INTRODUCTION

DIABETES HEALTH
IT'S IN YOUR HANDS

OBJECTIVES
The term diabetes was coined by Areteaus of Cappadocia. Thomas Willis added the word mellitus, from the Latin meaning "honey" a reference to the sweet taste of the urine. Areteaus did attempt to treat it but could not give a good prognosis, he commented that "life (with diabetes) is short, disgusting and painful" [Medvei, 1993].

Diabetes mellitus (DM), which literally means "sweet excessive urine", is a condition where the body is unable to maintain normal blood glucose level. Diabetes is becoming a global epidemic and the risk of both economic and human loss is estimated to be huge. Diabetes is a group of "metabolic diseases" characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both [Tierney et al., 2002]. Normally, blood glucose level is tightly controlled by insulin, a hormone produced by the pancreas. In patients with diabetes, the absence or insufficient production of insulin causes hyperglycemia. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia. Over time, diabetes can lead to blindness, kidney failure, and nerve damage. Diabetes is also an important factor in accelerating the hardening and narrowing of the arteries (atherosclerosis), leading to strokes, coronary heart disease, and other large blood vessel diseases.

Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia. Acute, life-threatening consequences of diabetes are hyperglycemia with ketoacidosis or the nonketotic hyperosmolar syndrome.

Diabetes is threatening to be the first major epidemic of the millennium. The prevalence varies from 2 to 5% in developing countries and 5 to 10% in developed countries. Globally, there will be about 300 million by the year 2025, whereas the estimated figure of the year 2000 was 151 million [Amos et al., 1997, King et al., 1998]. As per the overall estimates by International Diabetic Federation (IDF), diabetes affects approximately 40.9 million population of India and this is further set to rise to 69.9 million by the year 2025 (Fig 1) [Sicree et al., 2006]. In
developing countries, the majority of people with Diabetes is in the age group of 45-64 years hence they have more years of life to develop the complications of this disease i.e., adult blindness, amputation, renal failure, heart attacks and stroke. Developing countries like India and China where diabetes is more prevalent in adult population need immediate steps for the prevention and control at early stages.

Clinical diagnosis of diabetes requires a fasting plasma glucose concentration of > 7.8mM (140 mg/dl) on two separate occasions and plasma glucose concentrations of > 11.1mM (200 mg/dl) at two times during a 2-hour oral glucose tolerance test [Foster, 1998].

Diabetes is not one disease rather is a heterogeneous group of syndromes and categorized into two classes:

1. Juvenile or Insulin dependent Diabetes Mellitus (IDDM) or Type 1
2. Non-insulin dependent Diabetes Mellitus (NIDDM) or Type 2

Type 1 Diabetes Mellitus (T1DM) constitutes 10-20% and individuals with T1DM require insulin to avoid life threatening ketoacidosis. The disease is characterized by an absolute deficiency of insulin hormone caused by massive autoimmune attack on the β-cells in the islets of Langerhans (site of insulin secretion) of the pancreas. The main cause of the beta cell loss is a T-cell mediated autoimmune attack [Rother, 2007].

T1DM is fatal unless treated with insulin. Injection is the most common method of administering insulin; insulin pumps and inhaled insulin has been available at various times. Pancreas transplants have been used to treat type 1 diabetes, however, this procedure is currently still at the experimental trial stage [Patrick, 2008]. There is no preventive measure against developing type 1 diabetes. Most people who develop type 1 are otherwise healthy [Drexel, 2009]. Although the cause of type 1 diabetes is still not fully understood it is believed to be of immunological origin. T1DM can be distinguished from T2DM via a C-peptide assay, which measures endogenous insulin production. Type 1 treatment must be continued indefinitely in all cases. Treatment need not significantly impair normal activities, if sufficient patient training, awareness, appropriate care, discipline in testing and dosing of insulin is taken. However, treatment is burdensome for many people. Complications may be associated with both low blood sugar and high blood sugar. Low blood sugar may lead to seizures or episodes of unconsciousness and requires emergency treatment. High blood sugar may lead to increased tiredness and can also result in long term damage to other organs such as eyes and joints.
Type 2 Diabetes Mellitus (T2DM) is the most common form of diabetes affecting approximately 80% of the diabetic people [Thompson and Godin, 1995]. T2DM is largely determined by genetic as well as environmental factors and does not involve any type of autoimmunity and is thought to be due to a combination of two factors i.e., β-cells dysfunction and insulin resistance. In β-cells dysfunction cells fail to secrete enough insulin to correct the prevailing hyperglycaemia.

Insulin resistance is the non-responsiveness of target organs to insulin, e.g., insulin resistance in the liver leads to uncontrolled hepatic glucose production, whereas resistance in muscle and adipose tissue results in decreased glucose uptake by these tissues. Insulin resistance may develop because of a number of defects in signal transduction ranging from defects in insulin receptors or glucose transporters. The reasons behind this resistance to insulin vary widely, often involving environmental causes and genetics. T2DM in humans is frequently coupled with obesity and hyperlipidaemia as well as hyperglycaemia, many investigations have shown high levels of free fatty acids or other lipids might be harmful to islet function [Robertson et al., 2004]. There is ample evidence that fatty acids, which under normal circumstances are physiological fuels for the β-cells, become toxic when present at elevated concentrations for prolonged periods of time [McGarry and Dobbins, 1999]. Most patients with this form of diabetes are obese, and obesity itself causes some degree of insulin resistance [Kissebah et al., 1998; Butkiewicz et al., 1995]. Patients who are not obese may have an increased percentage of body fat by traditional weight criteria distributed predominantly in the abdominal region [Banerji et al., 1994]. Ketoacidosis seldom occurs spontaneously in this type of diabetes, when seen, it usually arises in association with the stress of another illness such as infection [Umpierrez et al., 1995; Zimmet, 1991]. This form of diabetes frequently goes undiagnosed for many years because the hyperglycaemia develops gradually and at earlier stages is often not severe enough for the patient to notice any of the classic symptoms of diabetes [Fujimoto et al., 1987; Kuusisto et al., 1994].

Diabetic patients have a several time greater risk than do non-diabetic individuals of developing atherosclerosis and its complications, which include stroke, myocardial infarction, and peripheral vascular disease [Kannel and McGee, 1979]. Several conditions have been proposed to explain the acceleration of atherosclerosis in diabetes, including hyperglycaemia, accelerated formation of advanced glycation endproducts (AGEs), increased oxidative stress, hypertriglyceridaemia, a high LDL-C/HDL-C ratio, hyperinsulinaemia, and genetic variables. Hypertension, abnormalities of lipoprotein metabolism and periodontal disease are often found in people with diabetes. The emotional and social impact of diabetes and the demands of therapy may cause significant psychosocial dysfunction in patients and their families.
Excess glucose or hyperglycemia, can be toxic to cells in several ways, four major molecular mechanisms have been proposed in hyperglycemia-induced oxidative stress, namely, activation of protein kinase C (PKC) via de novo synthesis of the lipid second messenger diacylglycerol (DAG), increased hexosamine pathway flux, increased advanced glycation end product (AGE) formation and increased polyol pathway flux [Kaneto et al., 1996; Evans et al., 2002]. These process entails the reduction of oxygen, producing oxidizing intermediates, such as $O_2^-$, $OH^-$ and $H_2O_2$ and $\alpha$-ketoaldehydes [Hunt et al., 1990]. These molecules can damage important biomolecules such as DNA, proteins and lipids and leading diabetic complications.

While the management of diabetes mellitus includes diet, exercise, oral hypoglycemic agents and insulin, these do not effectively prevent the complications of diabetes mellitus [Plumbo, 2001]. In modern medicine, there is still no satisfactory effective therapy available to cure diabetes [Piedrola et al., 2001]. Antidiabetics like sulphonylureas, biguanides, meglitinides, thiazolidinediones and insulin have been the mainstays of treatment for diabetes mellitus and are still in active use. Though the oral antidiabetic therapy are found to be relatively safe and effective in diabetes, each drug has its own range of side effects which may compromise the disease status or even worsen the condition in some cases (for example weight gain of sulphonylureas). Oral hypoglycemic agents produce a series of side-effects including hematological, gastro-intestinal reactions, hypoglycemic coma, and disturbances in liver and kidney metabolisms. In addition, these preparations are not ideal for use during pregnancy [Altan and Kilic, 1997].

Considering the fact that DM is a progressive disease with varied symptoms at each stage, treatment is also complicated, and usually the patients are prescribed with a combination of drugs once the disease attains a more chronic state. In view of the compelling evidence for a major role of oxidative stress in the development, progression, and complications of diabetes, antioxidants may serve as a potential therapy for ameliorating these [Cunningham, 1998; Maritim et al., 2003]. Thus, an ideal therapy for diabetes mellitus would be a drug that not only possesses antihyperglycemic effect, but also enhances or protects the antioxidant defense system which is usually compromised. Unfortunately, among the currently available hypoglycemic agents, the choice is very limited. Availability of new information about safety, efficacy, and tolerability of newer agents from diabetic clinical trials that would significantly affect the way drugs are prescribed is eagerly awaited. The last few years have been stagnant as far as new therapeutic options for oral agent for patients with DM are concerned and clearly there is a need for some newer specific and effective agents with action on multiple targets. This may result in better and more
efficient management of diabetes mellitus and its related complications. There has been a resurgent interest in the herbal treatments of diabetes. The growing public interest and awareness of natural medicines have led the pharmaceutical industry and academic researchers to pay more attention to medicinal plants [Day, 1998]. Even World Health Organization (WHO) has recommended that traditional plant treatments for diabetes warrant further evaluation [WHO, 1980]. This has led to the belief that natural products are safe because they are more harmonious with biological systems [Atal, 1983]. More than 1200 species have been reported for the treatment of this metabolic disorder [Marles and Farnsworth, 1995], although only a relatively small portion of them have been subjected to scientific and medical evaluations. Most of the drugs from plant sources are secondary metabolites (phenolics, flavonoids, tannins, saponins and sterols). These metabolites act in multiple ways to lower blood glucose level like restoring the integrity and function of β-cells, improvement in glucose uptake/utilization, having insulin releasing activity and antioxidant property. In recent times, focus on flavonoids and polyphenolic compounds, composing the major class of plant-derived antioxidants have received great attention as potential antiperoxidative agents as providing a potential therapeutic approach to prevent oxidative stress and hyperglycemia associated with DM [Kakkar et al., 1995]. Clinical trials on human populations show that antioxidants are associated with improved diabetic complications [Devaraj et al., 2002; Liu et al., 2004]. Initial studies have shown promise for this strategy. In many cases the direct scavenging of free radicals have been used as a strategy to prevent oxidative damage, and a variety of physiological and synthetic antioxidant molecules have been identified and synthesized. A number of agents with antioxidant activity have been shown to reduce progression of disease [Zibadi et al., 2008; Islam et al., 2008; Daisy et al., 2009]. Further studies of antioxidants as therapeutic intervention for DM, therefore, appear to be warranted.

Collectively, these evidences suggest that assessing and reducing oxidative damage may be a beneficial clinical strategy to prevent or inhibit the development and progression of DM. Therefore, in the present study we had used Butea monosperma and Terminalia arjuna and Pycnogenol after a comprehensive review of the available literature and the advice from experts of the practicing physicians. These herbs have not shown only free radical scavenging, antioxidant and anti-inflammatory effects but also have efficacy against several metabolic disorders on both human and animal. These observations prompted us to evaluate the effect of these herbal agents in rat model of DM. Glibenclamide, a sulfonylurea, is often used as a standard drug in streptozotocin (STZ)-induced moderate diabetic model to compare the antidiabetic properties of variety of compounds [Ivorra et
Section-I Introduction

J. So, the effects produced by extracts were compared with glibenclamide, a known hypoglycemic agent. Though sulfonylureas are valuable in treatment of diabetes, their use is restricted by their limited action, pharmacokinetic properties, secondary failure rates and accompanying side effects [Kameswara et al., 1997].

ANIMAL MODEL USED

Streptozotocin (STZ) is a deoxy-s [(methyl-nitrosoamino) carbonyl]-amino]-D glucopyranose molecule that produces a selective toxic effect on β cells and induces diabetes mellitus in most laboratory animals [Lown et al., 1979; Doux et al., 1986]. STZ is frequently used to induce diabetes mellitus in experimental animals through its toxic effects on pancreatic β-cells [Yamagishi et al., 2001; Stefek et al., 2002; Kim et al., 2003]. The cytotoxic action of STZ is associated with the generation of reactive oxygen species (ROS) causing oxidative damage [Szkudelski, 2001]. High doses of β-cell toxins like STZ induce insulin deficiency and T1DM with ketosis. However, doses calculated to cause a partial destruction of β-cell mass can be used to produce a mild insulin deficient state of T2DM, without a tendency to cause ketosis [Portha et al., 1989]. STZ is preferred because it has more specific β-cell cytotoxicity, but the sensitivity of this agent varies with species, strain, sex and nutritional state and there are batch differences in activity [Okamato, 1981]. Diabetes manifested by experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense system. Increased oxidative stress and changes in antioxidant capacity, observed in both clinical and experimental diabetes mellitus are thought to be the etiology of diabetic complications [Baynes, 1991].

USEFULNESS FOR DM PATIENTS

Development of animal models is essential for better understanding of the pathogenesis and progression of DM, and testing therapeutic agents for the treatment of DM patients. The treatment of disease will be done in the light of the pathogenesis of the disease. On the basis of the known mechanism, the drug selected for the rats may be used for DM patients.
AIMS & OBJECTIVES

Diabetes mellitus (DM) is currently a chronic disease, without a cure, and medical emphasis must necessarily be on managing/avoiding possible short-term as well as long-term diabetes-related problems. DM is almost irreversible and the goals of the existing therapies are to lower the blood glucose level, and drugs (sulfonylurea and biguanides as well as insulin administration) are effective in lowering blood glucose level only. A safe and effective therapeutic agent that can not only provide symptomatic relief but also can block or reduce the many harmful effects of DM—including hyperglycemia, polyuria, polydipsia, and weight loss is urgently needed. Therefore, it has become necessary to search for an economically and therapeutically effective treatment, especially for usage in developing and under developed countries. The search of the current therapy would not only provide symptomatic relief to the diabetic patients but also delay/stop its progression.

Keeping in mind all above facts study has been done with the following objectives and efficacy of selected herbs were also compared with a known standard drug glibenclamide

- To establish a model of insulin dependent diabetes mellitus (IDDM) and non insulin dependent diabetes mellitus (NIDDM)

- To search and test the efficacy of some potent herbal extracts (*Butea monosperma, Terminalia arjuna, Pycnogenol*) for the treatment of diabetes mellitus in animal models

- To check whether the required therapeutic dose is safe or produces some type of toxicity

- To study biochemical and morphological changes in treated and untreated models of diabetes mellitus
REVIEW OF LITERATURE
The prevalence of diabetes is rapidly rising all over the globe at an alarming rate! Over the past 30 yrs, the status of diabetes has changed from being considered as a mild disorder of the elderly to one of the major causes of morbidity and mortality affecting the youth and middle aged people. Millions of people have diabetes mellitus, commonly called diabetes. You may be surprised to know that many of these people don't even know they have it.

DIABETES MELLITUS

The term diabetes mellitus (DM) describes metabolic disorder of multiple aetiologies, characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. In diabetic condition, person's body does not produce or properly use insulin. Insulin is a hormone that is secreted by pancreas and needed to convert sugar and other foods for energy needed for daily life. The effects of DM include long-term damage, dysfunction and failure of various organs. DM may present with characteristic symptoms such as thirst, polyuria, blurring of vision and weight loss. In its most severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and in the absence of effective treatment, death. Often symptoms are not severe or may be absent and consequently hyperglycemia, sufficient to cause pathological and functional changes may be present for a long time before the diagnosis is made. The long-term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, charcot joints and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease [WHO, 1999].

