormones, and inflammatory biomarkers that activate a wide range of biochemical pathways responsible for the progression of the disease. All these diabetes-induced pathways and molecules damage both neuronal and vascular cells in the retina, leading to neurovascular damage in DR (Ola et al., 2012).

### 1.3.1.2 Diabetic nephropathy (DN)

DN is considered to be one of the major complications of DM. The pathological changes such as expansion of mesangial cells, accumulation of extracellular matrix protein, thickening of glomerular and tubular basement membranes, tubulointerstitial fibrosis, glomerulosclerosis and renal endothelial dysfunction are noted to occur in the diabetic kidney (Schrijvers et al., 2004; Kanwar et al., 2008;
Balakumar et al., 2008). DN is associated with albuminuria, proteinuria and reduction in glomerular filtration rate (Balakumar et al., 2008b). The elevated levels of serum creatinine and blood urea nitrogen are considered to be an index of DN (Arora and Singh, 2013; Balakumar et al., 2009). DN is the main cause of end stage kidney disease in developed countries. Worldwide, it is responsible for 25-50% of the patients who undergo dialysis treatment (Tang, 2010). Although the incidence of diabetic nephropathy has been decreasing, population studies reveal it occurrence in 10-40% of the individuals who have Type 1 DM (Bojestig et al., 1994; De carvalho et al., 2011) and 5-20% of the ones who have Type 2 DM (Adler et al., 2003). In general, the development of clinically manifested nephropathy is observed from 10 to 20 years after the DM diagnoses. Therefore, a substantial number of women who have DM that are planning to get pregnant may present DN (Landon et al., 2000). Pregnancy is a period of physiological changes may predispose the developing or changes in the course of DN. Furthermore, DN may predispose an increased risk of perinatal complications (Kitzmiller et al., 1981; Reece et al., 1988). In spite of a probable correlation between physiological adaptations in the pregnancy period and a raise of the kidney overload, the studies currently available are conflicting about the effect of the pregnancy over the development and progression of DN (Miodovnik et al., 1996; Purdy et al., 1996; Reece et al., 1990). DN is characterized by a series of ultrastructural changes in all renal compartments. The changes include basement membrane thickening, glomerular and tubular hypertrophy, mesangial expansion, glomerulosclerosis and tubulointerstitial fibrosis. While most attention has focused on glomerular changes, it is now increasingly recognized that tubules play an important role in the pathogenesis of diabetic nephropathy (Magri and Fava, 2009). The
proximal tubule is susceptible to a variety of metabolic, hemodynamic and inflammatory factors associated with hyperglycemia. Early tubular injury has been reported in patients with diabetes mellitus whose glomerular function is intact (Singh et al., 2008).

Chronic hypoxia could have a dominant pathogenic role in DN, not only in promoting progression but also during initiation of the condition. Early loss of tubular and peritubular cells reduces production of 1,25-dihydroxyvitamin D3 and erythropoietin, which, together with dysfunction of their receptors caused by the diabetic state, diminishes the local trophic effects of the hormones (Matheson et al., 2010). This diminution could further compromise the functional and structural integrity of the parenchyma and contribute to the gradual decline of renal function (Singh et al., 2008). Urinary markers, characteristic of tubular damage, are mainly composed of enzymes urinary or plasma proteins of low molecular weight that are normally freely filtered by the glomerulus (Matheson et al., 2010). Their increased excretion in urine results from the impaired reabsorption of plasma proteins by the tubular cells or from the increased secretion of urinary enzymes by tubular epithelia cells, leading to tubular proteinuria (Barratt and Topham, 2007).
1.3.1.3 Diabetic neuropathy

Diabetic neuropathy refers to a group of debilitating, diabetes-related nerve disorders (Boulton et al., 2005). It is one of the most common diabetes related complications, affecting from 50% to 66% of all patients with diabetes (Argoff et al., 2006; Boulton, 2005; Hall et al., 2006), and affects individuals with both type 1 and type 2 diabetes (Atins and Zimmet, 2010). Moreover, diabetic neuropathy has the highest morbidity and mortality rates of any diabetes-related complication, with diabetic neuropathy increasing the risk of amputation 1.7-fold and the 5- to 10-year mortality rate by 25-50% (Vinik et al., 2003). Diabetic neuropathy has serious detrimental effects on patients' physical, emotional and social functioning (Jensen et al., 2007). Many diabetic neuropathy patients experience pain or discomfort, activity limitations, anxiety, and depression and have lost workdays or decreased work productivity as a result of diabetic neuropathy (Gore et al., 2006). Neuropathy is the most prevalent long term complication of diabetes, affecting >50% of patients with long standing diabetes (Dyck et al., 1993; Pirart, 1978).

Oxidative stress has been investigated in neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotropic lateral sclerosis, memory loss and depression (Dyck et al., 1993). The brain is particularly vulnerable to oxidative damage because of its high oxygen utilisation, its high content of oxidisable polyunsaturated fatty acids, and the presence of redox active metals (Cu, Fe). Oxidative stress increases with age and therefore it can be considered as an important causative factor in several neurodegenerative diseases, typical for older individuals (Valko et al., 2007). The production of β-amyloid, a toxic peptide often present in the brain of Alzheimer's patients, is due to oxidative stress and plays a vital role in neurodegenerative diseases (Dyck et al., 1993). Apolipoprotein E, a lipid transport
molecule that has been linked to the pathogenesis of Alzheimer's Disease, has been found to be subject to free radical attack and a direct correlation exists between apolipoprotein E peroxidation and Alzheimer's disease (Butterfield et al., 2002). A majority of studies explored the effect of oxidative stress that contributes to the cascade of events leading to dopamine cell degeneration in parkinsonism disease (Tretter et al., 2004).

1.3.2 Macrovascular complications

Macrovascular complications include coronary artery disease (CAD), peripheral vascular disease (PVD), and cerebrovascular events (CVA). DM is an independent risk factor for the development of atherosclerosis. On the other hand, atherosclerotic or macrovascular disease is responsible for more than 50% of all deaths in patients with Type 2 DM. Cardiovascular disease accounts for most cases of diabetic macrovascular complications and the remaining are caused by cerebrovascular events and peripheral vascular disease (Leung and Lam, 2000). In India, escalating population levels of major coronary risk factors have contributed to the coronary heart disease epidemic. Several studies show that parallel to the increase in chronic heart disease (CHD) in Indian urban populations there has been an increase in prevalence of hypertension, diabetes, high LDL-C, low HDL-C and the metabolic syndrome (Gupta et al., 2007). Mortality among diabetic patients with CAD is higher than non-diabetic subjects. Studies have also shown that myocardial infarction in diabetics was more common than non-diabetics (Haffner et al., 1998). Indian seems to be more predisposed to both diabetes and CAD. PVD is defined as disease of any blood vessel that is not part of heart or brain. The more common form of PVD is observed in lower extremity which is termed as the lower extremity arterial disease. The simplest screening test for PVD is palpitation of peripheral pulses and
this is the usual clinical tool to assess the occlusive arteries in peripheries. Absence of peripheral, tibial, popliteal or femoral pulses on peripheral examination are clinically significant. Several techniques used for diagnosis of PVD are angiography, colour duplex ultrasound, and continuous waveform Doppler (Beach and Bedford, 1998).

Hypertension is the primary preventable cause of the two major causes of mortality: CAD and CVD. It increases the risk for CAD by two fold, CVD by seven folds and congestive heart failure by four fold. There is ample evidence for a consistent gradient relationship of blood pressure with CVD and CAD. Studies have also shown that an increase of blood pressure of 5 mm Hg is associated with a 34% increase in risk for CVD and a 21% excess risk for CAD (Kannel et al., 2003; Stamler, 1991).

1.3.3 Other complications

Depression is twice as much common in people with diabetes as in the general population and major depression is present in at least 15% of patients with diabetes. Depression is associated with poorer glycemic control, health complications, decreased quality of life and increased healthcare costs. People with diabetes should be screened for depression regularly, either with direct questioning or with a standardized questionnaire (Groot et al., 2001).

The other complication in diabetic men is ED and the prevalence was 34 to 45%. Risk factors include increasing age, duration of diabetes, poor glycemic control, cigarette smoking, hypertension, dyslipidemia and cardiovascular disease. Microvascular, macrovascular complications, psychological and situational factors may also cause or contribute to ED. In addition to this ED is a side effect of many drugs commonly prescribed to men with diabetes, such as antihypertensive
(beta-blockers and thiazide diuretics) and antidepressants. All adult men with diabetes should be periodically screened for ED with a sexual function history and screening for ED in men with Type 2 DM should begin at diagnosis of diabetes (Ofra et al., 2005).

1.4 Induction of DM in animal model

An animal model for biomedical research is one in which normative biology or behaviour can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans or other species of animals. According to this definition of the american national research council committee on animal models for research and aging, animal models used in biomedical research can be classified into five groups: a) spontaneous models in which diseases or conditions occur spontaneously in animals as in humans, b) experimentally and c) genetically modified models in which diseases or conditions are induced chemically/surgically or by genetic manipulation, respectively; d) negative models, including animals resistant to a particular condition or disease and e) orphan models, including animal models with disease unknown to human counterparts (1). In the past century, in an effort to minimize the number of animals used in research, Russell and Burch (2) proposed that the use of animals must follow the three -Rs‖: Replacement, substituting animals with non-animal (alternative) models; Reduction, reducing the numbers of animals used in research; and Refinement, following the best quality care that can be provided to the animal. The 4th -R‖, that of Responsibility, was added by Ronald Bank (3, 4).