TERMINOLOGY

The term 'diabetes' was coined by Aretaeus of Cappadocia. The Greek word diabainein literally means "passing through" or "siphon," a reference to one of diabetes' major symptoms-excessive urine production. The word became "diabetes" from the English adoption of the Medieval Latin diabetes. In 1675 Thomas Willis added mellitus from the Latin word for honey (mel in the sense of "honey sweet") when he noted that the blood and urine of a diabetic patient has a sweet taste. This had been noticed long before in ancient times by the Greeks, Chinese, Egyptians and Indians.
HISTORY
Although diabetes has been recognized since ancient time and various treatments have been known since the middle ages, the elucidation of the pathogenesis of diabetes occurred mainly in 20th century Oskar Minkowski and Joseph von Mering, first discovered the critical role of pancreas by removing the pancreas from a dog, the dogs developed all signs and symptoms of diabetes and died shortly afterward [Ahmed, 2002]. Dr. Banting with the assistance of Best, colleagues (particularly Collip) continued his research on de-pancreatized dogs, but went a step further and demonstrated that they could reverse induced diabetes in dogs by giving them an extract from the pancreatic islets of Langerhans of healthy dogs. For this, Banting et al. received the Nobel Prize in Physiology or Medicine in 1923; both shared their Prize money with others in the team who were not recognized. Insulin production and therapy rapidly spread around the world, largely as a result of this decision [Patlak, 2002]. In 1930s British clinician Harry Himsworth conducted series of experiments in both animals and humans (normal and diabetic patients) that led him to a conclusion that, diabetes could be caused not only by a lack of insulin but also by a lack of sensitivity to insulin. Himsworth’s experiments showed that there were two types of diabetes: type-1 and type-2. People with type-1 diabetes were sensitive to insulin and occurred at young age; those with type-2 were insensitive to insulin and tended to gradually develop a milder form of disease at middle age or older.

Other landmark discoveries include [Ahmed, 2002]
- Sulfonylureas were discovered by the chemist Marcel Janbon and his co-worker.
- The radioimmunoassay for insulin, discovered by Rosalyn Yalow and Solomon Berson (gaining Yalow the 1977 Nobel Prize in Physiology or Medicine).
- Identification of PPAR (peroxisome proliferator activated receptor) activator as effective antidiabetics in the 1990s.

EPIDEMIOLOGY/ PREVALENCE
Rates of diabetes are increasing worldwide. The International Diabetes Federation reports that in India 87.0 million people will have diabetes (20-79 yrs) by 2030 [IDF Diabetes Atlas, 2009]. The top 10 countries, in numbers of people with diabetes, are currently India, China, the United States, Indonesia, Japan, Pakistan, Russia, Brazil, Italy, and Bangladesh. The greatest percentage increase in rates of diabetes will occur in Africa over the next 20 years.

[9]
However, at least 80% of people in Africa with diabetes are undiagnosed, and many in their 30s to 60s will die from diabetes there. In 2005, people with diabetes were estimated to account for 7% of the US population, or approximately 20.8 million people [IDF, 2009]. Of these 20.8 million people, 14.6 million have a diagnosis of diabetes, and diabetes is undiagnosed in another 6.2 million. Approximately 5-10% have type 1 diabetes, and 90-95% have type 2, and 1-5% have other types. Additionally, an estimated 54 million people have pre-diabetes. According to the American Diabetes Association, approximately 18.3% (8.6 million) of Americans age 60 and older have diabetes. Thus prevalence of diabetes is increasing. In India, the prevalence rate of diabetes was estimated to be 1/5% [Rao et al., 1989]. In India, the prevalence of diabetes mellitus is increasing with 2.4% in rural population and 11.6% in urban population [Ramachandran, 2002]. According to the Diabetes Atlas 2006 published by the International Diabetes Federation, the number of people with diabetes in India currently around 40.9 million is expected to rise to 69.9 million by 2025 unless urgent preventive steps are taken [Sicree et al., 2006].

COSTS ON DIABETES

Diabetes mellitus is complex and costly disease that requires long-term medical attention both to limit the development of its devastating complications and to manage them when they do occur. The population with diabetes uses more health care services and is less productive than the population without diabetes. In the United states alone, it is estimated that about 12 to 15% of total health care expenditure is spent on the treatment of diabetes and its long term complications [Jonathan and Yen-Pin, 1995; Kenny et al., 1995]. The American Diabetes Association (ADA) estimated the national costs of diabetes in the USA for 2002 to be $US 132 billion, increasing to $US 192 billion in 2020. The American Diabetes Association reported that the direct healthcare cost of diabetes was $116 billion in 2007. Overall, direct health care costs of diabetes range from 2.5% to 15% of annual health care budgets, depending on local diabetes prevalence and the sophistication of the treatment available.

A study in China has estimated that for the urban population, the direct medical costs of diabetes are US$451/year for someone without complications, rising to US$1694/year for people with complications [Chen et al., 2003]. In Australia, costs are much higher at US$3012/year for those free of complications, US$5277/year for those with microvascular complications, US$6784/year for macrovascular complications, and US$7256/year for those with micro- and macrovascular complications [Colagiuri et al., 2003].
CLASSIFICATION
Although all forms of diabetes mellitus share hyperglycemia as a common feature, the pathogenic processes involved in the development of hyperglycemia vary widely. The previous classification of diabetes mellitus were based on the age at onset of disease or on the made of therapy; in contrast, the recently revised classification reflects our greater understanding of the pathogenesis of each variant. The etiologic classification of diabetes mellitus is given in [ADA, 2004].

Etiologic classification of diabetes mellitus
I. Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency, immune mediated and Idiopathic)
II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)

III. Other specific types
A. Genetic defects of β-cell function
   Examples: Chromosome 12, HNF-1α or 4α, Chromosome 7, glucokinase, Chromosome 20,
B. Genetic defects in insulin action
   Examples: Type A insulin resistance, Leprechaunism, Rabson-Mendenhall syndrome,
C. Diseases of the exocrine pancreas
   Examples: Pancreatitis, Trauma/pancreatectomy, Neoplasia, Cystic fibrosis, Hemochromatosis, Fibrocalculous pancreatopathy
D. Endocrinopathies
   Examples: Acromegaly, Cushing’s syndrome, Glucagonoma, Pheochromocytoma, Hyperthyroidism, Somatostatinoma, Aldosteronoma
E. Drug- or chemical-induced
   Examples: Pentamidine, Nicotinic acid, Glucocorticoids, Diazoxide, β-adrenergic agonists
F. Infections
   Examples: Congenital rubella, Cytomegalovirus
G. Uncommon forms of immune-mediated diabetes
   Examples: “Stiff-man” syndrome, Anti-insulin receptor antibodies
H. Other genetic syndromes sometimes associated with diabetes
   Examples: Down’s syndrome, Klinefelter’s syndrome, Turner’s syndrome, Wolfram’s syndrome, Friedreich’s ataxia, Huntington’s chorea, Laurence-Moon-Biedl syndrome

IV. Gestational diabetes mellitus (GDM)
CRITERIA FOR DIAGNOSIS

The following criteria are used to define blood sugar levels as normal or indicative of diabetes [Federal Bureau of Prisons Clinical Practice, 2008; WHO, 1999].

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<th>Normal</th>
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<tr>
<td>Fasting plasma glucose $&lt;100$ mg/dl or</td>
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<td>Oral glucose tolerance test (OGTT) 2-hr plasma glucose $&lt;140$ mg/dl</td>
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<th>Pre-diabetes</th>
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<td>Impaired fasting glucose (IFG) = fasting plasma glucose of 100–125 mg/dl or</td>
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<tr>
<td>Impaired glucose tolerance (IGT) = OGTT 2-hr plasma glucose of 140–199 mg/dl</td>
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<th>Diabetes</th>
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<td>1. Symptoms of diabetes and a casual plasma glucose $\geq200$ mg/dl (11.1 mmol/l).</td>
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<td>“Casual” is defined as any time of day, without regard to the time since the last meal. The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss. or</td>
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<tr>
<td>1. Fasting plasma glucose $\geq126$ mg/dl (7.0 mmol/l).</td>
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<td>Fasting is defined as no caloric intake for at least eight hours. or</td>
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<td>1. 2-h plasma glucose $\geq200$ mg/dl (11.1 mmol/l) during an oral glucose tolerance test.</td>
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<td>The test should be performed using a glucose load containing the equivalent of 75-g anhydrous glucose dissolved in water.</td>
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**TYPE 1 DIABETES MELLITUS**

Type 1 diabetes mellitus (T1DM) was previously called insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes. Type 1 diabetes develops when the cytotoxic T cells destroys pancreatic beta cells, the only cells in the body that make the hormone insulin that regulates blood glucose. This form of diabetes usually strikes children and young adults, although disease onset can occur at any age. Type 1 diabetes may account for 5% to 10% of all diagnosed cases of diabetes. The classical symptoms of type 1 diabetes include: polyuria (frequent urination), polydipsia (increased thirst), polyphagia (increased hunger), and weight loss [Cooke, 2008].

T1DM is a chronic autoimmune disease associated with selective destruction of insulin producing pancreatic β-cells. The onset of clinical disease represents the end stage of β-cells destruction leading to T1DM.

![Fig 4: Damaged pancreas tissues during type 1 diabetes](image-url)
Several features characterize T1DM as an autoimmune disease [Patlak, 2002]:

- Presence of immuno-competent and accessory cells in infiltrated pancreatic islets.
- Association of susceptibility to disease with the class II (immune response) genes of the major histocompatibility complex (MHC), human leucocyte antigens (HLA).
- Presence of islets cell specific auto-antibodies.
- Alteration of T cell mediated immunoregulation, particularly in CD4+ T cell compartment.
- Response to immunotherapy.

**Epidemiology**

Internationally rates of type 1 diabetes are increasing. The rate of type 1 diabetes is increasing in Europe (the Middle East and Australia) by 2-5% per year. Sardinia and Finland have the highest reported incidence of type 1 diabetes [Peter, 2007]. In the United States, Canada, and Europe, type 1 diabetes accounts for 5-10% of all cases of diabetes. It is estimated that about 5%-10% of North American diabetes patients have type 1. The fraction of type 1 in other parts of the world differs; this is likely due to both differences in the rate of type 1 and differences in the rate of other types, most prominently type 2. The longest surviving type I diabetes patient is Gladys Dull, who has lived with the condition for over 83 years.

**Risk Factors**

The exact cause of type 1 diabetes is unknown. Scientists do know that in most people with type 1 diabetes, their body's own immune system — which normally fights harmful bacteria and viruses — mistakenly destroys the insulin-producing (islet) cells in the pancreas. Genetics may play a role in this process, and exposure to certain viruses may trigger the disease. Whatever the cause, once the islet cells are destroyed, little or no insulin is produced. Main risk factors for type 1 diabetes may include genetic, environmental factors and some chemicals and drugs.

I. Genetics

A person whose parent or sibling has type 1 diabetes is at increased risk of developing the disease, compared to a person with no family history [Knip et al., 2005]. Type 1 diabetes may develop in people with a family history of type 1 diabetes, but may also develop in people with no family history of diabetes. In either case, the person has one or more genes that make them susceptible to the disease. Type 1 diabetes is a polygenic disease, meaning many different
genes contribute to its expression. Depending on locus or combination of loci, it can be dominant, recessive, or somewhere in between. The strongest gene, IDDM1, is located in the MHC Class II region on chromosome 6, at staining region 6p21. This is believed to be responsible for the histocompatibility disorder characteristic of type 1. Insulin producing pancreas cells (beta cells) display improper antigens to T cells. This eventually leads to the production of antibodies that attack these beta cells. Weaker genes are also located on chromosomes 11 and 18.

II. Environment
Environmental factors can strongly influence expression of type 1. A study showed that for identical twins, when one twin had type 1 diabetes, the other twin only had type 1 diabetes 30%-50% of the time. Despite having the exact same genome, one twin had the disease, where the other did not. This suggests that environmental factors, in addition to genetic factors, can influence disease prevalence. Environmental factors, such as exposure to certain viruses early in life, may trigger the autoimmune response [Knip et al., 2005]. Certain viruses have been associated with β-cell destruction. Diabetes occurs in some patients with congenital rubella. In addition, Coxsackie B, cytomegalovirus and other viruses (e.g. adenovirus and mumps) have been implicated in inducing the disease [WHO, 1999].

III. Some chemicals and drugs
Preferentially destroy pancreatic cells. Pyrinuron (Vacor, N-3-pyridimethyl-N-p-nitrophenyl urea), a rodenticide introduced in the United States in 1976, selectively destroys pancreatic β-cells, resulting in type 1 diabetes after accidental or intentional ingestion. Vacor was withdrawn from the U.S. market in 1979, but is still used in some countries. Zanosar is the trade name for streptozotocin, an antibiotic and antineoplastic agent used in chemotherapy for pancreatic cancer; it also kills beta cells, resulting in loss of insulin production. Other pancreatic problems, including trauma, pancreatitis or tumors (either malignant or benign), can also lead to loss of insulin production.

IV. Other risk factors include
Race
Type 1 diabetes is most common in people of non-Hispanic, Northern European descent (especially Finland and Sardinia), followed by African Americans, and Hispanic Americans. It is relatively rare in those of Asian descent.

Stress
The extra stress can cause diabetic ketoacidosis.
Sex
Type 1 diabetes is slightly more common in men than in women.

Age
Type 1 diabetes usually begins in childhood or young adulthood, but can develop at any age. Diabetes mellitus prevalence increases with age, and the numbers of older persons with diabetes are expected to grow as the elderly population increases in number. The National Health and Nutrition Examination Survey (NHANES III) demonstrated that, in the population over 65 years old, 18% to 20% have diabetes, with 40% having either diabetes or its precursor form of impaired glucose tolerance.

Pathophysiology of Type-1 Diabetes
The pathophysiological changes occurring in type-1 diabetes as a consequence of the severe insulin deficiency may be better understood by comparing the normal metabolic pathways, as summarized in fig. 7, with the abnormal situation present in type-1 diabetes, outlined in fig. 8 [Belfiore and Mogensen, 2000]. In type-1, the deficit of insulin and the prevalence of counter regulatory hormones, primarily glucagon, leads to the activation of glycogenolysis and gluconeogenesis in liver, with ensuing enhanced hepatic glucose output (HGO). In addition, the deficiency in insulin action results in reduced glucose utilization in peripheral insulin sensitive tissues (primarily muscle) as well as in activation of lipolysis in the adipose tissue, with enhanced release of FFA. The latter, although they cannot be directly converted into glucose in man, favor gluconeogenesis in the liver. Combination of enhanced HGO and reduced glucose utilization results in hyperglycemia. In addition, FFA exert anti-insulin effects at the muscle level, through the mechanism of the glucose-FFA cycle (Randle's cycle), which may cause resistance to the therapeutically administered insulin. It should also be considered that hyperglycemia itself favors glucose utilization (glucose effectiveness), perhaps by acting on non-insulin dependent glucose transporters (GLUT1 in gut, GLUT2 in liver and GLUT3 in brain), and that in type-1 diabetes this glucose effect may be reduced, i.e. there may be 'glucose resistance'.
TYPE-2 DIABETES MELLITUS

Type-2 diabetes mellitus (T2DM), also called non-insulin-dependent diabetes mellitus (NIDDM or adult-onset diabetes) occurs in approximately 90–95% of diabetic people in the Western world. T2DM resulting from insulin resistance and insufficient insulin secretion. Early symptoms of DM may be nothing more than chronic fatigue, generalized weakness and malaise (feeling of unease), excessive urine production, excessive thirst and increased fluid intake, blurred vision (typically from lens shape alterations, due to osmotic effects, e.g., high blood glucose levels), unexplained weight loss, lethargy, itching of external genitalia and excessive bowel movements.

The characteristic features of most patients with type 2 diabetes are the following:

- Insulin resistance in muscle cells.
- Normal or even excessive levels of insulin (to compensate for this resistance), eventually followed by a drop in insulin production.

In addition, researchers are trying to determine why the following events occur:

- Elevated levels of free fatty acids and the hormones resistin and leptin have been associated with insulin resistance at different phases. Such factors are also present in obesity. It is not known yet if elevated levels are simply a product of obesity or play some causal role in diabetes.
- Some researchers suggest that proteins called calpains may play an important role in both insulin secretion and insulin action.
- Elevated growth hormone during puberty appears to increase the risk for insulin resistance in overweight adolescents.
- Some experts theorize that abnormal regulation of certain important peptides (amylin and CGRP) may occur, thus affecting both the nervous and circulatory systems. One effect is to alter blood flow, which may contribute to insulin resistance. How each of these factors contributes to type 2 diabetes is under investigation.
- One 2001 study found high levels of interleukin 6 (IL-6) and C-reactive protein (CRP) in people with diabetes. Both of these substances are markers for inflammation and damage caused by an overactive immune response. Some researchers believe such inflammation may contribute to the disease process leading to diabetes.
Epidemiology

This type of diabetes accounts for between 90-95% of all diabetes cases, one third of whom are unaware of their condition. There were an estimated 23.6 million people in the U.S. (7.8% of the population) with diabetes with 17.9 million being diagnosed, [ADA, 2008] 90% of whom were type 2 [Inzucchi and Sherwin, 2005] About 90-95% of all North American cases of diabetes were type 2, [Zimmet et al., 2001] and about 20% of the population over the age of 65 have T2DM. The total prevalence of diabetes increased 13.5% from 2005-2007.