Animal models of diabetes provide crucial insight into human diabetic disease. Most of the available models are based on rodents, because they are small, easy to handle, and economically advantageous, and have a short generation interval. Rodent
models of type I diabetes include the alloxane-induced, the streptozotocin induced, and the NOD mouse models and the bio-breeding (BB) rat model. Type 2 diabetic models include the genetically altered zucker diabetic fatty rats, otsuka long evans tokushima fatt rats, goto kakizaki rats, spontaneously diabetic tori rats, $ob/ob^{+/+}$ mice, and $db/db^{+/+}$ mice, which feature insulin resistance and reduced $\beta$-cell mass (Kim et al., 1998).

1.4.1 Animal model of Type 1 DM

Type 1 DM, a multifactorial autoimmune disease involving genetic and environmental factors, is hallmarked by T-cell and macrophages-mediated destruction of pancreatic $\beta$-cells, resulting in irreversible insulin deficiency (Antonios chatzigeorgiou, 2009). Diabetic ketoacidosis, a Type 1 DM immediate consequence, can be fatal without treatment, while the long-term vascular Type 1 DM complications affecting several organs and tissues can significantly affect life expectancy. There is no doubt that Type 1 DM susceptibility is MHC-dependent and MHC genes account for approximately 50% of the total contribution to the disease. However, although to date studies corroborate that both HLA-DR and HLA-DQ genes are important in determining disease risk, the effects of individual alleles may be modified by the haplotypes on which they are carried (Fernando et al., 2008). Besides, immunological, genetic and molecular pathways' differences in the establishment of autoimmune diabetes between animal models indicate that human Type 1 DM can be probably generated from more than one loss of tolerance pathways.

In humans, type 1 DM is characterized by the specific destruction of pancreatic $\beta$ cells. Alloxane, a uric acid derivative used to induce type 1 DM in rodents, selectively destroys pancreatic $\beta$-cells via the induction of oxidative stress, resulting in insulin deficiency and hyperglycemia (Rerup, 1970). Streptozotocin
(STZ), a nitrosoare derivative isolated from *Streptomyces achromogenes*, also destroys pancreatic β-cells via the same mechanism as alloxane (Junod et al., 1967; Yamamoto et al., 1981). The NOD mouse and BB rat, which spontaneously develop type 1-like disease, are the two most commonly used animal models. In NOD mice, insulitis develops at 4-5 weeks of age, followed by subclinical β-cell destruction and decreased circulating insulin concentrations. Diabetes typically presents between 12 and 30 weeks of age (Kolb and Kroneke, 1993). BB rats develop weight loss, polyuria, polydipsia, hyperglycemia, and insulinopenia at approximately 12 weeks of age, often at the time of puberty (Nakhhooda et al., 1977).

### 1.4.2 Animal model of Type 2 DM

Animals exhibiting a syndrome of insulin resistance and type 2 diabetes, with characteristics similar to humans, comprise a wide range of species with genetic, experimental or nutritional causation. Both genetically and chemically induced type 2 animal models are available. Obese zucker rats are the most widely used animal model of genetic obesity. They become noticeably obese between the third and fifth week of life. They also show hyperphagia, insulin resistance, dyslipidemia, mild glucose intolerance and hyperinsulinemia (Zucker 1962; Zucker et al., 1972). OLETF rats develop diabetes slightly later, at around 18-25 weeks of age, and this trait is inherited mostly in males. They exhibit innate polyphagia, mild obesity, hyperinsulinemia, hypertriglycerideridemia and impaired glucose tolerance at approximately 16 weeks of age (Kawano et al., 1992). Kuo kondo (KK) mice, a polygenic model of obesity and type 2 diabetes, exhibit hyperphagia, hyperinsulinemia and insulin resistance (Reddi et al., 1988). They show moderate obesity by 2 months of age and reach maximum weight at 4-5 months. Insulin resistance precedes the onset of obesity. The *db/db* ^+/+^ (diabetic) mice possess a leptin receptor mutation and are spontaneously hyperphagic, obese, hyperglycemic, hyperinsulinemic and insulin resistant within the
first month of life; they later develop hypoinsulinemia and hyperglycemia, with peak expression at 3-4 months of age (Shafrir, 1992). The \( ob/ob^{+/+} \) (obese) mice possess a mutation in the leptin gene, which is manifested as obesity, hyperglycemia, mildly impaired glucose tolerance and severe hyperinsulinemia (Dubuc, 1976). Of the chemically induced type 2 animal models, STZ-induced diabetic are characterized by hyperglycemia, a reduced number of pancreatic \( \beta \)-cells and insulin resistance (Bonner-Weir et al., 1981). A single injection of STZ (100 mg/kg, i.p.) can also be used to generate a mouse model of non-insulin dependent diabetes (Ito et al., 2001). Currently, high-fat diet-fed and low-dose STZ-treated rats are used for type 2 diabetes research (Srinivasan et al., 2005).

1.5 Herbal treatment of DM

Plants have always been an exemplary source of drugs and herbal drugs which have been investigated all over the world to treat diabetes (Kaushik et al., 2010; Hnatyszyn, 2002). Till today more than 1200 species of plants have been screened for activity on the basis of traditional knowledge (Hillay et al., 2006). Table 1.5 displays a list of plants and/or their active compounds tested in diabetic animals (induced by either STZ or alloxan). The natural products display several effects besides lowering blood glucose in these experimental models. In view of the lack of parallel studies of their toxicity, these models of diabetes induced by either alloxan or STZ are considered a screening step in the search for drugs for the treatment of diabetes (Kecskemeti et al., 2002).
Table 1.5 List of plants and/or their active compounds with putative antidiabetic effects tested in vivo models (Frode and Medeiros, 2008).

<table>
<thead>
<tr>
<th>Plant (family)</th>
<th>Material</th>
<th>Treatment</th>
<th>Drug-induced diabetes</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aegle marmelos</em> (Rutaceae)</td>
<td>EtOH/leaves</td>
<td>i.p., 14d</td>
<td>STZ-rat</td>
<td>Glucose, glycosylated hemoglobin, C-peptide, glucose</td>
</tr>
<tr>
<td></td>
<td>AE/Fruits</td>
<td>p.o., 30 d</td>
<td>STZ-rat</td>
<td>Glucose, Insulin</td>
</tr>
<tr>
<td><em>Aloe vera</em> (Liliaceae)</td>
<td>EtOH, isolated compounds / leaves EtOH/leaves</td>
<td>p.o., 28d</td>
<td>db/db mice</td>
<td>Glycosylated hemoglobin Alc</td>
</tr>
<tr>
<td><em>Averrhoa bilimbi</em> (Oxalidaceae)</td>
<td>AE, BuOH, EtOAc, Hex/leaves</td>
<td>p.o., 14 d</td>
<td>STZ-rat</td>
<td>Glucose, lipids</td>
</tr>
<tr>
<td><em>Baccharis trimera</em> (Myrtaceae)</td>
<td>AE, EtOH, BuOH/leaves</td>
<td>p.o., 7 d</td>
<td>STZ-mice</td>
<td>Glucose</td>
</tr>
<tr>
<td><em>Bryophyllum pinnatum</em> (Crassulaceae)</td>
<td>AE/leaves</td>
<td>p.o., i.p., AT</td>
<td>STZ-rat</td>
<td>Glucose</td>
</tr>
<tr>
<td><em>Canarium schweinfurthii</em> (Burseraceae)</td>
<td>MeOH/CH₂Cl₂/ stem barks</td>
<td>p.o., 14 d</td>
<td>STZ-rat</td>
<td>Glucose</td>
</tr>
<tr>
<td><em>Chamaemelum nobile</em> (Asteraceae)</td>
<td>AE/leaves</td>
<td>p.o., 15 d</td>
<td>STZ-rat</td>
<td>Glucose</td>
</tr>
<tr>
<td><em>Coscinium fenestratum</em> (Menispermaceae)</td>
<td>AAE/stem barks</td>
<td>p.o., 12 d</td>
<td>STZ-rat</td>
<td>Glucose, glycosylated hemoglobin, glycogen, lipids, oxidative stress</td>
</tr>
<tr>
<td><em>Hintonia standleyan</em> (Rubiaceae)</td>
<td>MeOH, isolated compounds/ stem barks</td>
<td>p.o., AT</td>
<td>STZ-rat</td>
<td>Glucose</td>
</tr>
<tr>
<td><em>Lycium barbarum</em> (Solanaceae)</td>
<td>Isolated compound / fruits</td>
<td>p.o., 21-26d</td>
<td>STZ-rat</td>
<td>Glucose, oxidative stress, GLUT4, insulin</td>
</tr>
</tbody>
</table>

1.6 Biochemical estimations of DM

1.6.1 Carbohydrate metabolic enzyme

The basis of abnormalities in carbohydrate and protein metabolism in diabetes is deficient action of insulin on its target tissues, resulting from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action (Devendra et al., 2004). In experimental diabetes, enzymes of glucose metabolism are markedly altered and produce hyperglycemia, which leads to pathogenesis of diabetic complications (Algandaby et al., 2010). Hepatic glucose production is the net result of the breakdown of glycogen (glycogenolysis) and synthesis of new glucose molecules from lactate, amino acids and glycerol (glucogenogenesis) in liver. In type 2 diabetes, gluconeogenesis is a main cause of the elevated hepatic glucose output, contributing 50–60% of the released glucose (Hundal et al., 2000; Tayek and Katz, 1996). The rate of gluconeogenesis is regulated by the activity of the key gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-phosphatase. Insulin inhibits gluconeogenesis by repressing the mRNAs that encode gluconeogenic enzymes (Pilkis and Granner, 1992).