Risk Factors

I. Genetic Predisposition

T2DM is strongly favored by genetic predisposition. Although it shows familial aggregation as well as a high percentage (80%) in monozygotic twins, its mode of inheritance is not yet understood. It could be a polygenic disease. In any case, the risk of developing type 2 diabetes in offspring and siblings of diabetic patients is relatively elevated. History of previous impaired glucose tolerance (IGT) or impaired fasting glucose (IFG) and history of gestational diabetes mellitus or of delivering a baby with a birth weight of more than 9 pounds show the chances of occurrence of T2DM.

II. Environmental factors

In addition to the genetic predisposition, there are favoring environmental factors involved in increasing the chance of type 2 diabetes, such as excessive caloric intake, obesity associated with increased body fat in the abdominal (visceral) site, sedentary habit, etc. The insulin levels in type 2 diabetic patient may be normal or even increased (especially in presence of obesity) for a long time, but may decrease in the later stages of the disease.

VII. Medical conditions

Subclinical Cushing's syndrome (cortisol excess) may be associated with T2DM [Iwasaki, 2008]. The percentage of subclinical Cushing's syndrome in the diabetic population is about 9% [Chiodini, 2005]. Diabetic patients with a pituitary microadenoma can improve insulin sensitivity by removal of these microadenomas [Taniguchi, 2008]. Hypogonadism is often associated with cortisol excess, and testosterone deficiency is also associated with diabetes mellitus type 2 even if the exact mechanism by which testosterone improve insulin sensitivity is still not known. Although its incidence is far from universal, type 2 diabetes can arise from with the condition often termed Metabolic syndrome (it is also known as Syndrome X and Reavan's syndrome, or CHAOS) and Polycystic ovarian syndrome (which results in insulin resistance).
VIII. Medications

Some drugs, used for any of several conditions, can interfere with the insulin regulation system, possibly producing drug induced hyperglycemia. Some examples follow, giving the biochemical mechanism in each case.

**Niacin acid and glucocorticoids** - impair insulin action

**Atypical Antipsychotics** - Alter receptor binding characteristics, leading to increased insulin resistance.

**Beta-blockers** - Inhibit insulin secretion.

**Calcium Channel Blockers** - Inhibits secretion of insulin by interfering with cytosolic calcium release.

**Corticosteroids** - Cause peripheral insulin resistance and gluconeogenesis.

**Fluoroquinolones** - Inhibits insulin secretion by blocking ATP sensitive potassium channels.

**Niacin** - causes increased insulin resistance due to increased free fatty acid mobilization.

**Phenothiazines** - Inhibit insulin secretion.

**Protease Inhibitors** - Inhibit the conversion of proinsulin to insulin.

**Somatropin** - May decrease sensitivity to insulin, especially in those susceptible.

III. Other possible risk factors for developing T2DM include the following:

**Age**
Increasing age is a significant risk factor for type 2 diabetes. Risk begins to rise significantly at about age 45 years, and rises considerably after age 65 years. Although type 2 diabetes mellitus still occurs most commonly in adults aged 40 years or older, the incidence of disease is increasing more rapidly in adolescents and young adults than in other age groups [Kenneth et al., 2005].

**Obesity**
It is also seen more frequently now than before in young people, in association with the rising prevalence of childhood obesity.

**Sex**
The incidence of type 2 diabetes is essentially equal in women and men in all population.

**Race**
African Americans, Native Americans, Hispanic Americans, and Japanese Americans, have a greater risk of developing type 2 diabetes than non-Hispanic whites [Harris, 1995].

**Hypertension/High blood pressure (>140/90 mm Hg)**

**High-fat diet/High blood triglyceride** (fat) levels (high-density lipoprotein [HDL] cholesterol level <40 mg/dl or triglyceride level >150 mg/dl)
Pathophysiology

Type 2 diabetes is characterized by peripheral insulin resistance with an insulin-secretory defect that varies in severity. The natural history of T2DM has four stages. The first stage begins at birth, when glucose homeostasis is normal but individuals are at risk for T2DM because of genetic polymorphisms (diabetogenic genes). During stage 2, decrease in insulin sensitivity emerges probably as a result of a genetic predisposition and lifestyle (environmental), which are initially compensated for by an increase in β-cell function, so that glucose tolerance remains normal [Hales, 1994] but later both the β-cell function and insulin sensitivity, deteriorate, so that when challenged, as during a glucose tolerance test or a standardized meal, postprandial glucose tolerance becomes abnormal (stage of impaired glucose tolerance). At this point, β-cell function is clearly abnormal, but sufficient to maintain normal fasting plasma glucose concentrations. In stage 3, as a result of further deterioration in β-cell function and increased insulin resistance, fasting plasma glucose can increase due to an increase in basal endogenous glucose production, but the patient is still asymptomatic. Finally in stage 4, as a result of further deterioration in β-cell function, both fasting and postprandial blood glucose levels reach clearly diabetic levels and the patient becomes symptomatic. The ability of β-cells to adapt to insulin resistance depends on various genetic factors that determine the total β-cell mass, rates of replication and apoptosis of the cells, and the activity of key biochemical components of cells. Environmental factors (obesity) can probably aggravate the genetic predisposition leading to β-cell failure [Deffronzo et al., 1992]. The exact mechanism of β-cell failure however remains controversial and is probably regulated at the gene level [Deffronzo et al., 1992; Hamman, 1992; Gerich, 1998; Kahn, 2001].

The first phase of insulin secretion occurs in response to an increase in extracellular glucose and ATP and cAMP levels increase. This leads to closure of the ATP-sensitive potassium channels, which causes depolarization of the β-cell membrane and an influx of calcium through the voltage sensitive calcium channels; the resultant increase in intracellular calcium leads to the movement of insulin-containing granules towards the β-cell membrane, where they merge, incorporate, and melt into the membrane with the release of the granules contents (insulin, proinsulin and C-peptide). The second phase of insulin release involves synthesis of new insulin molecules as well as ATP-dependent mobilization of granules from a storage pool into a rapidly releasable pool. The normal β-cell response to an increase in glucose concentration is dependent on glucose entry
into the \( \beta \)-cell and its metabolism, synthesis of insulin and insulin granules, and other proteins necessary for moving the granules towards the \( \beta \)-cell membrane and facilitating their melting into the membrane so that their contents can be released. There is evidence that the integrity of these responses is necessary for maintenance of normal glucose homeostasis. Defect in this complex scheme that maintains normal glucose homeostasis leading to 12DM. Some forms of diabetes such as maturity onset diabetes of youth (MODY) have been found to be due to genetic defects. MODY has now been reclassified as individuals with a variety of defects in beta-cell function. This accounts for 2-5% of individuals with 12DM who present at a young age and have mild disease. The trait is autosomal dominant and can be screened for through commercial laboratories. The 6 currently identified mutation are; HNF-4-alpha, glycokinase gene, HNF-1-alpha, IPF-1, HNF-1-beta, and NEUROD1.

**DIAGNOSIS of DIABETES**

People with Type 1 diabetes mellitus, do not produce enough insulin to adequately maintain a normal blood glucose level. This is often due to their immune system mistakenly destroying pancreatic beta cells that produce insulin. Rarely, type 1 diabetes is diagnosed before symptoms develop. The diagnosis of diabetes is based upon a person's symptoms, the results of laboratory tests and immune destruction markers [Knip et al., 2005; WHO, 1999]. Most affected people are otherwise healthy and of a healthy weight when onset occurs.

**Symptoms** — Most people have symptoms of high blood sugar levels (hyperglycemia) before being diagnosed with both type of diabetes. These symptoms may include excessive thirst, fatigue, frequent urination, weight loss, or blurred vision. Less commonly, a person will develop signs and symptoms of diabetic ketoacidosis (DKA) at the time of diagnosis with type I diabetes and hyperosmolar nonketogenic coma with type 2 diabetes.

**Laboratory tests** — Several blood tests are used to measure blood sugar levels; this is the primary test for diagnosing diabetes. Additional tests can determine the type of diabetes and its severity. The blood tests must be repeated on another day to confirm that they remain abnormally high.

**Random blood sugar test** — For a random blood sugar test, blood can be drawn at any time throughout the day, regardless of when you last ate. A random blood sugar level of 200 mg/dl (11.1 mmol/l) or higher in a person who has the typical symptoms of hyperglycemia suggests a diagnosis of diabetes.

**Fasting blood sugar test** — Fasting blood sugar testing involves measuring the blood sugar level after not eating or drinking for 8 to 12 hours (usually overnight). Fasting blood sugar is normally less than 100 mg/dl (5.6 mmol/l); values of 126 mg/dl (7.0 mmol/l) or higher suggests diabetes. However, the test must be repeated on another day to confirm the diagnosis of diabetes.
Glycosylated hemoglobin test (HbA1c) — The HbA1c blood test measures the average blood sugar level during the past two to three months. It is used to monitor blood sugar control in people with known diabetes, but is not usually used to diagnose the disease. Normal values for HbA1c are usually 6.1 percent or lower (indicating an average blood sugar of 120 mg/dl [6.6 mmol/l]). Most healthcare providers recommend measuring HbA1c three to four times per year in people with type 1 diabetes.

Markers of immune destruction — Markers of immune destruction, including islet cell autoantibodies, and/or autoantibodies to insulin, and autoantibodies to glutamic acid decarboxylase (GAD) are present in 85-90 % of individuals with Type 1 diabetes mellitus when fasting diabetic hyperglycaemia is initially detected. These patients may also have other autoimmune disorders such as Graves’ disease, Hashimoto’s thyroiditis, and Addison’s disease [WHO, 1999].

Insulin resistance — In case of type 2 diabetes if treated with insulin, is more likely to require very large doses to control the blood glucose (e.g., >0.7 units/kg/day) due to insulin resistance. Such individuals frequently have characteristics associated with insulin resistance, including abdominal obesity, hypertension, lipid abnormalities, atherosclerosis, and hyperuricemia and also does not have evidence of anti-beta cell specific antibodies.

COMPLICATIONS
Diabetics are susceptible to three major acute complications [Mohan, 2000].

**Diabetic ketoacidosis**
Insulin deficiency combined with excess glucagon will lead to ketoacidosis. It is most exclusive complication of type-1 diabetes. It mainly occurs due to insufficient insulin intake and exposure to stress. Lack of insulin causes lipolysis in adipose tissue which causes the release of free fatty acids in to the plasma. These free fatty acids are taken up by liver where they are oxidized by enzyme acetyl coenzyme-A to ketone bodies (acetoacetic acid and β-hydroxybutyric acid), which results into imbalance between ketogenesis and the rate at which ketone bodies can be utilized by muscles and other tissues, leading to ketonaemia and ketonuria. Systemic metabolic ketoacidosis occurs if urinary excretion of ketone bodies is prevented by dehydration. Clinically this condition is characterized by anorexia, nausea, vomiting, deep and fast breathing, mental confusion and coma.

**Hyperosmolar nonketogenic coma**
Hyperosmolar nonketogenic coma is more common complication of type-2 diabetes. In patients with high blood glucose level, water will be osmotically driven out of the cells into the blood. The kidney will also be ‘dumping’ glucose into the urine, resulting in concomitant loss of water, causing an increase into the blood osmolarity. If the fluid is not replaced (orally or intravenously), the osmotic effect of high glucose levels combined with the loss of water
will eventually result into such high serum osmolarity (dehydration). The body's cells may become progressively dehydrated as water is drawn out from them and excreted. Electrolyte imbalances are also common. This combination of changes, especially if prolonged, will result in symptoms of lethargy (dulled or reduced level of alertness or consciousness) and may progress to coma. Thrombotic and bleeding complications are frequent due to high viscosity of blood. The mortality rate in hyperosmolar nonketotic coma is high.

**Hypoglycemia**

The problem of hypoglycemia is much more common in type-1 diabetes. Daytime hypoglycemic episodes are usually recognized symptoms: like the sweating, nervousness, tremor and hunger. Night time hypoglycemia may be without symptoms or manifest as night sweats, unpleasant dreams or early morning headache. Diabetic patients taking too much insulin, missing a meal, or over-exercising can result in hypoglycemia. It is imperative that a good relationship exists between the patient and the physician prescribing the insulin so that dosages can be gauged correctly. In response to the hypoglycemia, secretion of several hormones which raise blood glucose levels is increased: epinephrine, norepinephrine, growth hormone and cortisol. As a result, blood sugar levels will rebound and often lead to hyperglycemia. This phenomenon is commonly referred to as the Somogyi phenomenon.

On a long-term basis, the diabetic health condition is complicated by repeated elevations in blood glucose levels. Major chronic complications of diabetes include:

**Atherosclerosis**

The diabetic patient has a two to three fold higher risk of atherosclerosis than a non diabetic individual. The cause for atherosclerosis is not known but the possible contributing factors are hyperlipidemia, reduced HDL (high-density lipoprotein) levels, non-enzymatic glycosylation, increased platelet adhesiveness, obesity and associated hypertension in diabetes. The possible complications of diabetes in atherosclerosis are coronary artery disease, silent myocardial infarction, cerebral stroke and the gangrene of toes and feet.

**Diabetic microangiopathy**

Microangiopathy occurs due to recurrent hyperglycemia that causes increased glycosylation of hemoglobin and other proteins resulting in thickening of basement membrane of small blood vessels and capillaries of different organs and tissues such as skin, skeletal muscle, eye and kidney and non-muscular tissues such as peripheral nerves, renal tubules and Bowman's capsule.
Diabetic nephropathy
Diabetic nephropathy is the damage to the kidney which can lead to the chronic renal failure, eventually the death.

Four types of lesions are described in diabetic nephropathy:
- Diabetic glomerulosclerosis
- Vascular lesions
- Diabetic pyelonephritis and necrotizing renal papillitis
- Tubular lesions or Armanni-Ebstein lesion.

Diabetic neuropathy
The pathogenesis of diabetic neuropathy is not known, but it may be due to the increased glycosylation of hemoglobin and other proteins or may be due to accumulation of sorbitol and fructose as a result of hyperglycemia, leading to the deficiency of myoinositol. Diabetic neuropathy is mainly characterized by systemic peripheral neuropathy, but it may affect all parts of nervous system.

Diabetic retinopathy
Diabetic retinopathy is serious eye disease that can result in blindness. The retinopathic lesions are divided into background (consisting of microaneurysms, hemorrhage, exudates and retinal edema) and proliferative (with newly formed vessels, scaring, retinitis preoliferens, vitreous hemorrhage and retinal detachment). Besides retinopathy diabetes also predisposes the patient to the early development of cataract and glaucoma.

Infections
Diabetic patients are more susceptible to various infections such as tuberculosis, pneumonias, pyelonephritis, otitis, carbuncles and diabetic ulcers. This could be due to various factors such as impaired leucocyte functions, reduced cellular immunity, poor blood supply due to vascular involvement and hyperglycemia.

Other complications
Dental disease
Periodontal (gum) disease is more common among people with diabetes. Among young adults, those with diabetes have about twice the risk of those without diabetes. Almost one-third of people with diabetes have severe periodontal diseases with loss of attachment of the gums to the teeth measuring 5 millimeters or more.

Complications of pregnancy
Poorly controlled diabetes before conception and during the first trimester of pregnancy can cause major birth defects in 5% to 10% of pregnancies and spontaneous abortions in 15% to 20% of pregnancies. Poorly controlled diabetes during the second and third trimesters of pregnancy can result in excessively large babies, posing a risk to the mother and the child.
Foot damage

Nerve damage in the feet or poor blood flow to the feet increases the risk of various foot complications. Left untreated, cuts and blisters can become serious infections. Severe damage might require toe, foot or even leg amputation.

Hearing problems

Hearing impairments occur more often in people with diabetes.

STREPTOZOTOCIN & DIABETIC MODEL

Streptozotocin (STZ), 2-deoxy-2-(3-methyl-3-nitrosoure) 1-D-glupranose is a broad-spectrum antibiotic with oncolytic, oncogenic, and diabetogenic properties [Rossini et al., 1977]. It has been widely used to induce experimental diabetes in various laboratory animals. It was originally isolated from cultures of Streptomyces achromogenes in 1960 [Thulesen et al., 1997]. The diabetogenic response to STZ was first detected by Upjohn Laboratories during testing of potential antibiotics from this organism. However, Rakieten et al. [1963] were the first to describe that β-cells necrosis and the ensuing diabetic state could be produced after a single intravenous dose of STZ in rats and dogs.

Mechanism of the Diabetogenic effect of STZ

In chemical terms it is the glucose molecule in the structure which gives way to selective uptake of STZ into the beta cells, while the MNU nitrosourea moiety of STZ allows for its activity as an alkylating agent [Elshar et al., 2000]. STZ is taken up by pancreatic β-cells via glucose transporter GLUT2 (glucose transporter 2). A reduced expression of GLUT2, perhaps in combination with reduced cellular activity of β-cells at the time of administration of STZ may prevent the diabetogenic action of STZ [Thulesen et al., 1997].