1.6.2 Glycoproteins

Glycoproteins, a carbohydrate linked protein macromolecules found in the cell surface, serve as the principal component of animal cells. Alterations in glycoprotein level leads to the pathogenesis of diabetes mellitus (Michael and Fowler, 2008). Many studies confirm the involvement of glycoprotein in diabetic complications (Ramkumar et al., 2007). With increasing severity of diabetes, there is a parallel rise in glycoprotein levels (Gul Memon et al., 2008). During diabetes, utilization of glucose by insulin independent pathways leads to the synthesis of glycoprotein which may be a predictor of angiopathic complications (Neerati et al., 2011). An increase in
the biosynthesis and or a decrease in the metabolism of glycoproteins attributed to the
deposition of these materials in the basal membrane of pancreatic cells. In recent
times, many traditionally important medicinal plants have been tested for their
efficacy against impaired glycoprotein levels in diabetes (Sulaiman et al., 2012;
Suganya et al., 2012).

1.6.3 Lipid profile

Abnormalities in lipid profile are one of the most common complications in
diabetes mellitus found in 40% of diabetic cases (Kulkarni et al., 2012). Hyperlipidemia is metabolic complication of both clinical and experimental diabetes (Solano and Goldberg, 2006). Diabetes is known to affect large number of metabolic pathways, including lipid metabolism, by altering the activities of various enzymes involved in these pathways. Liver is an insulin dependent tissue, which plays a pivotal role in glucose and lipid homeostasis and is severely affected during diabetes. The insulin deficiency causes excessive breakdown of lipid in adipose depots, resulting in increased level of free fatty acids (Vijayaraghavan, 2010). Impairment of the biological action of insulin at the cellular level is believed to be a cardinal and possibly primary underlying metabolic defect in the development of the characteristic dyslipidemia observed in type 2 diabetes. The key components of diabetic dyslipidemia are elevated plasma LDL, VLDL, TGs, FFAs and HDL-C (Ansar et al., 2011). LDL is a strong risk factor for CVD, which is highly atherogenic and serves as a marker for atherosclerosis. Abnormalities in fatty acid metabolism may cause inappropriate accumulation of lipids and inflammatory mediators in muscle and liver as well as impairing β-cells function (Srinivasan and Pari, 2013).
1.6.4 Oxidative stress

Oxidative stress is thought to be a major risk factor in the onset and progression of diabetes. Many of the common risk factors, such as obesity, increased age, and unhealthy eating habits, all contribute to an oxidative environment that may alter insulin sensitivity either by increasing insulin resistance or impairing glucose tolerance. The mechanisms by which this occurs are often multifactorial and quite complex, involving many cell signaling pathways. A common result of both types of diabetes is hyperglycemia, which in turn contributes to the progression and maintenance of an overall oxidative environment (Rains and Jain, 2011). Macro and microvascular complications are the leading cause of morbidity and mortality in diabetic patients, but the complications are tissue specific and result from similar mechanisms (Ahmad et al., 2005), with many being linked to oxidative stress. There is a large body of clinical evidence correlating diabetic complications with hyperglycemic levels and length of exposure to hyperglycemia (Lasker, 1993). Oxidative stress can be defined as a state of imbalance toward the factors that generate reactive oxygen radicals (e.g., superoxide or hydroxyl radicals) and away from the factors that protect cellular macromolecules from these reactants including antioxidants like SOD, CAT, and Gpx. The factors that generate reactive oxygen species (ROS) exist as products of normal cellular physiology as well as from various exogenous sources. Mitochondria are thought to be the source of most cellular ROS, specifically superoxide radicals. The reactions that generate ATP in the mitochondria require electrons from reduced substrates to be passed along the complexes of the electron transport chain. In the presence of molecular oxygen, electrons that leak from this process react and form the free radical superoxide. Superoxide anions are significant mediators in numerous oxidative chain reactions and are also a precursor to many other ROS (Styskal et al., 2012). Other significant intracellular sources of ROS include NADPH oxidases (which generate superoxide), nitric oxide synthases
(nitric oxide), and lipoxygenases (fatty acid hydroperoxides) (Arshag et al., 2011; Halliwell and Gutteridge 1989). In addition, certain cell types within tissue systems may promote localized environments with elevated oxidative stress. For example, macrophages can produce localized oxidative stress as part of the inflammatory response (Federico et al., 2007). Thus, low levels of ROS are typical within both the cell and the higher order tissue and organ systems and some ROS (in particular superoxide and hydrogen peroxide) are required to support natural cellular function and regulate intracellular signaling (Pérez-Matute et al., 2009). However, excess ROS production (or reduced ROS regulation) can severely impair the cell and lead to macromolecular damage, dysfunction, and death. Table 1.6 shows biochemical estimations of DM.

**Table 1.6** shows biochemical estimations of DM.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate metabolic enzyme</td>
<td>Hexokinase, glucose-6-phosphatase, fructose-1,6-bisphosphatase, glycogen, glycogen phosphorylase and glycogen glycogen synthase</td>
</tr>
<tr>
<td>Glycoprotein</td>
<td>Hexose, hexosamine, fucose and sialic acid</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>TBARS, H₂O₂, SOD, CAT, GSH, GPx, GSSH</td>
</tr>
</tbody>
</table>

1.7 Streptozotocin
Streptozotocin (Streptozocin, STZ, CAS No. 18883-66-4) is a monofunctional nitrosourea derivative that was first isolated from *Streptomyces achromogenes* fermentation broth (Lewis and Barbiers, 1960; Herr et al., 1967; Vavra et al., 1960). It also has been synthesized by three different procedures: (i) from tetra-O-acetyl glucosamine hydrochloride (Herr et al., 1967), (ii) from d-glucosamine +N-nitrosomethyl carbamyl-azide (Hardegger et al., 1969), and (iii) from d-glucosamine N-methylurea (Hessler and Jahnke 1970). Its molecular structure, as described by Herr et al. (1967) is shown in Fig. 1.7, and corresponds to a 2-deoxy-D-glucose molecule substituted at C₂ with an N-methyl-N-nitrosourea group.

![Chemical structure of streptozotocin](image)

**Fig 1.7** Structure of streptozotocin

STZ is an antimicrobial agent and has also been used as a chemotherapeutic alkylating agent (Schein et al., 1967a and 1974b; White, 1963). In 1963, Rakieten et al. (Rakieten et al., 1963) reported that STZ is diabetogenic. Again, this insulinopenia syndrome, called 'STZ diabetes' (Schein et al., 1967a), is caused by the specific necrosis of the pancreatic β-cells and STZ has been the agent of choice for the induction of DM in animals ever since (Lenzen et al., 1996 and Arison et al., 1967).

### 1.7.1 Mechanism of action of STZ

STZ inhibits insulin secretion and causes a state of insulin dependent DM. Both effects can be attributed to its specific chemical properties, namely its alkylating potency in Table 1.7.1.

**Table 1.7.1** Chemical properties of STZ (Lenzen, 2008).
<table>
<thead>
<tr>
<th>STZ</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical name</td>
<td>2-Deoxy-2-(((methylnitrosoamino)carbonyl]amino)-D-glucopyranose</td>
</tr>
<tr>
<td>Chemical structure</td>
<td>Cytotoxic methylNitrosourea moiety (N-methyl-N-nitrosourea) attached to the glucose (2-deoxyglucose) molecule; glucosamine derivative</td>
</tr>
<tr>
<td>Chemical properties</td>
<td>Hydrophilic, β-cell toxic glucose analogue</td>
</tr>
<tr>
<td>Chemical reactivities</td>
<td>DNA alkylating agent, Protein alkylating agent, No donor</td>
</tr>
<tr>
<td>Mode of toxicity</td>
<td>DNA alkylation</td>
</tr>
</tbody>
</table>

**1.7.2 Beta cell selectivity of STZ**

STZ is a nitrosourea analogue in which the N-methyl-N-nitrosourea (MNU) moiety is linked to the carbon-2 of a hexose. The toxic action of STZ and chemically related alkylating compounds requires their uptake into the cells. Nitrosoureas are usually lipophilic and tissue uptake through the plasma membrane is rapid; however, as a result of the hexose substitution, STZ is less lipophilic. STZ is selectively accumulated in pancreatic beta cells via the low-affinity GLUT2 glucose transporter in the plasma membrane (Karunanayake et al., 1976; Tjälve et al., 1976). Thus, insulin producing cells that do not express this glucose transporter are resistant to STZ (Elsner et al., 2000). This observation also explains the greater toxicity of STZ compared with N-methyl-N-nitrosourea in cells that express GLUT2, even though both substances alkylate DNA to a similar extent (Elsner et al., 2000; Ledoux et al., 1986). The importance of the GLUT2 glucose transporter in this process is also shown by the observation that STZ damages other organs expressing this transporter, particularly kidney and liver (Weiss, 1982).

**1.7.3 Beta cell toxicity of STZ**
It is generally assumed that the toxicity of STZ is dependent upon the DNA alkylating activity of its methylnitrosourea moiety (Murata, et al., 1999; Ledoux et al., 1986; Uchigata et al., 1982), especially at the O6 position of guanine (Lenzen, 2007). The transfer of the methyl group from STZ to the DNA molecule causes damage, which along a defined chain of events (Pieper et al., 1999), results in the fragmentation of the DNA (Yamamoto et al., 1981). Protein glycosylation may be an additional damaging factor (Konrad and Kudlow 2002). In the attempt to repair DNA, poly (ADP-ribose) polymerase (PARP) is overstimulated. This diminishes cellular \( \text{NAD}^+ \), and subsequently ATP, stores (Uchigata et al., 1982 and Sandler and Swenne, 1983). The depletion of the cellular energy stores ultimately results in beta cell necrosis. Although STZ also methylates proteins (Bennett and Pegg, 1981; Wilson et al., 1988), DNA methylation is ultimately responsible for beta cell death, but it is likely that protein methylation contributes to the functional defects of the beta cells after exposure to STZ.

![Diagram](attachment://image.png)

**Fig. 1.7.3** Schematic representation of the toxic effects of the glucose analogues STZ in beta cells, which produce chemical diabetes (Lenzen, 2008).
Inhibitors of poly ADP-ribosylation suppress the process of DNA methylation. Thus, injection of nicotinamide and other PARP inhibitors in parallel with, or prior to the administration of STZ is well known to protect beta cells against the toxic action of STZ and to prevent the development of a diabetic state (Schein et al., 1967). Also, mice deficient in PARP are resistant to beta cell death mediated by STZ, in spite of DNA fragmentation. The absence of PARP prevents the depletion of the cofactor NAD\(^+\) and the subsequent loss of ATP (Pieper et al., 1999a,b; Masutani et al., 1999) and thus cell death.

The role of alkylation in beta cell damage has also been examined by the use of ethylating agents, which are less toxic than their methylating counterparts, on account of O\(^6\)-ethylguanine being less toxic than O\(^6\)-methylguanine (Delaney et al., 1995). The fact that N-ethyl-N-nitrosoureia and ethyl methane sulphonate are significantly less toxic to insulin-producing.

### 1.8 Antioxidant activity

Free radicals are highly reactive substances formed in the body's cells as a result of metabolic processes (Niki, 1992 and 2001). In 1954 Gerschman et al. first proposed the theory of free radical formation (Gerschman et al., 2001; Gutteridge and Halliwell, 2000). Free radicals are a molecule with one or more 35 unpaired electrons in its outer orbital (McCord, 2000). Many of these molecular species are oxygen (and sometimes nitrogen) centered. Oxygen free radicals and its non-radical products are associated with reactive oxygen species (Halliwell et al., 1992 and 1993). Free radicals are highly reactive, unstable molecules that react rapidly with adjacent molecules via a variety of reactions including: hydrogen abstraction (capturing), electron donation and electron sharing (McCord, 2000). Although free radicals play
an essential role in the body, they also can react with DNA, protein or lipids in the cell membrane and cause damage (Hawkins et al., 2001; Rodriguez-Amaya et al., 2010).

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants. Free radicals are responsible for causing a large number of diseases including cancer (Kinnula and Crapo, 2004), cardiovascular disease and diabetes (Singh and Jialal, 2006), neural disorders (Sas et al., 2007), alzheimer's disease (Smith et al., 2000), mild cognitive impairment (Guidi et al., 2006), parkinson's disease (Bolton et al., 2000), alcohol induced liver disease (Arteel, 2003), ulcerative colitis (Ramakrishna et al., 1997), aging (Hyun et al., 2006) and atherosclerosis (Upston et al., 2003).

In the human body, endogenous antioxidants help to protect tissues and organs from oxidative damage caused by reactive oxygen and reactive nitrogen species such as hydroxyl radicals (·OH), peroxyl radicals (·OOR), superoxide anion (O₂⁻), and peroxynitrite (ONO'O⁻). These endogenous antioxidative systems in the body include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and various nonenzymatic compounds such as selenium, α-tocopherol, and vitamin C (Wojcik et al., 2010).

ROS such as singlet oxygen (¹O₂), H₂O₂, O₂⁻ and OH radical are often generated as byproducts of biological reactions. These ROS create homeostatic imbalance which generate oxidative stress and cause cell death and tissue injury (Kakkar et al., 1992). Free radicals and ROS are well known inducers of cellular and pathological processes including diabetes, cell proliferation, inflammatory conditions and many disorders apart from aging processes (Aviram, 2000; Bland, 1995;
Halliwell, 1994; Senthilkumar et al., 2012). Some of the biological damage caused by ROS in the human body is outlined in Fig 1.8.

Fig. 1.8 Biological damage caused by ROS in human body (Lee et al., 2004).

ROS formed in vivo, such as superoxide anion, hydroxyl radical and hydrogen peroxide, are highly reactive and potentially damaging transient chemical species. These are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function. ROS are regulated by endogenous SOD, GPx and CAT but due to over production of reactive species, induced by exposure to external oxidant substances or a failure in the defense mechanisms, damage to cell structures, DNA, lipids and proteins (Anusha et al., 2011; Valko et al., 2006).
Enzymatic antioxidant defenses include SOD, GPx, CAT etc. Non-enzymatic antioxidants are ascorbic acid (vitamin C), \( \alpha \)-tocopherol (vitamin E), GSH, carotenoids, flavonoids, etc. All these act by one or more of the mechanisms like reducing activity, free radical scavenging and potential complexing of prooxidant metals and quenching of singlet oxygen (Stanner et al., 2004).

Synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) commonly used in processed foods have side effects and are carcinogenic (Branen, 1975; Ito et al., 1983). In recent years, the use of natural antioxidants present in food and other biological materials has attracted considerable interest due to their presumed safety, nutritional and therapeutic value (Ajila et al., 2007). Nutraceuticals are supposed to hold the key to a healthy society in the coming future. Antioxidants derived from fruits, vegetables, spices and cereals are very effective and have reduced interference with the body's ability to use free radicals constructively (Kahkonen et al., 1999; Wolfe et al., 2003). Natural antioxidants mainly come from plants in the form of phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols) ascorbic acid and carotenoids. The quest for natural antioxidants for dietary, cosmetic and pharmaceutical uses has become a major industrial and scientific research challenge over the last two decades.

Antioxidants play a vital role in both food systems as well as in the human body to reduce oxidative processes. In food systems, antioxidants are useful in retarding lipid peroxidation and secondary lipid peroxidation product formation, and thus help to maintain flavor, texture, and, in some cases, the color of the food product during storage. Antioxidants further reduce protein oxidation as well as the interaction
of lipid derived carbonyls with proteins that leads to alteration of protein functionality (Elias et al., 2008). Natural antioxidants such as vitamin C, tocopherols, herbal extracts like rosemary and sage, as well as tea extracts have already been commercialized as alternatives to synthetic antioxidants in food systems (Shahidi, 2000). Proteins and protein hydrolysates derived from sources like milk, soy, egg, and fish have also been shown to exhibit antioxidant activity in various muscle foods (Alam et al., 2013; Hagen and Sandnes, 2004; Pena-Ramos and Xiong, 2003; Sakanaka and Tachibana, 2006).

Apart from these, amino acids, peptides and proteins also contribute to the overall antioxidative capacity of cells and towards maintaining the health of biological tissues. For example, blood proteins are estimated to scavenge 10-50% of the peroxyl radicals formed in the plasma (Frei et al., 1988). Peptides such as carnosine, anserine, and glutathione are well-known for their endogenous antioxidative activity (Babizhayev et al., 1994). However, the antioxidant-prooxidant balance in human body can change with the progression of age and due to other factors such as environmental pollutants, fatigue, excessive caloric intake, and high fat diets. With advancing age, the plasma and cellular antioxidant potential as well as the absorption of nutrients, including antioxidants, gradually diminishes (Elmadfa and Meyer, 2008). Use of dietary antioxidants has been recognized as potentially effective to promote human health by increasing the body's antioxidant load.