At the intracellular level, three major phenomena are currently held responsible for β-cell death:

- Process of methylation
- Free radicals generation
- Nitric Oxide (NO) production.
Methylation

The deleterious effect of STZ results from the generation of highly reactive carbonium ions (CH$_3^+$), formed from decomposition of the nitroso-moiety. The CH$_3^+$ ions cause DNA breaks by alkylating DNA bases at various positions, resulting in activation of the nuclear enzyme poly (ADP-ribose) synthetase as part of the cell repair mechanism. As cellular pyridine nucleotide, particularly NAD$^+$ is utilized as substrate for the nuclear enzyme, a profound decline in NAD$^+$ occurs within 20 min. In effect, an abrupt and irreversible NAD$^+$ exhaustion leads to cessation of NAD$^+$-dependent energy and protein metabolism, ultimately leading to cell death. Inhibition of poly (ADP-ribose) synthetase by agent like 3-aminobenzamine and nicotinamide are known to protect $\beta$-cells from NAD$^+$ depletion and cell death after STZ exposure [Uchigata et al., 1982; Bennett and Pegg, 1981].

Free Radicals

Free radical involvement in STZ effect has also been investigated. Hydrogen peroxide has been shown to be produced in pancreatic islets upon STZ exposure in vivo and in vitro. Moreover, because superoxide dismutase, a free radical scavenger was demonstrated to provide some protection against the diabetogenic properties of STZ, it was concluded that oxidative stress could play a role in determining STZ toxicity [Gandy et al., 1982; Kubisch et al., 1994].

Nitric Oxide

NO generating has been shown to be the mechanism of STZ toxicity in diabetogenesis [Kwon et al., 1994]. Recently, an integrated hypothesis was proposed to explain the mechanism of action of STZ. In this hypothesis, STZ, through production of superoxide, would generate peroxynitrite. The latter would dissociate into NO and hydroxyl radicals, thus leading to $\beta$-cells DNA damage and apoptosis [Bedoya et al., 1996].

TREATMENTS

The prevalence of the disease has increased over the last 20 years adding urgency to the need to improve management. The pharmacological therapy of diabetes has recently undergone enormous expansion. With the recent development of new classes of antidiabetic agents, the opportunities and challenges of managing patients with diabetes have increased. The antidiabetic agents available for the management of diabetes are listed in table 4. many of the agents which are a mainstay of pharmacotherapy in diabetes have been shown to have antioxidant properties in addition to their primary pharmacological actions.
INSULIN

Chemistry

Insulin is a small protein, containing 51 amino acids arranged in two chains (A and B) linked by disulfide bridges. There are species differences in the amino acids of both chains. Proinsulin, a long single-chain protein molecule, is processed within the Golgi apparatus and packaged into granules, where, it is hydrolyzed into insulin and a residual connecting segment called C-peptide by removal of four amino acids. Insulin and C-peptide are secreted in equimolar amounts in response to all insulin secretagogues; a small quantity of unprocessed or partially hydrolyzed proinsulin is released as well. While proinsulin may have some mild hypoglycemic action, C-peptide has no known physiologic function. Granules within the \( \beta \)-cells store the insulin in the form of crystals consisting of two atoms of zinc and six molecules of insulin.

Insulin Secretion

Insulin is released from pancreatic \( \beta \)-cells at a low basal rate and at a much higher stimulated rate in response to a variety of stimuli, especially glucose. Other stimulants such as other sugars (e.g., mannose), certain amino acids (e.g., leucine, arginine) and vagal activity are recognized. One mechanism of stimulated insulin release is diagrammed in fig.12. As shown in the fig.12, hyperglycemia results in increased intracellular ATP levels, which close the ATP-dependent potassium channels. Decreased outward potassium efflux results in depolarization of the B cell and opening of voltage-gated calcium channels. The resulting increased intracellular calcium triggers secretion of the hormone. As noted below, the insulin secretagogue drug group (sulfonylureas, meglitinides and D-phenylalanine) exploits parts of this mechanism.

Principal type & duration of action of insulin preparations

Four principal types of insulin are available; (1) rapid-acting, with very fast onset and short duration; (2) short-acting, with rapid onset of action; (3) intermediate-acting; and (4) long-acting, with slow onset of action. Examples are regular insulin (Humulin R, Novolin R, others), insulin isophane (Humulin N, Novolin N), insulin lispro (Humalog), insulin aspart (NovoLog), insulin glargine (Lantus) and insulin detemir (Levemir). Depending on needs,
doctor may prescribe a mixture of insulin types to use throughout the day and night. Rapid-acting and short-acting insulin are dispensed as clear solutions at neutral pH and contain small amounts of zinc to improve their stability and shelf-life. All other commercial insulin have been modified to provide prolonged action and are, with the exception of insulin glargine, dispensed as turbid suspensions at neutral pH with either protamine in phosphate buffer or varying concentrations of zinc in acetate buffer (ultralente and lente insulin). Insulin glargine is the only soluble long-acting insulin. Current regimens generally use intermediate or long-acting insulin to provide basal or background coverage and rapid-acting or short-acting insulin to meet the mealtime requirements. The latter insulin is given as supplemental doses to correct high blood sugars.

Treatment with insulin
The current classification of diabetes mellitus identifies a group of patients who have virtually no insulin secretion and whose survival depends on administration of exogenous insulin. This insulin dependent group (type-1) represents 5–10% of the diabetic population in the USA. Most type-2 diabetics do not require exogenous insulin for survival, but many need exogenous supplementation of their endogenous secretion to achieve optimum health. Currently, the only options for getting insulin into the body are injection or insulin pump infusion.

Complications of insulin therapy
• Hypoglycemia
Mechanisms and Diagnosis
Hypoglycemic reactions are the most common complication of insulin therapy. They may result from a delay in taking a meal, inadequate consumption of carbohydrate consumed, unusual physical exertion or a dose of insulin that is too large for immediate needs. Rapid development of hypoglycemia in individuals with intact hypoglycemic awareness causes signs of autonomic hyperactivity, both sympathetic (tachycardia, palpitations, sweating, tremulousness) and parasympathetic (nausea, hunger) and may progress to convulsions and coma if untreated. When patients lack the early warning signs of low blood glucose, they may not take corrective measures in time. In patients with persistent, untreated hypoglycemia, the manifestations of insulin excess may develop—confusion, weakness, bizarre behavior, coma, seizures—at which point they may not be able to procure or safely swallow glucose-containing foods. Hypoglycemic awareness may be restored by preventing frequent hypoglycemic episodes. An identification bracelet, necklace or card in the wallet or purse, as well as some form of rapidly absorbed glucose, should be carried by every diabetic who is receiving hypoglycemic drug therapy.
• Hypokalemia: insulin draws K+ into the cell with glucose (hyperglycemia with normal K+).
• Lipodystrophy at injection site
• Weight gain
• Injection complications

**Oral hypoglycemic agents**

These are used to treat the type-2 diabetes mellitus.

1. **Biguanides (e.g.: metformin)** — Metformin (Glucophage®, Gumerza®, Riomet®, Fortamet®) improves the body response to insulin to reduce high blood sugar levels. Metformin is a pill that is usually started with a dose of 500 mg with the evening meal; a second dose may be added one to two weeks later (500 mg with breakfast). The dose may be increased every one to two weeks thereafter. Common side effects of metformin include nausea, diarrhea, and gas. Patients with certain types of kidney, liver, and heart disease, and those who drink alcohol excessively should not take metformin.

2. **Sulfonylureas and other drugs** — that stimulate insulin secretion (e.g.: tolbutamide, glibenclamide, nateglinide): Sulfonylureas have been used to treat type 2 diabetes for many years. They work by increasing insulin production, and can lower blood sugar levels by approximately 20 percent. Sulfonylureas are generally used if metformin does not adequately control blood sugar levels when taken alone, but may be used first in people who have liver, kidney, or heart disease and in those who drink alcohol excessively. Patients who take sulfonylureas are at risk of low blood sugar, known as hypoglycemia. This can cause sweating, shaking, hunger, and anxiety, and must be treated quickly by eating 10 to 15 grams of fast-acting carbohydrate (eg, fruit juice, hard candy, glucose tablets). All currently available sulfonylureas bind to specific receptors on B-cells, resulting in closure of potassium ATP channels. As a result, calcium channels open, leading to an increase in cytoplasmic calcium that stimulates insulin release [Pilipson and Steiner, 1995].

3. **Thiazolidinediones (TZDs) (e.g: rosiglitazone, pioglitazone)** — these increase tissue insulin sensitivity by affecting gene expression. They are taken in pill form and usually used second line, in combination with other medications such as metformin, a sulfonylurea, or insulin. Of these TZDs, rosiglitazone-users had shown more weight gain and edema than non-users [Kahn, 2006]. Rosiglitazone may increase risk of death from cardiovascular causes though the causal connection is unclear [Steven and Kathy, 2007]. Pioglitazone and rosiglitazone may also increase the risk of fractures [Aubert et al., 2010].

4. **GLP-agonists** — Exenatide (Byetta®), an injectable medication that is taken twice per day. It is not a first-line medication, but may be considered for people whose blood sugar is not controlled on the highest dose of one or two oral medications. It may be especially
helpful for overweight patients who are gaining weight on oral medications. It must be taken in addition to an oral medication. Exenatide does not usually cause low blood sugar. Exenatide may promote weight loss, but can also cause bothersome side-effects, including nausea, vomiting, and diarrhea.

5. Meglitinides — Meglitinides include repaglinide (Prandin®) and nateglinide (Starlix®). The mechanism of meglitinides to control blood sugar levels is similar to the sulfonylureas, and it can be used in people who are allergic to sulfonylureas drugs. They are taken in pill form. Nowadays, meglitinides are not generally used as a first-line treatment because they are more expensive than sulfonylureas and are short-acting.

6. Alpha-glucosidase inhibitors — These agents, which include acarbose (Precose®) and miglitol (Glyset®), interfere with absorption of some glucose containing nutrients, reducing (or at least slowing) the amount of glucose absorbed. The mechanism by which they control glucose rise is, interference with the absorption of carbohydrates in the intestines. However these agents are not as effective as metformin or the sulfonylureas. They can be combined with other medications if the first medication does not control blood sugar levels sufficiently. There are some side effects associated with these agents including — gas (flatulence), diarrhea, and abdominal pain; starting with a low dose may minimize these side effects.

Table 4. Some anti-diabetic agents for present and future uses. (Source: Chehade & Mooradian, 2000)

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Examples</th>
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<tr>
<td>Oral agents</td>
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<tr>
<td>Sulphonylureas</td>
<td>1st generation</td>
<td>Acetohexamide, Chlorpropamide, Tolazamide, Tolbutamide</td>
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<tr>
<td></td>
<td>2nd generation</td>
<td>Glimperide, Glipizide, Glibenclamide (or Glyburide), Gliclazide, Glibidone</td>
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<tr>
<td>Biguanides</td>
<td></td>
<td>Metformin, Phenformin (available in some countries)</td>
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<tr>
<td>Thiazolidinediones</td>
<td></td>
<td>Tiroglitazone, Pioglitazone, Rosiglitazone</td>
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<tr>
<td>Alpha-glucosidase inhibitor</td>
<td></td>
<td>Acarbose, Miglitol, Voglibose</td>
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<tr>
<td>Meglitinide analogues</td>
<td></td>
<td>Repaglinide, Nateglinide</td>
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<tr>
<td>Body weight reducing agents</td>
<td>Anorectics</td>
<td>Sibutramine, Phentermine, Fenfluramine, Benfluorex</td>
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<td></td>
<td>Lipase inhibitors</td>
<td>Orlistat</td>
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<td>Parent agents</td>
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<tr>
<td>Insulin</td>
<td></td>
<td>Regular/Semilente, NPH/Lente, Ultralente</td>
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<tr>
<td>Insulin analogues</td>
<td></td>
<td>Lispro insulin, neutral protamine, lispro (NPL) insulin, insulin aspart</td>
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<tr>
<td>Amylin agonist</td>
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<td>Pramlintide</td>
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<td>Glucagon-like peptide (7-36)-amide</td>
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Table 5. General mechanisms of action of various anti-diabetic drugs.

<table>
<thead>
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<th>S.No.</th>
<th>Drug Groups</th>
<th>Mechanism</th>
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<tbody>
<tr>
<td>1</td>
<td>Sulphonylureas, Meglitinide analogues</td>
<td>Stimulation of insulin release [Smit et al., 1996]</td>
</tr>
<tr>
<td>2</td>
<td>Biguanides</td>
<td>Reduction of hepatic glucose output and enhancement of insulin effect [Dunn and Peters, 1995]</td>
</tr>
<tr>
<td>3</td>
<td>Thiazolidinediones</td>
<td>Enhancement of insulin sensitivity [Day, 1999]</td>
</tr>
<tr>
<td>4</td>
<td>β-glucosidase inhibitors, glucagon-like-peptide-1 (GLP-1), amylin analogues</td>
<td>Prolongation of glucose absorption [Krentz et al., 1994]</td>
</tr>
<tr>
<td>5</td>
<td>Insulin formulations and analogues</td>
<td>Insulin replacement [Bailey, 1992]</td>
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SHORTCOMINGS IN AVAILABLE TREATMENTS

Oral hypoglycemic agents, which were introduced in the 1950s, established an indispensable role in the treatment of NIDDM for last 37 years, along with the basic treatments such as diet and exercise therapies. However, they have still certain shortcomings which need to be checked [Kaneko, 1994]. Treatment of diabetes by insulin and oral hypoglycemic drugs fails to prevent complications in many patients, warranting additional research for effective alternative treatments. The pharmacological agents currently employed, such as sulphonylureas (e.g., glibenclamide), biguanides (e.g., metformin), thiazolidinediones (e.g., pioglitazone) and alpha-glucosidase inhibitors (e.g., acarbose) act to selectively modulate a specific pathological pathway [Rang and Dale, 1991; Krentz and Bailey, 2005]. As a result, these drugs control blood glucose levels provided they are regularly administered. Even though these drugs may be valuable in the management of diabetes mellitus, they have limitations due to undesirable adverse effects such as hypoglycemia, weight gain, secondary failure, and inability to arrest pancreas degeneration [Aspuland et al., 1983; Harrower, 1994; Sodojembers et al., 1970] or diabetic complications which have been linked to oxidative stress [Baynes, 1991]. Moreover, these therapies only partially compensate for metabolic derangements seen in diabetics and do not necessarily correct the fundamental biochemical lesion [Taylor and Agius, 1988].

1) They can not treat diabetes associated complications. Oral hypoglycemices are indicated only in uncomplicated NIDDM, when not controlled by diet and exercise.

2) They are associated with adverse reaction: hypoglycemia being the common side effect of sulphonylureas and lactic acidosis with biguanides.

3) They can not treat insulin resistance.

4) There is no drug to treat impaired insulin secretion when β- cells of pancreas are damaged by auto-antibodies.
WHAT IS OXIDATIVE STRESS?

Oxidative stress results from an imbalance between radical-generating and radical-scavenging systems, i.e. increased free radical production or reduced activity of antioxidant defenses or both [Turko et al., 2001; Maritim et al., 2003]. Reactive oxygen species (ROS) include free radicals such as superoxide (\(\bullet O_2\)), hydroxyl (\(\bullet OH\)), peroxyl (\(\bullet RO_2\)), hydroperoxyl (\(\bullet HRO_2\)) as well as nonradical species such as hydrogen peroxide (\(H_2O_2\)) and hydrochlorous acid (\(HOCl\)) [Turko et al., 2001; Evans et al., 2002]. Production of one ROS may lead to the production of others through radical chain reactions. \(\bullet O_2\) is produced by one electron reduction of oxygen by several different oxidases including NAD (P)H oxidase, xanthine oxidase, cyclooxygenase and even eNOS under certain conditions as well as by the mitochondrial electron transport chain during the course of normal oxidative phosphorylation, which is essential for generating ATP [Evans et al., 2003; Griendling and Fitzgerald, 2003; Griendling and FitzGerald, 2003b; Taniyama and Griendling, 2003]. Under normal conditions, \(\bullet O_2\) is quickly eliminated by antioxidant defense mechanisms. \(\bullet O_2\) is dismutated to \(H_2O_2\) by manganese superoxide dismutase (Mn-SOD) in the mitochondria and by copper (Cu)-SOD in the cytosol [Evans et al., 2003]. \(H_2O_2\) is converted to \(H_2O\) and \(O_2\) by glutathione peroxidase (GSH-Px) or catalase (CAT) in the mitochondria and lysosomes, respectively. \(H_2O_2\) can also be converted to the highly reactive \(\bullet OH\) radical in the presence of transition elements like iron and copper. Excessive levels of ROS lead to the damage of proteins, lipids, and DNA [Melov et al., 1999; Melov, 2000]. If cellular antioxidants do not remove free radicals, radicals attack and damage proteins, lipids, and nucleic acids. The oxidized or nitrosylated products of free radical attack have decreased biological activity, leading to loss of energy metabolism, cell signaling, transport, and other major functions. Accumulation of such injury ultimately leads a cell to die through necrotic or apoptotic mechanisms. In addition to their ability to directly inflict damage upon cellular macromolecules, ROS play a significant role in activating stress sensitive signaling pathways that regulate gene expression resulting in cellular damage [Droge, 2002; Dalton et al., 1999; Allen and Tresini, 2000]. Thus, the aforementioned endogenous antioxidant systems exist within cells to neutralize ROS, and these systems are critical to maintaining proper cellular function. A major cellular antioxidant is reduced glutathione (GSH), which is regenerated most efficiently by glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate [Lu, 2000].