The antioxidant testing can reveal various mechanisms of action, depending on features of the particular assay. Simple methods include free radical scavenging with use of colored, artificial stable free radicals such as 2,2 and prime;-azinobis-(3-ethylbenzothiazoline- 6-sulfonate) ABTS (Re et al., 1999) and DPPH (Molyneux,
2004), as well as transition metal reduction that can be monitored by colorimetry. The metal based methods include the reduction of ferric ions: FRAP and ferric thiocyanate assays (Aruoma, 2003; Halliwell, 1995), or molybdenum ion phosphomolybdenum assay (Prieto et al., 1999). These tests are easy and affordable and can be used in high throughput screening. Their main drawback is that their relevance to the real oxidizing life is somewhat limited. The first issue is the chemical context of the assays, which use artificial compounds or are conducted in unrealistic conditions. This problem is eliminated in methods based on naturally occurring ROS, but the fate of a free radical is observed either indirectly with chromophore reagents or with more expensive ESR techniques. The scavenging of (O$_2$), (OH), or NO can be observed (Aruoma, 2003; Darmon et al., 1992; Kim et al., 2010; Sreejayan and Rao, 1997).

Several assays based on a substrate degradation inhibition are available which can be used to find out whether the tested compound can really protect biomolecules from oxidative damage. Polyunsaturated lipids, proteins, components of nucleic acids, cellular membranes, microsomal fractions from different organs can serve as model systems to be protected. The oxidation can be initiated by various chemical, physicochemical or biological approaches.

1.9 Allyl sulfur compounds

Garlic (*Allium sativum*) is used traditionally as a complementary therapy in the treatment of several diseases such as diabetes, several forms of cancer and neurodegenerative conditions such as ischemic stroke (Banerjee and Moulik, 2002; Rahman, 2003; Saravanan and Ponmurugan, 2012). In addition, garlic has been reported to possess range of cardiovascular effects such as lowering of plasma cholesterol; inhibition of platelet aggregation as well as reducing of arterial blood
pressure (Ali and Thomson 1995). S-allyl cysteine sulphoxide (SACS), a main bioactive constituent of garlic is an organosulphur-containing amino acid (Kim et al., 2006 a,b). Similar to garlic extract, SACS is reported to be antioxidative (Herrera-Mundo et al., 2006); anti-cancer (Chu et al., 2007); antihepatotoxic (Hsu et al., 2006) and can also reduce the incidence of stroke (Kim et al., 2006a,b). In the cardiac context, Padmanabhan and Prince (2006) have reported that SACS mediates cardioprotection in myocardial infarction via its antioxidative properties by decreasing lipid peroxide products.

The active substance allicin (diallyl thiosulphate) is responsible for the typical pungent smell and for its therapeutic properties (Macpherson et al., 2005; Li, 2000; Song and Milner, 2001). Major sulfur containing compounds present in garlic are gamma-glutamyl-s-allyl-cysteine and S-allyl-L-cystein sulfoxides (alliin). These also act as precursors of several other compounds (Matsuura, 1997).

1.9.1 S-allyl-L-cysteine (SAC)

It is a water soluble organic compound. Its concentration tends to increase during extraction in aqueous medium as well as during long term storage. The pharmacokinetic property of SAC in animal studies has suggested that it was highly dependent on oral doses of SAC, which is a metabolite of SAC was detected in the urine of dogs and humans. The bioavailability of SAC was found to be 103% in mice, 98.2% in rats and 87.2% in dogs (Nagae et al., 1994).

1.9.2 Role of sulphur allyl compounds in antidiabetic activity

Diabetes is a common endocrine disorder which leads to metabolic disturbances (Ahmed and Ahmed, 2006; Pendsey, 2002). It is a known fact that adequate insulin secretion is required for curing diabetes. Investigators have been
trying alternative therapies using medicinal plants as an additional treatment beside insulin (Demerdash et al., 2005). Garlic contains variety of compounds whose beneficial effects have been already proven through various studies involving human and animal models. Most of the studies have shown that garlic can help reduce blood glucose levels in diabetic rats, mice, rabbits and increases plasma insulin secretion in diabetic rats (Banerjee and Maulik, 2002; Grover et al., 2002; Jamison, 2003; Patumraj et al., 2000). Allicin helps to increase the amount of CAT and GPx. It is also reported that another compound SACS (S-allyl cysteine sulfoxide) stimulates insulin secretion in β-cells of normal rats (Liu et al., 2005; Thomson et al., 2007).

It has been reported that long term consumption of natural flavonoids such as quercetin helps to prevent glycation of collagens, which is believed to be a leading causative factor for the development of cardiovascular complication in diabetic patients (Urios et al., 2007). A study was conducted on diabetic women from USA to assess the use of natural remedies for treating type II diabetes. Garlic obtained the highest response as a commonly used vegetable (Johnson et al., 2006).

The bioactive components from garlic such as SACS exhibited hypoglycemic effect by stimulating insulin production or interfering with dietary glucose absorption (Srinivasan, 2005).

1.10 Molecular docking

Docking aims to accurately predict the structure of a ligand within the constraints of a receptor binding site and to correctly estimate the strength of binding (Waszkowycz et al., 2011).
Molecular recognitions including enzyme-substrate, drug-protein, drug-nucleic acid, protein-nucleic acid, and protein-protein interactions play important roles in many biological processes such as signal transduction, cell regulation, and other macromolecular assemblies. Therefore, determination of the binding mode and affinity between the constituent molecules in molecular recognition is crucial to understanding the interaction mechanisms and to designing therapeutic interventions. Due to the difficulties and economic cost of the experimental methods for determining the structures of complexes, computational methods such as molecular docking are desired for predicting putative binding modes and affinities. In molecular docking, based on the protein structures, thousands of possible poses of association are tried and evaluated; the pose with the lowest energy score is predicted as the –best match‖, i.e., the binding mode. Since Kuntz and colleagues‘ pioneering work (Kuntz et al., 1982), significant progress has been made in docking research to improve the computational speed and accuracy. Among them, protein-ligand docking is a particularly vibrant research area because of its importance to structure-based drug design (Brooijmans and Kuntz, 2003; Halperin et al., 2002; Kitchen et al., 2004; Kolb et al., 2009; Muegge and Rarey, 2001; Shoichet et al., 2002; Sousa et al., 2006).
Fig 1.10.2. Classification of the methods for protein-ligand docking (Kolb et al., 2009).

A protein-ligand docking program consists of two essential components, sampling and scoring. Sampling refers to the generation of putative ligand binding orientations/conformations near a binding site of a protein and can be further divided into two aspects, ligand sampling and protein flexibility. Scoring is the prediction of the binding tightness for individual ligand orientations/conformations with a physical or empirical energy function.

Fig 1.10.3. Molecular docking flow chart (Shoichet et al., 2002).

The top orientation/conformation, namely the one with the lowest energy score, is predicted as the binding mode. Here, the recent advances in protein-ligand docking on three important aspects: protein flexibility, ligand sampling, and scoring function, as illustrated in Figure 1.10.2 and 1.10.3.
Table 1.10 List of docked natural and synthetic compounds against α-Glucosidase.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Enzyme</th>
<th>Aminoacid Interacting to ligand</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pistagremic acid</td>
<td>α-glucosidase</td>
<td>Asp60, Arg69 and Asp 70</td>
<td>Uddin et al., 2012</td>
</tr>
<tr>
<td>Aryl-1,2,3-triazoles</td>
<td>α-glucosidase</td>
<td>Asp 114 and Arg89</td>
<td>Zhou et al., 2008.</td>
</tr>
<tr>
<td>Salacinol</td>
<td>α-glucosidase</td>
<td>Asp327 and His600</td>
<td>Nakamura et al., 2010.</td>
</tr>
<tr>
<td>2-aminopyrimidines</td>
<td>α-glucosidase</td>
<td>Asp 123 and Arg 81</td>
<td>Nakamura et al., 2010.</td>
</tr>
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<td>D- and L-isofagomine</td>
<td>α-glucosidase</td>
<td>Asp127 and Trp179</td>
<td>Kato et al., 2011</td>
</tr>
<tr>
<td>N-(1,3-Diaryl-3-oxopropyl)amides</td>
<td>α-glucosidase</td>
<td>Lys771, Phe1013, and Met770</td>
<td>Nepali et al., 2011.</td>
</tr>
<tr>
<td>Kotalanol</td>
<td>α-glucosidase</td>
<td>Trp406 and Phe450</td>
<td>Tanabe et al., 2012.</td>
</tr>
</tbody>
</table>

1.11 Histopathology

Histology is the study of the microscopic anatomy of cells and tissues of plants and animals. It is commonly performed by examining cells and tissues by sectioning and staining, followed by examination under a light microscope or electron microscope. Histological studies may be conducted via tissue culture, where live cells can be isolated and maintained in a proper environment outside the body for various research projects. The ability to visualize or differentially identify microscopic structures is frequently enhanced through the use of histological stains. Histology is an essential tool of biology and medicine (Mitchell et al., 2009). Histopathology, the microscopic study of diseased tissue, is an important tool in anatomical pathology,
since accurate diagnosis of diabetes, cancer and other diseases usually requires histopathological examination of samples.

Considering the above advantages of allyl sulphur compounds in therapeutics application, the present works was undertaken to synthesis and explore the potential utilization of allyl sulphur compound with the following objectives.

**Aim**

The aim of this study is to design and synthesize 2-allyl amino 4-methyl sulfanyl butyric acid (AMSB) and to evaluate its antidiabetic role in STZ induced experimental diabetic rats.
Objectives of the study

- To synthesize and characterize 2-allyl amino 4-methyl sulfanyl butyric acid (AMSB) and to evaluate its \textit{in vitro} antioxidant and antidiabetic properties (\(\alpha\)-amylase and \(\alpha\)-glucosidase inhibitory activity).

- To study the docking of AMSB with \(\alpha\)-glucosidase.

- To induce DM in male albino wistar rats using STZ (\textit{in vivo} model) and study the effect of AMSB on the following
  
  (i) Effect of AMSB on the carbohydrate metabolic enzymes and lipid profile

  (ii) Evaluating the effect of AMSB on protein metabolism and glycoproteins components and

  (iii) To evaluate AMSB on the oxidative stress in tissues such as pancreas, liver and kidney.
Synthesis, characterization and evaluation of in vitro antioxidant and antidiabetic properties of novel 2-allyl amino 4-methyl sulfanyl butyric acid

ABSTRACT

In the present study 2-allyl amino 4-methyl sulfanyl butyric acid (AMSB) was synthesized in good yield. AMSB was characterized by FTIR, NMR (\textsuperscript{1}H and \textsuperscript{13}C) and LCMS. The radical scavenging activity and reducing power assay of AMSB was assessed using DPPH, ABTS and FRAP assay and was found to be 44.1, 34.71 and 41.7 \textmu g/ml respectively. The synthesized compound showed effective inhibition against \textalpha-amylase and \textalpha-glucosidase. AMSB was identified to be a reversible mixed noncompetitive inhibitor of \textalpha-amylase and \textalpha-glucosidase. The molecular docking study was carried out to evaluate the specific groove binding properties and affords valuable information of AMSB binding mode in the active site of \textalpha-glucosidase. AMSB has strong potential to be further investigated as a new lead compound for better management of diabetes.
1.1 INTRODUCTION

DM is a chronic metabolic disorder characterized by hyperglycemia, resulting from insufficient or inefficient insulin secretion, with alterations in carbohydrate, protein and lipid metabolism. The reports indicate that hyperglycemia could induce non-enzymatic glycosylation of various proteins, resulting in the development of chronic complications in diabetes (Lebovitz 2001). Therefore control of postprandial blood glucose is critical for treatment of diabetes and reducing chronic vascular complications (Lebovitz, 2001; Baron, 1998). The \( \alpha \)-amylase and \( \alpha \)-glucosidase inhibitors may help to reduce postprandial hyperglycemia by partially inhibiting the enzymatic hydrolysis of complex carbohydrates, and hence may delay the absorption of glucose. Acarbose, voglibose and miglitol are widely used, either alone or in combination with insulin secretagogues, for patients with type II diabetes (Saito et al., 1998). The \( \alpha \)-amylase inhibitors were used as a target for drug-designing in the treatement of diabetes, obesity and hyperlipdemia (Heidari et al., 2005). Disaccharides, iminosugars, carbasugars, thiosugars and non-sugar derivatives are among the various types of glycosidase inhibitors (Braun et al., 1995; Withers and Umezawa, 1991).

Antioxidants are important in diabetes, with low levels of plasma antioxidants implicated as a risk factor for the development of the disease (Facchini et al., 2000; Pisanti et al., 1988; Salonen et al., 1995) and circulating levels of radical scavengers impaired throughout the progression of diabetes (Godin et al., 1988). Many of the complications of diabetes, including retinopathy and atherosclerotic vascular disease, the leading cause of mortality in diabetics, have been linked to oxidative stress (Baynes, 1991). Reactive oxygen species (ROS) induce programmed cell death or necrosis, induce or suppress the expression of many genes and activate cell signaling
cascades (Hancork and Desikan 2001). Because of the significance of free radicals and ROS in the pathogenesis of multifarious diseases antioxidants are gaining considerable attention as drug candidates to counter these diseases.

Sulphur is one of the essential nutrients required for plant growth (Ahmad and Abdin, 2000) and forms an important component of sulphur compounds including amino acids (Scherer 2001). S-allylcysteine is a sulphur containing amino acid derived from garlic, has been reported to have antidiabetic and anti-lipidemic activities (McKenna et al., 2002), antioxidant (Herrera-Mundo et al., 2006), anticancer (Chu et al., 2007) and neurotrophic properties (Moriguchi et al., 1997). A design of effective α-amylase and α-glucosidase inhibitors based on allyl sulphur with a high specificity and potency may revolutionize the treatments of DM. The present study was designed to synthesize antidiabetic molecule with sulphur containing amino acid, methionine. The resultant molecule synthesized by combining the L-methionine (2-amino-4-(methyl thio) butanoic acid) and 3-bromopropene was identified to be 2-allyl amino 4-methyl sulfanyl butyric acid (AMSB) which was found to be a promising candidate for in vitro α-amylase and α-glucosidase inhibition activity.

1.2 MATERIALS AND METHODS

1.2.1 Chemicals

DPPH, ABTS, α-amylase (EC 3.2.1.1), α-glucosidase (EC 3.2.1.20) and p-nitrophenyl-α-D-glucopyranoside (PNPG) were purchased from Sigma-Aldrich, U.S.A. 3-bromopropene and L-methionine was obtained from Himedia chemicals, India and all other chemicals were of analytical grade.
1.2.2 Materials and instrumentation

Melting point (Mp) of synthesized compound (AMSB) was determined in open capillary with an Electrothermal MEL-TEMP 3.0. FTIR analysis was carried out using Perkin Elmer Spectrophotometer and changes in % transmission at different wave lengths were observed from 4000-400 cm\(^{-1}\). The structure of the product (AMSB) was determined by \(^1\)H and \(^{13}\)C NMR spectra using Bruker, UltraShield plus 400 (400 MHz) NMR spectrometer. The mass spectrum was recorded on LC-MS-UPLC-TQD (ESI and APCI) instrument.

1.2.3 Synthesis of 2-allyl amino 4-methyl sulfanyl butyric acid (AMSB)

2-allyl amino 4-methyl sulfanyl butyric acid was synthesized by the method of Nishimura and Mizutani (1975) with slight modification. The ice cooled solution of 2-amino-4-(methylthio) butanoic acid, 10.0 g, 0.082 M in NH\(_3\)OH (2 M, 240 ml) was added 3-bromopropene (15.0 g, 0.12 M) with vigorous stirring. The mixture was stirred at 0\(^\circ\)C for 40 min and filtered. The filtrate was concentrated in vacuo (40\(^\circ\)C) to a small volume, and again filtered. The solid was washed repeatedly with ethanol, dried in vacuo, and recrystallized with water / ethanol (5:6) to yield white crystal needles of AMSB.

1.2.4 Determination of antioxidant activity

1.2.4.1 Determination of 1-1-diphenyl 2-picryl hydrazyl (DPPH) radical-scavenging activity

The free radical scavenging activity of the AMSB was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH (Blios, 1958). Solution of DPPH in ethanol (0.1 mM) was prepared and 1.0 ml of this solution was added to 2.0 ml of AMSB solution at different concentrations
(20-100 μg/ml). Thirty minutes later, the absorbance was measured at 517 nm. α-tocopherol and ascorbic acid were used as the positive control. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula:

\[
D = \frac{(A_{ini} - A_{obs})}{A_{ini}} \times 100
\]

Where \( A_{ini} \) was the absorbance of the control (blank, without AMSB) and \( A_{obs} \) was the absorbance in the presence of the AMSB.

1.2.4.2 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) radical cation assay

The spectrophotometric analysis of ABTS radical-scavenging activity was determined according to the method of Re et al., (1999). The ABTS cation radical was produced by the reaction between 7 mM ABTS in H₂O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12-16 h. Before usage, the ABTS solution was diluted to get an absorbance of 0.450 ± 0.001 at 745 nm with water. Different concentrations of AMSB (20-100 μg) were prepared. A total of 20 μl of each were added to 180 μl of ABTS solution (in triplicate) and absorbances were recorded for ABTS, every 5 min during a 20 min period. The assay was first carried out on α-tocopherol and ascorbic acid which served as standard. The percentage of inhibition was measured by the following formula:

\[
\text{Inhibition (\%)} = \frac{A_o - A_t / A_o}{100}
\]

Where \( A_o \) was the absorbance of the control (blank, without AMSB) and \( A_t \) was the Absorbance in the presence of the AMSB.
1.2.4.3 Ferric ion reducing antioxidant power assay (FRAP)

The reducing power of AMSB was determined using the method of Yen and Chen, (1995). The different concentrations of samples (20-100 µg/ml) suspended in 0.2 M, phosphate buffer (pH 6.6) were mixed with 0.125 ml of potassium ferricyanide (1%, w/v) and incubated at 50°C. At 20 min, 0.125 ml of trichloroacetic acid (10%, w/v) was added to the mixture to terminate the reaction. The solution was mixed with 1.5 ml of ferric chloride (0.1%, w/v), and the absorbance was measured at 700 nm. An increased absorbance of the reaction mixture indicated increased reducing power.