PRODUCTION OF ROS IN DIABETES

There are convincing experimental and clinical evidences that the generation of reactive oxygen species is increased in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress [Johansen et al., 2005; Rosen et al., 2001]. Abnormally high levels of free radicals and simultaneous decline of antioxidant defense systems can lead to the
damage of cellular organelles and enzymes, increased lipid peroxidation and development of complications of diabetes mellitus [Mariim et al., 2003; Bayens, 1991]. Also this is particularly relevant and dangerous for the beta islet, which is among those tissues that have the lowest levels of intrinsic antioxidant defenses [Grodsky et al., 1982; Lenzen et al., 1996; Robertson, 2004; West, 2000]. Chronic hyperglycemia causes oxidative stress in tissues prone to complications in patients with diabetes [Rosen et al., 2001; Greene et al., 1992]. There are multiple sources of oxidative stress in diabetes including non-enzymatic, enzymatic and mitochondrial pathways. Five main hypotheses about how hyperglycemia causes diabetic complications included: stress-signaling pathway activation, increased polyol pathway flux; increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux. Until recently, there was no unifying hypothesis linking these five mechanisms. In each case, activation of these pathways appears to be linked to a hyperglycemia-mediated rise in ROS production and consequent increase in oxidative stress [Nishikawa et al., 2000; Nishikawa et al., 2000]. Oxidative biochemistry of glucose can give rise to many non-enzymatic sources of oxidative stress. Hyperglycemia itself can generate increased ROS generation. During hyperglycemia glucose can undergo autoxidation and generate *OH radicals [Turko et al., 2001]. In addition, glucose reacts with proteins in a nonenzymatic manner causing glycosylation of protein and leading to the development of amadori products followed by formation of AGESs. ROS is generated at multiple steps during this process [Cameron and Cotter, 1999; Mullarkey et al., 1990; Bierhaus et al., 1998; Schmidt and Stern, 2000]. During hyperglycemia, there is enhanced metabolism of glucose through the polyol pathway, also called sorbitol pathway, which also results in enhanced production of *O₂ [Stevens et al., 1993]. Excess dihydroxyacetone formed during hyperglycemia undergo reduction to glycerol-3-phosphate and subsequent acylation to yield diacylglycerol (DAG). DAG then activates PKC [Koya and King, 1998; Xia et al., 1994]. There is evidence that PKC is also stimulated in diabetes via multiple mechanisms, i.e. polyol pathway and Ang II, activates NAD(P)H oxidase [Amiri et al., 2002]. Once activated, PKC activates the MAPKs that phosphorylated transcription factors and thus alter the balance of gene expression [Tomlinson, 1999]. Several lines of evidence have established that the excessive flux of glucose or FFA into a variety of cell types results in the activation of the hexosamine biosynthetic pathway [Marshall et al., 1991; Hawkins et al., 1997; Schleicher and Weigert, 2000]. It has been proposed that the activation of this pathway leads to insulin resistance and the development of late complications of diabetes [Marshall et al., 1991; Hawkins et al., 1997; Schleicher and Weigert, 2000]. In mesangial cells, overexpression of GFAT increased NF-κB-dependent promoter activation [James et al., 2002]. One major intracellular target of hyperglycemia and oxidative stress is the transcription factor NF-κB [Mohamed et al., 1999; Baldwin, 2001]. NF-κB plays a critical role in mediating immune and
inflammatory responses and apoptosis. The aberrant regulation of NF-κB is associated with a number of chronic diseases including diabetes and atherosclerosis. NF-κB regulates the expression of a large number of genes, including growth factors [e.g., vascular endothelial growth factor (VEGF)], proinflammatory cytokines [e.g., TNF-α and IL-1β], RAGE, adhesion molecules [e.g., vascular cell adhesion molecule-1], and others. JNK/SAPK and p38MAPK are known as stress-activated kinases [Tibbles and Woodgett, 1999]. JNK/SAPK and p38MAPK is activated by hyperglycemia-induced oxidative stress and is likely involved in apoptosis mediated by hyperglycemia in human endothelial cells [Ho et al., 2000; Dunlop and Muggli, 2000]. Interestingly, H₂O₂ generation, JNK/SAPK hyperglycemia could be suppressed by the antioxidant [Ho et al., 2000, Natarajan et al., 1999]. Taken together, these recent data suggest that the NF-κB, JNK/SAPK, and p38MAPK pathways are candidate stress-sensitive signaling systems that can chronically lead to the late complications of diabetes.

Enzymatic sources of augmented generation of reactive species in diabetes include NOS, NAD(P)H oxidase and xanthine oxidase [Guzik et al., 2000; Guzik et al., 2002; Aliciguzel et al., 2003]. Guzik et al. (2002) investigated •O₂⁻ levels in vascular specimens from diabetic patients and probed sources of •O₂⁻ using inhibitors of NOS, NAD(P)H oxidase, xanthine oxidase and mitochondrial electron transport chain.

Fig 13: Mechanism of action
The mitochondrial respiratory chain is a source of non-enzymatic generation of ROS. Under normal conditions, \( \text{O}_2 \) is immediately eliminated by natural defenses. Therefore, when glycolysis, electron transfer, and oxidative phosphorylation are chronically or acutely overloaded, excess \( \text{O}_2 \) produces oxidative stress. A recent study demonstrated that hyperglycemia-induced generation of \( \text{O}_2 \) at the mitochondrial level is the initial trigger of vicious cycle of oxidative stress in diabetes [Nishikawa et al., 2000; Brownlee, 2001]. In mitochondria it critically alters energy regulation and survival through at least three mechanisms. First, physiological levels of NO reversibly compete with molecular oxygen for binding to cytochrome (cyt) c oxidase, producing reversible inhibition and acting as a regulatory switch for electron transfer. In contrast, in the presence of excess \( \text{O}_2 \), NO is converted to \( \text{ONOO}^- \), which competes with molecular oxygen for irreversible binding to cyt c oxidase and affects mitochondrial function by inhibiting ATP synthesis [Ghafourifar et al., 2001, Brown, 2001]. Second, mitochondrial oxidative stress through excess \( \text{O}_2 \) And \( \text{ONOO}^- \) production inhibits the import of essential proteins to the mitochondria [Wright et al, 2001]. Finally, oxidative damage of existing inner membrane proteins induces membrane permeability transition, which precedes cyt c release and apoptosis [Kowaltowski et al., 2001].

**ROLE OF AKT IN THE PATHOPHYSIOLOGY OF INSULIN RESISTANCE**

Akt (also termed PKB or Rac) is a 60 kDa serine threonine kinase, which is stimulated by a wide range of receptor tyrosine kinase [Burgering and Coffer, 1995]. Akt is though to be a downstream target of phosphatidylinositol (PI) 3-kinase [Datta et al., 1996]. Several lines of evidence suggest that PI 3-kinase is both necessary and sufficient for insulin activation of Akt activity [Burgering and Coffer, 1995]. Furthermore, Akt/protein kinase B (Akt) has been implicated in regulation of a number of cell functions including glucose uptake, glycogen synthesis, cell growth, survival, apoptosis, protein synthesis, and endothelial nitric oxide production [Shiojima and Walsh, 2002]. Recently, Akt activity has been reported to be essential for activation of glycogen synthase by insulin [Kitamura et al., 1998]. Considering the major role of PI3K-Akt pathway in insulin signaling, it is not surprising that this system has been investigated as a possible site of insulin resistance (IR), the hallmark of T2DM. Numerous studies in various experimental settings, exploring insulin-sensitive cell types and tissues, have suggested that this assumption is correct [Rondinone et al., 1999; Carvalho et al., 2000; Song et al., 1999]. Defects in GLUT4 translocation and expression were associated with the defective Akt phosphorylation [Carvalho et al., 2000, Tremblay et al., 2001]. Defects in Akt activation may be, in part, secondary to factors characteristic for diabetic milieu. One factor implicated in impaired Akt activation in diabetic tissues is oxidative stress [Tirosh et al., 1999]. Overproduction of \( \text{H}_2\text{O}_2 \) in adipocytes leads to 90% reduction of Akt phosphorylation. Finally, TNF-\( \alpha \), an inflammatory cytokine, has been identified as one of possible mediators of IR [Teruel et al., 2001].
BIOMARKERS OF OXIDATIVE STRESS

Lipid Peroxidation

Diabetes produces disturbances of lipid profiles, especially an increased susceptibility to lipid peroxidation [Lu, 1999], which had been responsible for increased incidence of atherosclerosis [Giugliano et al., 1995], a major complication of diabetes mellitus [Steiner, 1985]. An enhanced oxidative stress has been observed in these patients as indicated by increased free radical production [Hitamatsu and Arimori, 1988], lipid peroxidation and diminished antioxidant status. Peroxyl radicals can remove hydrogen from lipids, producing hydroperoxides that further propagate the free-radical pathway [Halliwell and Gutteridge, 1990]. Induction of diabetes in rats with streptozotocin (STZ) or alloxan uniformly resulted in an increase in thiobarbituric acid reactive substances (TBARS), an indirect evidence of intensified free-radical production. Preventing the formation of hydroxyl radicals would be an efficient means to reduce hydroxyl induced damage, and several compounds have been tested as antioxidants in diabetic animals with varying success. For example, the increase in TBARS associated with diabetes was prevented by treatment with antioxidants [Jang et al., 2000; Montilla et al., 1998; Pierrefiche et al., 1993; Mohan and Das, 1998; Kocak et al., 2000; Mekinova et al., 1995].

Glutathione Levels

Reduced glutathione is a major intracellular redox buffer that may approach concentrations up to 10 mM [Meister et al., 1983]. Glutathione functions as a direct free-radical scavenger, as a co-substrate for glutathione peroxidase activity, and as a cofactor for many enzymes, and forms conjugate in endo- and xenobiotic reactions [Josephy, 1997; Gregus et al., 1996]. Glutathione concentration has been found to be decreased in various tissues of chemically induced diabetic animals and normalized by several antioxidant [Thompson and McNeill, 1993; Abdel-Wahab and Abd-Allah, 2000; Aragno et al., 1999; Montilla et al., 1998; El-Missiry and Gindy, 2000; Mohan and Das, 1998; Mekinova et al., 1995], when these antioxidants are given prior to or at the same time as the diabetogen.

Glutathione Peroxidase and Glutathione Reductase

Glutathione peroxidase (GPx) and reductase (GR) are two enzymes that are found in the cytoplasm, mitochondria, and nucleus. GPx metabolizes hydrogen peroxide to water by using reduced glutathione as a hydrogen donor [Sies, 1993; Santini et al., 1997]. Glutathione disulfide is recycled back to glutathione by GR, using the cofactor NADPH generated by glucose 6-phosphate dehydrogenase. Decreased activity of these two enzymes was seen in diabetic animals, which was reversed by treatment with antidiabetic agents [Refai et al., 2009; Kakkar et al., 1998; Bhor et al., 2004; Kaul et al., 1995; Kaul et al., 1996].
Catalase
Catalase (CAT), located in peroxisomes, decomposes hydrogen peroxide to water and oxygen. Several reports describing the alterations of CAT activity due to diabetes were normalized by treatment with antioxidants [Rauscher et al., 2001; Maritim et al., 1999; Sanders et al., 2001, Sugiura et al., 2006; Refai et al., 2009].

Superoxide Dismutase
Isoforms of superoxide dismutase (SOD) are variously located within the cell. CuZn-SOD is found in both the cytoplasm and the nucleus. Mn-SOD is confined to the mitochondria, but can be released into extracellular space [Reiter et al., 2000]. SOD converts superoxide anion radicals produced in the body to hydrogen peroxide, thereby reducing the likelihood of superoxide anion interacting with nitric oxide to form reactive peroxynitrite. Alterations of SOD activity in diabetic animals were normalized by pharmacotherapy [Kaul et al., 1995; Kaul et al., 1996, Mohan and Das, 1998; Sugiura, et al., 2006] all of which were administered prior to or concomitant with the diabetogen.

Nitrite Level
Increased oxidative stress and subsequent activation of the transcription factor NF-κB have been linked to the development of late diabetic complications. NF-κB enhances nitric oxide production, which is believed to be a mediator of islet beta-cell damage. Nitric oxide may react with superoxide anion radical to form reactive peroxyl nitrite radicals. A number of studies are continuing to examine the role of nitric oxide in diabetes mellitus. A number of studies are continuing to examine the role of nitric oxide in diabetes mellitus. Nitric oxide concentration in STZ-diabetic rats was restored after treatment to levels significantly higher than normal [Maritim et al., 1999; Komers et al., 2000].

Diabetic dyslipidemia
Central characteristics of dyslipidemia in patients with type II diabetes are an elevated cholesterol level, triglyceride level, LDL-C, VLDL-C levels and decreased HDL-C level [Levey et al., 1998; Mulec et al., 1990; Soltani et al., 2007]. Patients with type II diabetes typically have a preponderance of smaller, denser, oxidized LDL particles, which may increase atherogenicity even if the absolute concentration of LDL-C is not elevated. High levels of total cholesterol and more importantly LDL-C in blood are major coronary risk factors. Insulin has an inhibitory action on HMG-CoA reductase, a key rate-limiting enzyme responsible for the metabolism of cholesterol-rich LDL particles. Acute insulin deficiency initially causes an increase in free fatty acid mobilization from adipose tissue. This results in increased production of cholesterol rich LDL particle [Balasee et al., 1972; Taskinen, 1987; Murali et al., 2002].
NATURAL DEFENSE AGAINST OXIDATIVE STRESS AND ANTIOXIDANTS

Reactive species can be eliminated by a number of enzymatic and nonenzymatic antioxidant mechanisms. As discussed above, SOD immediately converts \( \cdot \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \), which is then detoxified to water either by catalase (CAT) in the lysosomes or by glutathione peroxidase (GSH-Px) in the mitochondria. Another enzyme that is important is glutathione reductase (GR), which regenerates glutathione that is used as a hydrogen donor by GSH-Px during the elimination of \( \text{H}_2\text{O}_2 \). Maritim and colleagues recently reviewed in detail that diabetes has multiple effects on the protein levels and activity of these enzymes, which further augment oxidative stress by causing a suppressed defense response [Martim et al., 2003]. Non-enzymatic antioxidants include GSH, vitamins A, C and E, \( \alpha \)-lipoic acid, carotenoids, trace elements like copper, zinc and selenium, coenzyme Q10 (CoQ10), and cofactors like folic acid, uric acid, albumin, and vitamins B1, B2, B6 and B12. Alterations in the antioxidant defense system in diabetes have recently been reviewed [Vega-Lopez, 2004].

Evidence from experimental models

In addition to an increase in ROS, a decrease in antioxidant capacity occurs in diabetes mellitus [Opara et al., 1999; Shigeta et al., 1961; Godin et al., 1988]. A decline in important cellular antioxidant defense mechanisms, including the glutathione redox system, vitamin C-vitamin E cycle, and the LA/dihydrolipoic acid (DHLA) redox pair significantly increases susceptibility to oxidative stress. Thus, attempts have been made to reduce oxidative stress-dependent cellular changes in patients with diabetes by supplementation with naturally occurring antioxidants. A multitude of in vivo studies have been performed utilizing antioxidants in experimental diabetic models [Mekinova et al., 1995; Kedziora-kornatowska et al., 2003; Cinar, 2001; Obrosova, 2000; Kocak et al., 2000]. The effects of antioxidants on oxidative stress are measured through certain observable biomarkers. These markers include the enzymatic activities of CAT, SOD, GSH-Px, and GR, as well as thiobarbituric acid reactive substances (TBARS) levels, an indirect measurement of free-radical production that has been shown to be consistently elevated in diabetes. Normalization of the activity levels of any of these markers, and ultimately, the balance of free-radical production/removal, would be an effective method to reduce ROS-induced damage.

STANDARD DRUG USED IN THE PRESENT STUDY

Glibenclamide (GL), a sulphonylurea, was used as a reference antidiabetic drug in this study. Chemically, GL (also known as glyburide) is a derivative of sulfonyl cyclohexylurea [Reich et al., 1987]. It is known to promote insulin secretion through inhibition of ATP-sensitive K' (K\text{ATP}) channels in the pancreatic cells [Schmid-Antomarchi et al., 1987]. The inhibition of ATP sensitive channels leads to membrane depolarization, activation of voltage-gated Ca\text{\textsuperscript{2+}}
channels, increased $Ca^{2+}$ influx, a rise in cytosolic ($Ca^{2+}$) and thereby insulin release. Thus, it produces hypoglycemic effect through increased release of insulin from pancreatic $\beta$-cells and enhancement of insulin action on target tissues [Jackson and Bressler, 1981]. Though sulfonylureas are valuable in treatment of diabetes, their use is restricted by their limited action and accompanying side effects (Weight gain, hyperinsulinenia, and tolerance of sulphonylureas). Thus, any plant secondary metabolite or chemical constituent that is capable of affecting the pancreatic $\beta$-cells or insulin action will be a good mimic of sulphonylureas. Due to the minimal, or lack of, side effects of plant based drugs, these can be better candidates for the treatment of diabetes mellitus. The hypoglycemic activity of a number of plants and plant products has been evaluated and confirmed in animal models [Gupta et al., 2005; Ruzaidi et al., 2005; Kesari et al., 2006] as well as in human beings [Jaouhari et al., 1999; Herrera-Arellano et al., 2004; Jayawardena et al., 2005].

**IDEAL ANTI-DIABETIC DRUG**

In view of the compelling evidence for a major role of oxidative stress in the development, progression, and complications of diabetes, antioxidants may serve as a potential therapy for ameliorating these [Maritim et al., 2003]. Thus, an ideal therapy for diabetes mellitus would be a drug that not only possesses antihyperglycemic effect, but also enhances or protects the antioxidant defense system which is usually compromised. Unfortunately, among the currently available hypoglycemic agents, the choice is very limited. An ideal anti-diabetic drug should have following characteristics:

1. It should orally active.
2. It should improve diabetes associated complications.
3. It should ameliorate impaired insulin secretion.
4. It should decrease insulin resistance.
5. It should cause only a small degree of adverse reactions. It should not cause hypoglycemia.
6. It should have specific effects on the impaired metabolism of liver, muscle and fat tissue, intending for the extra pancreatic action.
7. It should supplement dietary management and should not replace it
8. It should have anorectic action to aid weight reduction.

**HERBS/HERBAL DRUGS USED IN STUDY**

These drugs were screened for their effect on DM and best was selected for the detailed study.

1). *Butea monosperma*
2). *Terminalia Arjuna*
3). *Pycnogenol*
**BUTEA MONOSPERMA**
Scientific Name(s): *Butea monosperma*.
Family: Fabaceae
Common Name(s): Dhak, Palash, Flame of the forest, Parrot tree

**Botany:** *Butea monosperma* (BM) (Lam.) is commonly known as Flame of forest, belongs to the family Fabaceae [Pati et al., 2006]. It is a medium sized tree native of the mountainous regions and common throughout India, Burma and Ceylon except in very acrid parts. It is also found in other asian countries Bangladesh, Bhutan, Cambodia, China, Indonesia, Java, Laos, Myanmar, Nepal, Pakistan, Sri Lanka, Thailand, Vietnam. Generally it grows gregariously on open grasslands and scattered in mixed forest. It is a medium sized dry season-deciduous tree, growing to 15 m tall. It is a slow growing tree, young trees have a growth rate of a few feet per year. The leaves are pinnate, with an 8–16 cm petiole and three leaflets, each leaflet 10–20 cm long. The flowers are 2.5 cm long, bright orange-red, and produced in racemes up to 15 cm long. The fruit is a pod 15–20 cm long and 4–5 cm broad.

**History**
Almost all the parts of the plant are being used since decades in medicine and for other purposes. It is well-documented medicinal plant, which is used in Ayurvedic system for liver ailments. Bark fibers are obtained from stem for making cordage [Kirtikar and Basu, 1935]. Stem bark powder is used to stupefy fishes. Young roots are used for making ropes [Publication and Information Directorate, CSIR, New Delhi, 1988]. Green leaves are good fodder for domestic animals. Leaves are used for making platters, cups, bowls and beedi wrappers [Ambasta, 1994]. Leaves are also used for making Ghongda to protect from rains and are eaten by buffaloes and elephants. Tribals use flowers and young fruits as vegetables. Flowers are boiled in water to obtain a dye [Pati et al., 2006]. Orange or red dye is used for colouring garments and for making skin antiseptic ointments [Agarwal, 1990]. Fresh twigs are tied on horns of bullocks, on occasion of ‘pola’ and dry twigs are used to feed the sacred fire [Pati et al., 2006]. Free radical scavenging activity of various extracts of flowers by using different in-vitro models like reducing power assay, scavenging of 2,2 diphenyl-1-picrylhydrazyl (DPPH) radial, nitric oxide radical, super oxide anion radical, hydroxyl radical and inhibition of erythrocytes hemolysis using 2,2’ azo-bis (amidinopropane) dihydrochloride (AAPH) have been done earlier [Lavhale et al., 2007]. The antistress activity of flowers could be due to higher phenolic contents in the extracts [Bhatwadekar et al., 1999]. Due to thyroid inhibitory and insulin stimulatory nature of stigmasterol, isolated from *B. monosperma*, suggest its anti-diabetic and antiperoxidative properties [Sharma and Garg, 2009; Panda et al., 2009]. Some preliminary study on the antihyperglycemic and antihyperlipaemic effects of BM in NIDDM rats have been reported [Somani et al., 2006; Bavarva and Narasimhacharya, 2008].
Phytochemistry

The phytochemical analysis showed that major chemical constituents of BM were sterols, poly-phenols, flavanoids, ascorbic acid and saponins. **Flower:** Triterpene [Kasture et al., 2002], butein, butin, isobutrin, coreopsin, isocoreopsin (butin 7-glucoside), sulphurein, monospermoside (butein 3-α-D-glucoside) and isomonospermoside, chalcones, aurones, flavonoids (palasitrin, prunetin) and steroids [Lavhale and Mishra, 2007; Gupta et al., 1970].

**Seed:** Oil (yellow, tasteless), proteolytic and lypolytic enzymes, plant proteinase and polypeptidase. (Similar to yeast tripsin) [Publication and Information Directorate, CSIR, New Delhi, 1988].

**Resin:** Jalaric esters I, II and laccijalaric esters III, IV.; Z-amyrin, e-sitosterone its glucoside and sucrose; lactone-nheenicosaonic acid--lactone (11,12).

**Sap:** Chalcones, butein, butin, colourless isomeric flavanone and its glucosides, butrin [Publication and Information Directorate, CSIR, New Delhi, 1988].

**Leaves:** Glucoside, Kino-oil containing oleic and linoleic acid, palmitic and lignoceric acid [Nadkarni’s, 2002].

**Bark:** Kino-tannic acid, Gallic acid, pyrocatechin (K.M. Nadkarni’s, 2002). The plant also contains palasitrin, and major glycosides as butrin, alanind, allophanic acid, butolic acid, cyanidin, histidine, lupenone, lupeol, (-)-medicarpin, miroestrol, palasimide and shelloelic acid [Nadkarni’s, 2002; Indurwade et al., 2005; Shah et al., 1992; Madhav et al., 1967; Porwal et al., 1988; Robinson et al., 1937; Bandara et al., 1990; Schoeller et al., 1938].

**Stem:** 3-Z-hydroxyeuph-25-ene and 2,14-dihydroxy-11,12-dimethyl-8-azo-octadec-11-enylcyclohexane [Guha et al., 1990]. Stigmasterol-α-D-glucopyranoside and nonacosoanoic acid [Shukla et al, 2000].

**Beneficial effect of flower**

*Butes monosperma* flowers has been shown to possess a number of properties being bitter, aphrodisiac, expectorant, tonic, emmenagogue, diuretic, good in biliousness, inflammation and gonarthoea. Earlier flowers has been used as astringent to bowel, cure “Kapha”, leprosy, strangury, gout, skin diseases, thirst, burning sensation, eye diseases [Burli and Khade, 2007]. Flowers are boiled in water to obtain a dye, [Patil et al., 2006] which is used for colouring garments and for making skin antiseptic ointments [Agarwal, 1980]. Flowers have been reported as depurative, as they are used to
disperse swelling and to promote menstrual flow and also useful to prevent pus from
urinogenital tracts of males [Burdi and Khade, 2007]. Furthermore, flowers crushed in milk
and sugar has been used to reduce body heat and chronic fever. Flowers soaked in water
overnight has been used against leucorrhoea till cure [Patil et al., 2006, Kirtikar and Basu,
1935]. Flowers of *Butea monosperma* are boiled in water to obtain a dye and also used as an
antiseptic, against infections of round worms, thread worms and Giardiasis [Badhe and
Pande, 1988].

**SOME BIOLOGICAL ACTIVITIES**

**Antistress activity**

Extract has been shown to attenuate water immersion stress, induced elevation of brain
serotonin and plasma corticosterone levels [Bhatwadekar et al., 1999; Kasture et al., 2002].

**Hepatoprotective and antitumorogenic properties**

An extract from the flowers of *Butea monosperma* is used in India for the treatment of liver
disorders and two antihypototoxic flavonoids, isobutrin and butrin have been isolated from
the extract [Wagner et al., 1986]. Chemopreventive effects of extract has been shown on
hepatic carcinogenesis and on tumor promoter induced markers and oxidative stress
[Sehrawat and Sultana, 2006] and confined these effect may be due to presence of its
constituents isobutrin and butrin. Recently, aqueous extract of flowers of *Butea monosperma*
has been evaluated for its hepatoprotective efficacy against acute liver injury to validate its use
in traditional medicines [Sharma and Shukla, 2011]. Moreover, hepatoprotective and anti-
cancer activities of the aqueous extract of *Butea monosperma* flowers were evaluated [Tenzin
Choedon et al., 2010]. Recently, the aqueous extract of *B. monosperma* flowers has been
reported to be acted not only hepatoprotective but also antitumorogenic by preserving the
nuclear morphometry of the liver [Mathan et al., 2011].

**Anticonvulsive activity**

Kasture et al. [2000] reported effect of flowers in memory and behaviour mediated via
monoamine neurotransmitters [Kasture et al., 2000]. Anticonvulsive activity may be due to
the presence of a triterpene [Kasture et al., 2000; Kasture et al., 2002]. Further studies are
required to investigate its usefulness in the treatment of epilepsy.

**Antiestrogenic and antifertility activity**

Shah et al., [1990] reported antiestragenic activity of flowers. Significant inhibition of uterus
weight gain, vaginal epithelium cornification and characteristic histological changes were
observed [Shah et al., 1990].
Free radical scavenging

Free radical scavenging activity of various extracts of flowers evaluated by using different in-vitro models like reducing power assay, scavenging of 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical, nitric oxide radical, superoxide anion radical, hydroxyl radical and inhibition of erythrocytes hemolysis using 2,2' azo-bis (amidinopropane) dihydrochloride (AAPH) [Lavhale and Mishra, 2007]. Methanolic extract along with its ethyl acetate and butanol fractions showed potent free radical scavenging activity. The observed activity could be due to higher phenolic contents in the extracts [Schoeller et al., 1940].

Anti-inflammatory activity of Butea monosperma flowers

Methanolic extract of Butea monosperma flowers was studied for anti-inflammatory activity against carrageenan induced paw edema and cotton pellet granuloma in albino rats [Shahavi and Desai, 2008]. Recently, anti-inflammatory activity of the aqueous extract of Butea monosperma flowers was investigated [Tenzin Choedon et al., 2010]. Furthermore, anti-inflammatory effects of BM and its constituent’s butrin, isobutrin, and butein were investigated for the treatment of mast cell-mediated inflammation. This was the first report identifying the molecular basis of the reported anti-inflammatory effects of BM and its constituents [Zafar Rasheed et al., 2010] and may be valuable treatment for inflammatory and other diseases in which activated mast cells play a role.

Antidiabetic activity

Several preliminary studies have been carried out to investigate the antidiabetic activity of BM using different part of the extract like seed, leaves, bark and flowers but none meets to upto mark. Some preliminary studies have shown the hypoglycemic property of BM flowers [Budhe et al., 1996; Shiva, 1998; Somani et al., 2006]. One preliminary study on the antihyperglycemic and antihyperlipaemic effects of Butea monosperma seeds in NIDDM rats have been reported [Bavarva and Narasimhacharya, 2008]. Moreover, antidiabetic and antioxidant activity of leaves of BM have been investigated [Sharma and Garg, 2009]. Stigmasterol, isolated from B. monosperma bark has been suggested to posses anti-diabetic and antiperoxidative properties due to thyroid inhibitory and insulin stimulatory nature [Panda et al., 2009]. Recently, antihyperglycemic and antioxidative potential of Butea monosperma flowers in alloxan-induced diabetic mice [Sharma and Garg, 2009].
**TERMINALIA ARJUNA**

Scientific Name(s): *Terminalia arjuna*. Family: Combretaceae  
Common Name(s): Arjun, Arjuna, Koha, Kahu, Arjan, Vellamatta

**Botany:** *Terminalia arjuna* (TA) is a deciduous and evergreen tree, standing 20–30m above ground level. It belongs to Combretaceae family. It is found in abundance throughout Indo-sub-Himalayan tracts of Uttar Pradesh, South Bihar, Madhya Pradesh, Delhi and Deccan region near ponds and rivers. It is also found in forests of Sri Lanka, Burma and Mauritius.  
TA was introduced in Mauritius by early Indian migrants [Pettit et al., 1996; Gurib-Fakim et al., 1997].

**Phytochemistry**

TA holds a reputed position in both Ayurvedic and Yunani Systems of medicine. active constituents of TA include tannins, triterpenoid saponins (arjunic acid, arjunolic acid, arjungenin, arjunglycosides), flavonoids (arjunone, arjunolone, luteolin), gallic acid, ellagic acid, oligomeric proanthocyanidins (OPCs), phytosterols, calcium, magnesium, zinc and copper [Bone, 1996; Kapoor, 1990].

(A) Stem bark

1. Triterpenoids: arjunin, arjunic acid, arjunolic acid, *arjungenin, **terminic acid [Row et al., 1970a; *Honda et al., 1976a; **Anjaneyulu and Prasad, 1983]
3. 3β-Sitosterol [Ghosh, 1926; Anjaneyulu and Prasad, 1983].
4. Flavonoids: arjunolone, arjunone, bicalein, *luteolin, gallic acid, ethyl gallate, quercetin, kempferol, pelargonidin, oligomeric proanthocyanidins [Sharma et al., 1982; *Pettit et al., 1996; Anonymous, 1999].
5. Tannins: pyrocatechols, punicallin, punicalagin, terchebulin, terflavin C, castalagin, casuarin, casuarinin [Dymock et al., 1891; Ghoshal, 1909; Chopra and Ghosh, 1929; Takahashi et al., 1997; Lin et al., 2001].
(B) Roots
1. β-Sitosterol [Anjaneyulu and Prasad, 1983].
2. Triterpenoids: arjunic acid, arjunolic acid, oleanolic acid, terminic acid [Anjaneyulu and Prasad, 1983].
3. Glycosides: arjunoside I, arjunoside II, arjunoside III, arjunoside IV, \( \alpha, \beta \)-dihydroxy-3-oxo-olean-12-en-28-oic acid28-C-\( \beta \)-d-glucopyranoside [Anjaneyulu and Prasad, 1982a,b; Choubey and Srivastava, 2001].

(C) Leaves and fruits
1. Glycosides
2. Flavonoids: luteolin [Pettit et al., 1996].

SOME BIOLOGICAL ACTIVITIES OF TA BARK
Cardioprotective and antioxidant activities
It was reported that extracts of TA contain several antioxidants and cardioprotective agents such as napthanol glycoside, rjunaphthanoloside [Ali et al., 2003], arjunic acid, arjunigenin and their glucosides, arjunetin and arjunoglucoside II [Pawar and Bhutani, 2005]. Sun et al. [2008] identified arjunic acid, a strong free radical scavenger from the bark of Terminalia arjuna [Sun et al., 2008]. The bark of TA is used since ancient times for the treatment of cardiac disorders [Ram et al., 1997; Gauthaman et al., 2001].