1.2.5 α-amylase inhibition assay

The α-amylase inhibition was determined by a modified method of Worthington Biochemical Corp., (1993a). A total of 20-100 µl of AMSB and 500 µl of 20 mM sodium phosphate buffer (pH 6.9) containing α-amylase solution (1.0 U/ml) were incubated at 25°C for 10 min. After preincubation, 500 µl of a 1% starch solution in 20 mM sodium phosphate buffer (pH 6.9) was added to each tube at timed intervals. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1 ml of dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 15 ml of double distilled water, and absorbance was measured at 540 nm. The reference sample included all other reagents and the enzyme with the exception of the test sample. The α-amylase inhibitory activity was expressed as percentage inhibition. The α-amylase inhibitory activity was calculated according to the equation below:
\[ D = \frac{A_{ini} A_{obs}}{A_{ini}} \times 100 \]

Where \( D \) is the (%) inhibition of AMSB, \( A_{ini} \) is the area under the curve of the absorption spectrum at 540 nm at time zero and \( A_{obs} \) is the area under the curve of the absorption spectrum at 540 nm at a determined time.

### 1.2.6 \( \alpha \)-glucosidase inhibition assay

Briefly, appropriate dilutions of the AMSB (20-100 μl) and 100 μl of \( \alpha \)-glucosidase (1.0 U/ml) in 100 mM phosphate buffer (pH 6.9) solution were incubated at 25°C for 10 min. Then, 50 μl of 5 mM p-nitrophenyl-\( \alpha \)-D-glucopyranoside (PNPG) in 100 mM phosphate buffer (pH 6.9) solution was added. The mixtures were incubated at 25°C for 5 min, before reading the absorbance at 405 nm in the uv-vis spectrophotometer (Apostolidis et al., 2007). The \( \alpha \)-glucosidase inhibitory activity was expressed as percentage inhibition.

\[
\% \text{ inhibition} = \left[ \frac{(A_{405} - A_{e405})}{A_{405}} \times 100 \right]
\]

Where \( A_{405} \) = absorbance without compound of AMSB; \( A_{e405} \) = absorbance with AMSB.

### 1.2.7 Kinetics of inhibition

The kinetic inhibition of \( \alpha \)-amylase and \( \alpha \)-glucosidase by AMSB was determined by using lineweaver-burk (LB) and Dixon plot equations. Soluble starch, in the range 0.5-2 % and PNPG, in the concentration range 1-4 mM, were used as substrates for \( \alpha \)-amylase and \( \alpha \)-glucosidase, respectively. Enzyme activities were determined in the absence or presence of different concentrations of AMSB. The concentrations of AMSB used for the inhibitory kinetics of \( \alpha \)-amylase and \( \alpha \)-glucosidase were 20-80 μg/ml. The values of initial velocities (\( V_o \)) were determined
from the slope of the linear part of the progress curves. The values of kinetic parameters \( K_m, V_{\text{max}}, \) and \( K_i \) were determined according to the type of inhibition for each enzyme reaction with different AMSB concentrations by both LB and dixon plot (Bowden, 1974 and Dixon, 1953).

1.2.8 Protein structure and synthetic compounds

The Structure for \( \alpha \)-glucosidase (PDB ID: 3CTT) and synthetic drugs were retrieved from protein data bank (www.rcsb.org/pdb) and pubchem (http://www.ncbi.nlm.nih.gov/pccompound) database respectively.

1.2.8.1 Binding site prediction

The binding site for the retrieved structure (PDB ID: 3CTT) was performed using online server Qsite-finder (http://www.modelling.leeds.ac.uk/qsitefinder/).

1.2.8.2 Docking

Flexible docking of synthetic compounds (already in use for diabetes) onto the active site of the \( \alpha \)-glucosidase protein was performed using GLIDE software package (http://www.schrodinger.com/). Glide searches for favorable interactions between one or more typically small ligand molecules and a larger receptor molecule usually a protein. Each ligand must be a single molecule, while the receptor may include more than one molecule e.g. a protein and a cofactor. GLIDE can be run in rigid or flexible docking modes; the later automatically generates conformation for each input ligand. The combination of positions and orientation of the ligand relative to the receptor, along with its conformation in flexible docking, is referred to as a ligand pose. The ligand poses that GLIDE generates pass through a series of hierarchical filters that evaluate the ligand interaction with the receptor. The initial filters test the spatial fit of the ligand to the defined active site and examine the complimentarity of ligand-
receptor interactions using the GRID based method after the empirical chemscore function.

1.2.9 Statistical analysis

Analysis at every time point from each experiment was carried out in triplicates. The results were statistically analysed by one way ANOVA and Duncan’s multiple range tests. Statistical significance was accepted at a level of $P < 0.05$.

1.3 RESULTS AND DISCUSSION

1.3.1 Synthesis and spectroscopy

Scheme 1.1 illustrates the method used for the preparation of new synthetic compound, AMSB. The AMSB was prepared according to the method of Nishimura and Mizutani (1975) reported for the synthesis of S-(2-Propenyl)-L-cysteine and was obtained as white needles with a yield of 79%. Structural conformation of AMSB was done using FTIR, NMR ($^1$H and $^{13}$C), Mass spectra and elemental analysis. The melting point (Mp) was determined to be 259-265°C. Fig 1.1 shows the FTIR spectrum of compound AMSB whose absorption band at 3783 cm$^{-1}$ corresponds to N-H stretching frequency, the peaks at 2931 and 2591 cm$^{-1}$ represents C-H stretching bands. The absorption peaks at 1589, 1506 and 1407 cm$^{-1}$ corresponds to COO stretching and absorption at 753 cm$^{-1}$ corresponds to S-C stretching frequency. The NMR ($^1$H and $^{13}$C) spectrum of the compound was recorded in (D$_2$O) solvent. The $^1$H NMR spectra of AMSB showed multiplet at $\delta$ 5.39-5.4 indicating the presence of allyl –CH$_2$ proton and multiplet appearing at $\delta$ 5.8, shows the presence of =C-H proton. The singlet appearing at $\delta$ 3.12 indicate the presence of CH$_3$-S proton attached to the sulphur, besides triplet at $\delta$ 3.5-3.54 indicated the presence of CH$_2$ proton. The appearance of triplet at $\delta$ 2.52-2.55 was due to CH proton. The CH$_2$ proton appeared
as multiplet at about 2.00-2.11 ppm (Fig 1.2 a and b). Moreover, $^{13}$C NMR spectra showed the signals in the range of δ 174.16 ppm and at δ 127.22 ppm, which may be due to the presence of -COOH and =C-H carbon, respectively. The peak appearing in the range of δ 124.18 and 60.45 ppm are due to =CH$_2$ and -CH carbon, besides the peak range at 28.65-53.88 ppm corresponds to -CH$_2$ carbon (Fig. 1.3). Fig 1.4 shows the AMSB structure confirmation by mass spectrum through the appearance of molecular ion peak at m/z: 189.08 [$M^+$] correlating with the calculated molecular weight of empirical formula C$_8$H$_{15}$NO$_2$S. The elemental analyses of AMSB were found to be C, 50.77; H, 7.76; S, 16.55; N, 7.10. FTIR, NMR, mass spectra and elemental analysis results are in agreement with the proposed structure.

1.3.2 Biological activities

The newly synthesized AMSB was screened for their in vitro biological activities such as antioxidant (DPPH, ABTS and FRAP) and α-amylase and α-glucosidase inhibition assay. The mode of inhibition of AMSB on α-amylase and α-glucosidase were studied using LB and Dixon plot.

1.3.2.1 In vitro antioxidant activities

Antioxidants have been recognized to exhibit protective functions against oxidative damage and are associated with reduced risk of chronic diseases (Liu and Adom, 2002). Fig. 1.6 and 1.7 shows free radical scavenging activity of AMSB at varying concentration of 20-100 μg/ml. α-tocopherol and ascorbic acid were taken as a positive control (standard) and L-methionine was used as negative control. AMSB shows an IC$_{50}$ values of 44.1 μg/ml and 34.71 μg/ml towards DPPH and ABTS respectively. The positive control, ascorbic acid and α-tocopherol show an IC$_{50}$ value of 16 μg/ml and 18 μg/ml respectively. L-methionine was used as negative control and showed no free radical scavenging activity (Fig. 1.5 and 1.6). The results showed
antioxidant activity of AMSB in dose dependent manner. The antioxidant activity of AMSB was also determined by measuring its ability to transform Fe$^{3+}$ to Fe$^{2+}$. Fig. 1.7 shows the dose response curve for the reducing activity of AMSB, α-tocopherol, ascorbic acid and L-methionine. The inhibition concentration (IC$_{50}$) of AMSB was found to be 41.7 μg/ml, whereas 20 μg/ml and 18 μg/ml were needed for α-tocopherol and ascorbic acid respectively. Several reports reveal the antioxidant capacity of sulfur containing amino acids with allyl group (Atmaca, 2004; Kim et al., 2006; Powolny and Singh, 2008).