The cardioprotective effects of TA bark has been studied in isoproterenol-induced myocardial ischemia model in rats, rabbits and mice by several authors [Dwivedi et al.,...]

[Fig 15. Chemical structure of important flavonoids detected in TA: (a) apigenin and bioleolin; (b) kempferol and quercetin; (d) pelargonidin and cyanidin]

[Fig 16. Chemical structure of terpenoids and glycosides found in TA: (a) arjunic acid; arjunoside I, arjunoside II and arjunoside IV; (b) terminic acid; (c) terminal acid]
1988; Antani et al., 1991; Tandon et al., 1995]. Effect of arjunolic acid derived from TA on antiplatelet activity, electrocardiographic changes, serum marker enzymes, antioxidant status, lipid peroxide and myeloperoxidase (MPO) were measured. Cardioprotection conferred by arjunolic acid could possibly be due to the protective effect against the damage caused by myocardial necrosis [Sumitra et al., 2001]. Role of TA as an antioxidant agent on ischemic perfused rat heart has been studied very recently. It was thus concluded that crude bark of TA augments endogenous antioxidant compounds of rat heart and prevents it from oxidative stress [Gauthaman et al., 2001]. Recently arjungenin an oleanane terpenoid derived from TA bark and its glucoside, arjunglucoside II, have been demonstrated to exert free radical scavenging activities in human polymorphonuclear cells [Pawar and Bhutani, 2005]. Few other experiments carried on using extract of TA bark has been demonstrated to protect the oxidant damage to the liver, kidney and heart following carbon tetrachloride challenge [Manna et al., 2006; Manna et al., 2007] and N-nitrosodiethylamine-induced liver injury in rats [Sivalokanathan et al., 2006]. These observations clearly indicate endogenous antioxidant activity of the TA. If we integrate above observations with that of the earlier ones [Gauthaman et al., 2001; Karthikeyan et al., 2003], there is reasonably sufficient experimental evidence that TA plays a significant role in the prevention of oxidative injury to the heart as well as to the LDL cholesterol. It has been described that cardioprotective role of TA bark extract may be mediated through alterations in thyroid hormones [Parmar et al., 2006]. Furthermore, studies have suggested the cardioprotective effect of bark extract due to its antioxidant property [Sinha et al., 2008; Singh et al., 2008]. The mechanistic clues for the cardioprotective effect of *Terminalia arjuna* bark extract has been reported in isoproterenol-induced chronic heart failure in rats [Parveen et al., 2011]. The bark and leaf extract has been reported to exhibit the antioxidant activity and reducing oxidative stress in diabetic animals also respectively [Raghavan and Kumari, 2006; Biswas et al., 2011].

**Cardiotonic activities**

Early physiological studies carried on the isolated frog and rabbit heart revealed that the bark of TA had cardiotonic and stimulant actions [Ghoshal, 1909]. It was subsequently found that intravenous administration of the glycoside, obtained from the bark of TA, resulted in rise in blood pressure [Ghosh, 1926]. Later on it was detected that the bark powder, in addition to cardiotonic property, also possessed diuretic properties [Caious et al., 1930]. Subsequent experimental studies in isolated frog heart and isolated rat atria revealed that the aqueous extract of the bark had chronotropic and inotropic activities [Chopra et al., 1958; Radhakrishnan et al., 1993; Karamsetty et al., 1995]. Recently, cardiotonic activity of TA bark extract has been investigated in healthy young adults [Sandhu et al., 2010].
Coronary flow
Injection of the aqueous extract of the bark in isolated rabbit heart preparation (Langendorff's) was noted to produce increase in coronary flow [Bhatia et al., 1998]. The antiplatelet activity has been evaluated in healthy subjects and patients with coronary artery disease using TA ethanolic bark extract [Malik et al., 2009].

Hypotensive effects
Singh et al. [1982] reported dose-dependent sustained hypotension and bradycardia. Further, hypotensive effect was supported by other studies of aqueous extract of TA [Srivastava et al., 1992; Bhatia et al., 2000; Nammi et al., 2003]. A study on the rat thoracic aorta using aqueous extract revealed constriction of isolated rat thoracic aorta followed by relaxation. The aqueous extract as well as the fraction of the extract containing tannin-related compounds produced hypotensive effects [Takahashi et al., 1997]. Increased Prostaglandins activity may possibly explain the pharmacological basis of the increased coronary flow following TA infusion [Bhatia et al., 1998]. This may also be contributing to the beneficial effect of TA in CAD patients.

Effects on lipids
A case controlled study in rabbits fed on high cholesterol diet and administered TA bark has shown that rabbits receiving TA had a marked reduction in total cholesterol than control rabbits [Tiwari et al., 1990]. These findings were further confirmed in a later work [Pathak et al., 1990; Khanna et al., 1996]. Among three Terminalias, TA was observed to be the most potent hypolipidemic agent [Dwivedi et al., 2000; Dwivedi and Gupta, 2002; Khalil, 2005]. Interestingly it also raised high-density lipocholesterol. Besides hypolipidemia it also induced partial inhibition of aortic atherosclerosis, thus showing anti-atherogenic properties [Shaila et al., 1998].

Anti-ischemic activities
The utility of TA therapy in ischemic stroke and ischemic heart disease has been extensively studied by several group of workers [Dwiedi et al., 1988; Wahal, 1991; Jain et al., 1992; Dwivedi and Agarwal, 1994; Kumar et al., 1999; Dwivedi et al., 2005a, b]. Dwivedi and Aggarwal, described the role of TA for the treatment of T2DM associated with hypertension, ischemic heart disease, and/or dyslipidemia [Dwivedi and Aggarwal, 2009].

Effect on endothelial dysfunction
On account of its rich bioflavonoid content it is considered to be a strong antioxidant and an ideal agent for correction of endothelial dysfunction [Bhamni et al., 2004].

Anticancer activity
Due to its antioxidant and immunomodulatory activity it has been described to prevent liver disorders [Huseini et al., 2005]. Moreover, it plays a beneficial role on hepatocellular carcinoma in vivo and in vitro [Sivalokanathan et al., 2006a, b] and possess anti carcinogenic activity [Verma and Vinayak, 2009]. Furthermore, it has profound effects against DLA tumour cells [Ganesan et al., 2010].
Antiulcer property
Bark of TA has been shown to possess antiulcer and ulcer healing property probably due to its free radical scavenging activity [Devi et al., 2007; Devi et al., 2007].

Wound healing property
Some studies have evaluated the wound healing property of TA [Mukherjee et al., 2003; Rane and Mengi, 2003; Chaudhari and Mengi, 2006].

PYCNOGENOL

Scientific Name(s): Pinus maritima Mill.; Pinus pinaster Ait. Family: Pinaceae
Common Name(s): Pycnogenol

Botany
Pycnogenol (PYC) is the brand name of British company Horphag Research, Ltd. for a standardized herbal extract from the bark of the French maritime pine tree. The Maritime Pine (Pinus pinaster) is a pine native to the western Mediterranean region. The largest man-made forest in the world, the 900,000 hectare Les Landes on the Atlantic coast of southwestern France, is populated almost entirely by P. pinaster. The range extends from Portugal and Spain north to southern and western France, east to western Italy, and south to northern Morocco, with small outlying populations in Algeria and Malta (possibly introduced by man). It generally occurs at low to moderate altitudes, mostly from sea level to 600 m, but up to 2000 m in the south of its range in Morocco. PYC is known to be one of the most potent anti-oxidants currently known, and is many times more powerful than both vitamins C and E [Nelson et al., 1998; Packer et al., 1999]. As well as being an extremely potent anti-oxidant it actually recycles vitamins C and E, allowing them to be re-used, and prolonging their life span. In addition, PYC increases the production of our own natural anti-oxidant defenses [Wei et al., 1997].

History
The use of PYC may be traced back to ancient traditional medicine in both the Old World and in the Americas. The bark of the pine has been utilized for more than 2000 years. Indications of the therapeutic use of the pine bark can be found in the 4th century B.C, when Hippocrates mentioned its use against inflammatory diseases [Kollesch et al, 1994]. In the Thesaurus Medicaminum, compiled by the pharmacist H. Minner in 1479, pine bark was considered helpful for wound healing [Minner, 1479]. In old Europe pine bark was utilized against inflammation and to overcome the symptoms of scurvy [Dragendorff et al., 1898] the latter may be considered an early suggestion for the strong interplay of flavonoids with
Section-II  
Review of literature

Ascorbic acid. It was also used as an emergency food in northern Europe until World War II [Helsinki, 1979]. Other uses of PYC were suggested by the naturalist Hieronymus Boch and included topical application on skin ulcers and general use against skin disorders [Hoppe et al., 1969]. In the New World, Native Americans utilized the bark of the pine as a food, a beverage, and a remedy for various conditions, such as inflamed wounds or ulcers, now recognized to have free radical involvement [Chandler, 1979; Fielder, 1975; Youngken, 1924]. It is worth noting that pine bark was used for many conditions that are now known to involve vitamin C deficiency, such as scurvy, skin disorders, and wound healing. This indicates an interaction between vitamin C and flavonoids, which was also suggested by Szent-Gyorgyi and coworkers [Bentosath, 1936]. They understood the complex nature of this interaction and the important role that polyphenols (which were proposed to be named vitamin P) play in the recycling and interplay of other antioxidants, and, ultimately, in human health and disease. An extensive overview of the historical landmarks in the pharmacologic use of pine bark and French maritime pine bark has recently been compiled by Drehsen [Drehsen, 1998].

Chemistry

French maritime pine (Pinus maritima) bark extract, Pycnogenol® (PYC), a patented combination of bioflavonoids, has a high antioxidant potential [Rohdewald, 1998]. A concentrate of polyphenols, mainly phenolic acids, and procyanidins, the extract is used extensively as a dietary supplement. Using a patented process, pine bark is boiled with saturated sodium chloride, cooled, and extracted with ethyl acetate. After concentration, the solution is precipitated with chloroform. This process is repeated several times to remove condensed tannins from the product. Pycnogenol-related compounds are also designated as procyanidol oligomers (PCOs). The oligomers range from monomers to dodecamers [Suzuki et al., 2002]. PYC is primarily composed of proanthocyanidins (80-85%), the monomers catechin and taxifolin (5%), and phenolic acids (2-4%). The phenolic acids are derivatives of benzoic and cinnamic acids.
Pycnogenol Research

The pharmacokinetics of PYC constituents have been studied in human volunteers. A total of 15 compounds were found to be rapidly absorbed and metabolized by phase 2 enzymes [Grimm et al., 2006]. Other studies of PYC bioavailability and efficacy have been reviewed [Williamson et al., 2005]. Research has demonstrated many positive benefits from PYC supplementation including: 1) prevention of blood clots; 2) protects DNA from damage; 3) lowers blood sugar levels; 4) lowers blood pressure; 5) reduces the risk of cancer; 6) protects cells from the damage of UV radiation; 7) protects against the damaging effects of cigarette smoke; 8) improves sperm quality; 9) improves wound healing; 10) improves lung function in asthmatics; 11) increase the break down of fats; 12) reduces the storage of fats within fat cells. It may also be of benefit to sports people by reducing the amount of damage caused by free radicals during exercise.

Antioxidant

PYC assists in the treatment of hypoxia following atherosclerosis and cardiac or cerebral infarction, and reduces tumor promotion, inflammation, ischemia, alterations of synovial were found to inhibit matrix metalloproteinases more potently than the parent compound [Grimm et al, 2004]. Inhibition of xanthine oxidase by PYC was found to be a selective effect on the enzyme, [Moini et al., 2000] which is not mediated by redox activity as was initially proposed [Elstner and Kleber, 1989]. An electron spin resonance study of procyanidin B3 observed formation of a free radical species [Guo et al., 1999]. An ex vivo study with human plasma showed that pycnogenol could enhance the antioxidative capacity of the plasma if combined with whey proteins [Janisch et al., 2002].

Effects in defined cellular systems have also been detected. PYC was capable of quenching NO formation in activated macrophages and inhibited both induced nitric oxide synthase (iNOS) mRNA expression and iNOS activity [Packer et al., 1999]. It protected endothelial cells from oxidant-induced injury, possibly by upregulating radical scavenging systems [Wei et al., 1997]. Similar effects were seen in macrophages, with inhibition of oxidative burst, lipoprotein oxidation, and hydroxyl radical-induced DNA damage [Nelson et al., 1998]. HT-4 neuronal cells were protected against glutamate-induced cytotoxicity [Kobayashi et al., 2000]. PYC preparations have even been used in toothpastes [Battino et al., 2005] and to limit lipid oxidation in cooked beef products [Ahn et al., 2006]. A large number of studies on cultured cells have demonstrated that PYC can limit damage caused by nitrosamine metabolites in liver and lung microsomes, [Huynh et al., 1998] maintain tocopherol levels in endothelial cells in the face of reactive nitrogen species, [Virgili et al., 1998a] and scavenge free radicals in macrophages [Virgili et al., 1998b]. Rat cerebellar granule cells were protected against ethanol-induced apoptosis, [Siler-Marsiglio et al., 2004a] and the protective mechanisms were found to involve reduction of reactive oxygen species and a variety of other biochemical effects [Siler-
Marsiglio et al., 2004b]. PYC also protected PC12 rat cells from amyloid-beta peptide-induced apoptosis, [Peng et al., 2002] as well as from hydrogen peroxide injury [Horakova et al., 2003]. Streptozotocin-induced diabetic rats given PYC demonstrated reduced blood glucose and altered biochemical markers of oxidative stress [Maritiin et al., 2003].

Inflammation

PYC is capable of inhibiting inflammatory mediators [Sharma et al., 2003]. PYC reduced the production of the proinflammatory cytokine IL-1 in macrophage cell lines, blocked activation of NF-kB and activator protein (AP)-1, and abolished lipopolysaccharide-stimulated (LPS)-induced IaB destruction [Cho et al., 2000; 2001]. Pycnogenol had a protective effect in a rat model of inflammatory bowel disease [Mochizuki and Hasegawa, 2004]. Inhibition of NF-kB activation and matrix metalloproteinase (MMP)-9 secretion was found in plasma of humans after 5 days of oral PYC administration [Grimm et al., 2006].

Platelet function

The effect of PYC on the platelet function of smoking and nonsmoking patients with coronary artery disease has been studied [Wang et al., 1999; Putter et al., 1999; Araghi-Niknam et al., 2000]. PYC decreased platelet aggregation ex vivo in all 3 studies. In the nonsmoker study, platelet activating factor (PAF)-stimulated aggregation was not affected, ruling out a role for PAF in the mechanism. This work is also the subject of a patent [Rohdewald, 1998]. PYC has also proved to be effective at reducing the risk of blood clots [Belcaro et al., 2004; Putter et al., 1999; Araghi-Niknam et al., 2000]. A reduced risk of blood clots is particularly important since when blood clots can cause strokes and heart attacks and therefore present a very serious health threat.

Blood pressure control

PYC was able to significantly lower blood pressure in moderately hypertensive patients [Hossein et al., 2001a]. The researchers believed that it was PYC ability to elevate NO production that was the primary reason for reduced blood pressure. Previous research has shown that PYC can increase NO production [Witzpatrick, 1998]. NO causes blood vessels to relax, increasing blood flow, and decreasing blood pressure. Further research also supports the use of PYC in moderately high blood pressure patients [Liu et al., 2004a]. PYC is able to bind to collagen — a key protein that gives strength and elasticity to blood vessels — protecting it from damage caused by free radicals. It can also help to repair worn out capillaries, reducing the leakage of fluids [Gulati, 1999]. As such it has proved effective in the treatment of the painful swelling around the ankles during long haul flights [Cesarone et al., 2005] and is often taken to reduce the risk of developing thread veins and to slow their progression.
Increase the breakdown of fats
Researchers have found that PYC inhibits the oxidation of LDL cholesterol and therefore helps to prevent the build up of fatty deposits on the inner lining of blood vessels [Nelson et al., 1998; Hasegawa, 1999; Hasegawa, 2000; Mochizuki and Hasegawa, 2004].

Control blood sugar
It also appears to be effective at controlling blood sugar levels. Research by Liu et al., [2004 a, b] found that PYC significantly lowered blood sugar levels in Type 2 diabetes patients.

Improves lung function in asthmatics
Asthmatics may also benefit from PYC. Researchers studied the effects of consuming Pycnogenol in asthmatic patients [Hosseini et al., 2001b]. The researchers found that the subjects total lung volume increased significantly following PYC consumption. Asthma is known to be caused by an immune reaction that reduces airflow into the lungs. The researchers found that improved airway function corresponded with a reduced immune reaction following supplementation. Therefore, PYC could prove effective in the treatment in other inflammatory immune diseases such as the inflammatory skin disorder psoriasis [Rihn et al., 2001].