**1.3.3 Inhibitory effect of AMSB against α-amylase and α-glucosidase**

Inhibition of enzymes involved in the metabolism of carbohydrates such as α-amylase and α-glucosidase are important therapeutic approach for reducing postprandial hyperglycemia (Shim et al., 2003). The inhibitory effects of AMSB, acarbose (standard) and L-methionine (control) on α-amylase and α-glucosidase are shown in Fig.1.8 and 1.9. The results revealed that, AMSB inhibited α-amylase and α-glucosidase activity by dose dependent manner (20-100 μg/ml), the IC$_{50}$ values of AMSB on α-amylase (51.2 μg/ml) and α-glucosidase (49.6 μg/ml) are shown in Table 1.1. L-methionine was used as negative control and showed no inhibitory activity (Fig. 1.8 and 1.9). Ghosh et al., (2012) reported that α-amylase and α-glucosidase inhibitors are class of compounds that help in control of diabetes by diminishing the absorption of glucose. α-amylase and α-glucosidase are two key enzymes related to carbohydrate digestion and elevation of blood glucose. It is now believed that inhibition of these enzymes can be an important strategy in the management of hyperglycemia by retarding the postprandial increase of blood glucose level after a mixed carbohydrate diet (Puls et al., 1977). According to numerous in vivo studies,
inhibition of α-amylase and α-glucosidase is believed to be one of the most effective approaches for diabetes care (Etxeberria et al., 2012; van de Laar, 2008).

1.3.4 Kinetic mechanism of α-amylase and α-glucosidase inhibition of AMSB

In order to study the type of inhibition for α-amylase and α-glucosidase enzymes, AMSB was used as the target compound for the determination of kinetic parameters ($K_m$, $V_{max}$ and $K_i$). The LB plot was performed to identify the mode of inhibition of AMSB. Fig. 1.10A and B shows that double reciprocal plots with straight lines were intercepted at a single point in the second quadrant exhibiting mixed non competitive inhibition. Starch and PNPG were used as substrates for α-amylase and α-glucosidase respectively, to identify the $K_m$ and $V_{max}$ by LB plot (Table 1.1), subsequently inhibitor constant $K_i$ was found by Dixon plot. The $K_i$ value of α-amylase and α-glucosidase were found to be 28.8 and 18.8 μg respectively (Fig. 1.11A and B). Inhibition of α-amylase and α-glucosidase by different classes of compounds were described in the literature (Kim et al., 2005; Shim et al., 2003; Tadera et al., 2006). Apostolidis et al., (2007) reported that kinetic inhibition of porcine pancreatic α-amylase by acarbose, maltose and maltotriose was mixed non-competitive type inhibition.
1.3.5 Active site prediction

In *in vitro* study of AMSB showed high inhibition activity of AMSB against α-glucosidase when compared to α-amylase (please refer Fig. 1.8 and 1.9). Based on this result α-glucosidase was selected for molecular docking study with AMSB. Three dimensional structure of α-glucosidase (PDB ID: 3CTT) was retrieved from protein data bank (Fig. 1.12). The ligand molecule, glimepiride and N-Acetyl-D-glucosamine bound to the structure were removed for further studies. The active site of α-glucosidase protein was found using Q site finder. The active site residues are PRO284, ALA285, LEU286, PRO287, SER288, ALA291, ARG520, SER521, PHE522, ILE523, LEU524, GLY533, LYS534, PHE535, ALA536, ALA537, HIS538, GLY564, ILE565, PRO566, MET567, PHE641, and LYS776. The active site volume of α-glucosidase was found to be 365 Å³ of the total 84383 Å³ volume of the protein.

1.3.5.1 α-glucosidase-ligand interaction

Rational drug design helps to facilitate and speedup the drug designing process, which involves variety of methods to identify novel compounds. One such method is the docking of the drug molecule with the receptor (target). The α-glucosidase was docked with AMSB and other synthetic drugs using Glide. The α-glucosidase was preprocessed before docking and a grid was generated for the active sites of protein to which the ligand is to be bound. Docking was performed in extra precision and the binding of ligand to the protein were listed out in the XP visualizer. The interaction of the ligand with the α-glucosidase was shown and the number of hydrogen bond, glide score, bond length and residues of interaction were noted for every ligand with the target α-glucosidase. The result was tabulated in the Table 1.2. AMSB has the lowest docked binding energy of -5.23 Kcal/mol and forms
three hydrogen bonds with active site residue SER288, SER521 and ILE523 respectively (Fig 1.13A). Fig 1.13B shows glimepiride having binding energy of -5.18 kcal/mol and forms two hydrogen bonds with active site residues PHE535 and PHE535 whereas, N-acetyl-D-glucosamine binding energy of -5.06 kcal/mol and forms one hydrogen bond with active site residue LYS534 respectively (Fig 1.13C). These interactions are in conjunction with other glucosidase inhibitor studies (Bharatham et al., 2008; Rodbard et al., 2009). The drug developing industries uses synthetic drugs glimepiride and N-Acetyl-D-Glucosamine against the $\alpha$-glucosidase protein (3CTT). Through the docking analysis, we have showed that the new drug namely AMSB showing much better results than the glimepiride and N-acetyl-D-glucosamine.

**1.4 CONCLUSION**

In conclusion we have successfully designed and synthesized AMSB. The AMSB exhibited potent free radical scavenging ability and reducing power assay as evident by DPPH, ABTS and FRAP assays. The results from *invitro* study clearly indicated AMSB had inhibitory activity against $\alpha$-amylase and $\alpha$-glucosidase. The kinetic study of AMSB shows effective mixed noncompetitive inhibiton against $\alpha$-amylase and $\alpha$-glucosidase. In the present study we conclude that AMSB is one of the potential drug target compound similar to available commercial drugs by performing *in silico* docking studies.
Scheme 1.1 Reaction protocol for the synthesis of 2-allyl amino 4-methyl sulfanyl butyric acid (AMSB).

Fig 1.1 FTIR spectrum of AMSB.
Fig 1.2 (a) $^1$H NMR spectrum of AMSB.

Continuation of Fig 1.2 (a)

(b)

Fig 1.2 (b) $^1$H NMR spectrum of AMSB.
Fig 1.3 $^{13}$C NMR spectrum of AMSB.

Fig.1.4 The molecular mass of the AMSB, mass spectrum determined by LC-ESI-MS with the range of the scan at m/z 100-540.
**Fig. 1.5** DPPH radical scavenging activities of AMSB in comparison with \( \alpha \)-tocopherol, ascorbic acid and L-methionine.

**Fig 1.6** ABTS radical scavenging activities of AMSB in comparison with \( \alpha \)-tocopherol, ascorbic acid and L-methionine.
Fig 1.7 Antioxidant activity of FRAP in comparison with α-tocopherol, ascorbic acid and L-methionine.

Fig 1.8 Inhibitory effect of different concentrations of AMSB on α-amylase.
Fig 1.9 *In vitro* effect of AMSB on α-glucosidase.

Fig 1.10 LB plot analysis of the inhibition kinetics of A) α-amylase and B) α-glucosidase inhibitory effects by AMSB [I].
Fig 1.11 Dixon plot for determining the kinetic constants for A) α-amylase and B) α-glucosidase, substrate concentration [S] were indicated.

Fig 1.12 Three dimensional structure of α-glucosidase (PDB ID: 3CTT)
**Fig 1.13** Docking of (A) AMSB, (B) glimepiride and (C) N-acetyl-D-glucosamine with α-glucosidase showing ligand forming hydrogen bonds.
Table 1.1 Kinetic properties and type of inhibition of AMSB on α-glucosidase and α-amylase.

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<th>Parameters</th>
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<tr>
<td>$K_i$</td>
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</tr>
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<td>IC$_{50}$</td>
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Table 1.2 Docking of AMSB and synthetic compounds against α-glucosidase (3CTT).

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<th>S. No</th>
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<td>-3.52</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Acarbose (Nakamura et al., 2010)</td>
<td>-6.63</td>
<td>2</td>
<td>GLU276 and ASP349</td>
</tr>
</tbody>
</table>
Effect of AMSB on carbohydrate and lipid metabolism in STZ induced experimental diabetic rats

ABSTRACT

The male albino wistar rats were randomly divided into four groups with six animals in each group. DM was induced by intraperitoneal injection of streptozotocin (STZ) (55mg/kg). After induction of diabetes, rats were treated with AMSB (150 mg/kg body weight) for 45 days. The glucose level was studied after 72 hrs to ratify the development of DM. Before the post treatment, the blood glucose level was increased whereas body weight was decreased. The biochemical estimations like lipid profile, fatty acid, phospholipids of plasma and tissues (liver and kidney) and carbohydrate metabolic enzymes were performed. Administration of AMSB resulted in significant reduction in blood glucose, TC, TG, LDL, VLDL, free fatty acids and phospholipids. In addition, significant elevation in body weight and HDL was also observed in AMSB treated rats. On the other hand, the activity of hexokinase, glycogen and glycogen synthase were significantly increased and reduction in glucose-6-phosphatase, fructose-1,6-bisphosphatase and glycogen phosphorylase were also observed. The antihyperlipidemic effect of AMSB was compared with glibenclamide a well known antidiabetic and antihyperlipidemic drug. The results demonstrate that AMSB possesses antihyperlipidemic effect in addition to its antihyperglymeic effect on STZ induced diabetic rats.