Other benefits
Researchers have shown that PYC may reduce the risk of some types of cancer effectively [Peng et al., 2000; Simme and Reeve, 2004]. It also appears to help to prevent sunburn and photoaging caused by ultraviolet radiation [Salieu et al., 2001]. It may be beneficial for reducing cramps and pain associated menstrual disorders [Kohana and Suzuki, 1999], and also both sperm quality and sperm function, and could therefore be of benefit to couples looking to improve their fertility naturally [Roseff, 2002].

Research has shown that free-radicals caused by exhaustive exercise can cause muscle cells to completely rupture [Vina et al., 2000]. By taking a potent anti-oxidant like PYC you can counteract the damage caused by free-radicals and thus gain more benefit from exercise [Pavlovic, 1999]. One of the most interesting pieces of research with regards to sport was a paper looking at the effects of PYC on human growth hormone (HGH) [Buzzard et al., 2002]. The researchers found that PYC was extremely effective at enhancing growth hormone secretion in cultured cells. Enhancing human growth hormone naturally, with PYC, would be very beneficial for anyone looking to improve recovery from exercise. Since growth hormone increases lean muscle mass, and decreases fat mass, PYC could be of benefit to people looking to increase muscle mass and decrease fat mass. Rats with experimental wounds healed more rapidly with topical PYC than controls and showed reduced scarring [Blazso et al., 2004]. PYC had a variety of positive immunomodulatory effects in mice infected with a murine retrovirus or dosed with ethanol [Cleshier et al., 1996]. It also reduced the toxicity of antitumor drugs to mice [Feng et al., 2002] and protected rats from ionizing radiation damage [De Moraes Ramos et al., 2006; Sime and Reeve, 2004].
METHODOLOGY
MATERIALS AND METHODS

Chemicals and reagents
Oxidized and reduced glutathione (GSSG & GSH), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 2, 2-diphenyl-1-picryl hydrazyl (DPPH), thiobarbituric acid (TBA), 1-chloro-2, 4-dinitrobenzoic acid (CDNB), ethylene diamine tetra acetic Acid (EDTA), trichloroacetic acid (TCA), and 2, 4-dinitrophenyhydrazine (DNPH) and streptozotocin (STZ) were purchased from Sigma-Aldrich, Chemicals Pvt. Ltd. India. Sulfsalicylic acid (SSA), p-nitrophenyl phosphate (PNPP), anthrone reagent, bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) were purchased from Srl and Merck Chemical Pvt. Ltd. India. Other chemicals were of analytical reagent grade.

Plant material
The B. monosperma (BM) flowers and T. arjuna (TA) bark were procured from Saiba industries, Mumbai, INDIA and authenticated by Dr. Mohd. Mujeeb, Department of Pharmacognosy and Phytochemistry, Jamia Hamdard, New Delhi. Pycnogenol (PYC) was procured from Horphag Research, Gevena, Switzerland.

Selection of herbal drugs
Many of the herbs act as anti-hyperglycemic, anti-lipidemic and used for DM in animals as well as human. But it is not clearly known whether these herbs act directly on the DM or it has some other effects which directly or indirectly are related with the DM. The Following herbs/herbal drugs were selected after a detail search with their therapeutic importance in Indian system of medicine. These drugs were screened for their effect on DM and the best was selected for the detailed study.

1) Butea monosperma
2) Terminalia arjuna
3) Pycnogenol

Preparation of the active phytochemical constituents of Butea monosperma
The powdered flower material was extracted exhaustively in a Soxhlet apparatus. Briefly, 500 g dried powdered plant material was Soxlet extracted with methanol for 20 h. The extract was filtered and then solvent was removed under reduced pressure in rotatory evaporator, to obtain an orange brown powder (yield 25%). The dried extract was dissolved in normal saline and used for experimental work.
Preparation of the active phytochemical constituents of *Terminalia arjuna*

The powder material of *T. arjuna* was extracted exhaustively in a Soxhlet apparatus. Briefly, 500 g dried powdered plant material was Soxlet extracted with methanol for 20 h. The extract was filtered and then solvent was removed under reduced pressure in rotatory evaporator. Dark brownish red shiny crystals were obtained and the yield was 24%. The dried extract was dissolved in normal saline and used for experimental work.

Preliminary phytochemical screening

The extracts may be considered as biosynthetic laboratory for a multitude of compounds like alkaloids, glycosides, volatile oils, and tannins etc that exert physiological effects. The compounds that are responsible for therapeutic effect are usually the secondary metabolites. The extracts of the plant material were subjected to preliminary phytochemical screening for the detection of various plant constituents [Kokate et al., 1996].

Tests for sterols

a) *Salkowski reaction*

Few mg of the residue of each extract was taken in 2 ml of chloroform and 2 ml of conc. sulphuric acid was added from the side of the test-tube. The test-tube was shaken for few minutes. The development of red color in the chloroform layer indicated the presence of sterols.

b) *Liebemann-Burchards reaction*

Few mg of residue was dissolved in chloroform and few drops of acetic anhydride were added to it, followed by concentrated sulphuric acid from the side of the tube. A transient color development from red to blue and finally green indicated the presence of sterols.

Tests for alkaloids

Few mg of residue of extract was taken separately in 5 ml of 1.5% v/v hydrochloric acid and filtered. These filtrates were then used for testing alkaloids with following reagents:

a) *Dragendorff’s reagent*

It was prepared by mixing solution A (17 gm of bismuth subnitrate + 200 gm tartaric acid + 800 ml distilled water) and solution B (160 gm potassium iodide + 4(x) ml distilled water) in 1:1 v/v proportion. From this solution working standard was prepared by taking 50 ml of this solution and adding 100 gm of tartaric acid and making upto 500 ml with distilled water.

The above Dragendorff’s reagent was sprayed on whatmann No. 1 filter paper and the paper was dried. The test filtrate after basification with dilute ammonia was extracted with chloroform and the chloroform extract was applied on the filter paper, impregnated with Dragendorff’s reagent, with the help of a capillary tube. Development of an orange red color on the paper indicated the presence of alkaloids.
b) *Mayer's reagent* (*potassium mercuric iodide reagent*)
The Mayer's reagent was prepared as follows: 1.36 gm of mercuric chloride was dissolved in 60 ml of distilled water. Both the solutions were mixed and diluted to 100 ml with distilled water. To a little of the test filtrate, taken in a watch glass, a few drops of the above reagent were added. Formation of cream colored precipitate showed the presence of alkaloids.

c) *Wagner's reagent* (*Iodine-potassium iodide*)
1.27 g of iodine and 2 g of potassium iodide were dissolved in 5 ml of water and the solution was diluted to 100 ml with water. When few drops of this reagent were added to the test filtrate, a brown flocculent precipitate was formed indicating the presence of alkaloids.

Tests for saponins

a) *Foam test*
A few mg of the test residue was taken in a test tube and shaken vigorously with a small amount of sodium bicarbonate and water. It is a stable, characteristic honeycomb like froth is obtained, saponins are present.

b) *Haemolysis test*
A little of the test residue was dissolved in normal saline in such a way that 5 ml of the solution represented 1 gm of the crude drug. In a series of 5 test-tubes, doses of 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1ml were added and volume was made up to 1ml in each case with normal saline. 1ml of diluted blood (0.5 ml of rabbit's blood diluted to 25 ml with normal saline) was added to each tube and changes observed. If haemolysis of blood occurs, than saponins are present.

Tests for tannins

The test residue of each extract was taken separately in water, warmed and filtered. Tests were carried out with the filtrate using following reagents.

a) *Ferric chloride reagent*
A 5% w/v solution of ferric chloride in 90% alcohol was prepared. Few drops of this solution were added to a little of the above filtrate. If dark green or deep blue color is obtained, tannins are present.

b) *Lead Acetate test*
A 10% W/v solution of basic lead acetate in distilled water was added to the test filtrate. If precipitate is obtained, tannins are present.

Tests for flavanoids

a) *Shinoda test*
A small quantity to test residue was dissolved in 5 ml ethanol (95% v/v) and reacted with few drops of concentrated hydrochloric acid and 0.5 g of magnesium metal. The pink, crimson or magenta color is developed within a minute or two, if flavonoids are present.
Section-III Materials & Methods

b) Porter test
A small quantity of residue was dissolved in HCl/n-butanol and reacted with ammonium iron (III) sulfate, which stabilizes the reaction. Then solution was heated which leads color change and indicates presence of procyanidin. It is an acid hydrolysis, which splits larger chain units (dimers and trimers) into single unit monomers and oxidizes them.

Tests for proteins
a) Biuret test
A few mg of the residue was taken in water and 1ml of 4% sodium hydroxide solution was added to it. This was followed by a drop of 1% solution of copper sulphate. Violet color is formed if proteins are present.

b) Xanthoproteic test
A little residue was taken with 2ml of water and 0.5 ml of concentrated nitric acid was added to it. Yellow color is obtained if proteins are present.

Tests for amino acids
a) Ninhydrin test
The Ninhydrin reagent is 0.1% w/v solution of ninhydrine in n-butanol. A little of this reagent was added to the test extract. A violet or purple color is developed if amino acids are present.

Tests for sugars & carbohydrates
a) Molisch's test
The Molisch's reagent was prepared by dissolving 10g of α-naphthol in 100 ml of 95% alcohol. A few mg of the test residue was placed in a test-tube containing 0.5 ml of water, and it was mixed with 2 drops of Molisch's reagent. To this solution, was added 1 ml of concentrated sulphuric acid from the side of the inclined test-tube, so that the acid formed a layer beneath the aqueous solution without mixing with it. If a purple ring appears at the common surface of the liquids, sugars are present.

b) Barford's test
This reagent was prepared by dissolving 13.3 gm of crystalline neutral copper acetate in 200 ml of 1% acetic acid solution. The test residue dissolved in water and heated with a little of the reagent. If a red precipitate of cuprous oxide is formed within two minutes, monosaccharides are present.

Test for phenolic compounds
a) Ferric chloride solution
The extracts were taken in water and warmed; to this 2ml of ferric chloride solution was added and observed for the formation of green and blue color.

b) Lead acetate solution
To the extract (2ml) lead acetate solution was added and observed for the formation of precipitate.
Test for resins
a) Distilled water (5.0ml) was added to the extract and observed for turbidity.
b) A mixture of extract in acetone (3ml) and HCl (3 ml) was heated on a water bath for 30 minutes and observed for pink color.

Test for glycosides
A small portion of the extract was hydrolysed by boiling with dilute hydrochloric acid for a few minutes and the hydrolysate was subjected to the following tests.

a) Legal’s test
The hydrolysate was dissolved in pyridine and a solution of sodium nitroprusside was added to it and made alkaline. Formation of pink or red color indicates the presence of cardiac glycosides.

c) Bornträger’s test
An organic solvent such as ether or chloroform was added to the hydrolysate and the contents were shaken. The organic layer was shaken and treated with a solution of ammonia. The development of a pink color indicates the presence of anthraquinone glycosides.

Test for lipids/fats
Rub a small quantity of powdered drug on a paper and observed for a permanent translucent stain.

DETERMINATION OF TOTAL PHENOLIC CONTENT
For the determination of total phenolic content the method of Pourmorad et al. [2006] was used. Phenolic compounds are a class of antioxidant agent which acts as free radical terminators. 10 mg/ml solution of extract was prepared in methanol followed by addition of 5 ml Folin- ciocalteau reagent and 4 ml Na₂CO₃ solution. Absorbance was taken at 765 nm after 15 minutes. Gallic acid was used as standard for calculation.

DETERMINATION OF TOTAL FLAVONOID CONTENT
For determination of total flavonoid content the method of Pourmorad et al. [2006] was used. The mechanism of action of flavonoids is through scavenging or chelating process. 10 mg/ml solution of extract was prepared in methanol. 0.5 ml of solution was taken and added 1.5 ml methanol. To this, 0.1 ml of AlCl₃ and 0.1 ml of CH₃COONa and 2.8 ml distilled water were added and kept for 30 minutes. Absorbance was taken at 415 nm. Rutin was used as standard for calculation.
IN VITRO ANTI-OXIDANT ACTIVITY

**Method 1: In vitro antioxidant activity by reducing power method**

The reducing power of extract was determined according to the method described [Oyaizu, 1986]. Different concentrations of extract (10 μg/ml – 50 μg/ml) in 1 ml of distilled water were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide. The mixture was incubated at 50°C for 20 min. 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml distilled water and ferric chloride (0.5 ml, 0.1%). The absorbance was measured at 700 nm (Shimadzu UV-Vis 1601). Ascorbic acid was used as a reference standard.

**Method 2: In vitro antioxidant activity by hydrogen peroxide scavenging method**

The ability of extract to scavenge hydrogen peroxide was determined according to the method of [Ruch et al., 1989]. A solution of hydrogen peroxide (2 mM) was prepared in phosphate buffer (pH 7.4) and concentration was determined spectrophotometrically at 230 nm (Shimadzu UV-Vis 1601). Extract (5 μg/ml – 25 μg/ml) in distilled water was added to a hydrogen peroxide solution (0.6 ml, 2 mM) and the absorbance of hydrogen peroxide at 230 nm was determined after 20 min against a blank solution in phosphate buffer without hydrogen peroxide.

**Method 3: In vitro antioxidant activity by 2, 2-diphenyl-1-picryl hydrazyl radical DPPH scavenging method**

The free radical scavenging capacity of the extract was determined using DPPH [Hasan et al., 2006]. DPPH solution (0.004% w/v) was prepared in 95% methanol. Methanolic extract of plant was mixed with 95% methanol to prepare the stock solution (1 mg/ml). Freshly prepared DPPH solution was taken in test tubes then extract was added followed by serial dilutions (10 μg to 50 μg) to every test tube so that the final volume was 3 ml. After 10 min, absorbance was read at 515 nm using a spectrophotometer (Shimadzu UV-Vis 1601). Ascorbic acid was used as a reference standard.

ANIMALS STUDIES

**Animals**

All the experiments were carried out in male Wistar rats weighing 160-200 g. They were kept in the Central Animal House of Jamia Hamdard (Hamdard University) in colony cages at an ambient temperature of 25 ± 2°C and relative humidity of 45–55% with 12 h light/dark cycles. They had free access to standard rodent pelleted diet (Hindustan Lever Ltd., Bombay, India) and water ad libitum, prior to the dietary manipulation. All procedures using animals were reviewed and approved by the Institutional Animal Ethical Committee that is fully accredited by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA) Chennai, India.
Diabetic models used

*Induction of type 1 diabetes mellitus (T1DM)*

Experimental T1DM was induced by a single intraperitoneal injection of STZ to animals fasted overnight at a dose of 60 mg/kg body weight (fresh solution in 0.1 N citrate buffer; pH 7.5) [Suresh Babu and Srinivasan, 1998]. The rats had free access to 5% of glucose water and basal diet ad libitum during the next 24 hours. Blood samples were obtained from retro-orbital plexus in STZ injected animals at 72 h, after an overnight fast. After 4 days the STZ injection the rats with the fasting blood glucose (FBG) of ≥140 mg/dl were considered diabetic and selected for further studies.

*Induction of type 2 diabetes mellitus (T2DM)*

The model of T2DM was established according to the method reported by Reed et al. [2000] with modification. Rats were allocated with high fat diet (HFD; 40% fat, 18% protein and 41% carbohydrate, as a percentage of total kcal) ad libitum, for the initial period of 2 weeks. After the 2 weeks of dietary manipulation, animals were injected intraperitoneally with low dose of STZ (40 mg/kg in 0.1N citrate buffer; pH 4.5). After 6 days the STZ injection the rats with the FBG of ≥140 mg/dl were considered diabetic and selected for further studies.

Experiment- I: Evaluation of therapeutic dose of BM, TA and PYC against type 1 diabetes mellitus and check whether the required therapeutic dose is safe or produces some type of toxicity

Experiments were carried out to evaluate the therapeutic dose for all three herbs against T1DM. Experiments involving antihyperglycemic activity of these extracts was performed. For comparative studies, glibenclamide (GL) was used as standard. Fasting blood glucose level was measured in each control, diabetic, herbal and standard treated groups. For BM, forty five rats were divided into nine groups of five animals each: group I (control (C) group) rats were fed standard diet and only vehicle treated; group II, III, IV (C + BM) rats were fed standard diet and given three different doses of BM (200mg/kg, 300 mg/kg, 400 mg/kg body weight for 4 weeks; group V (STZ group) rats were injected with STZ and then allowed 5% glucose for next 24 hour; group VI, VII, VIII (STZ + BM) rats were injected with STZ and then supplemented with three different doses of BM for 4 weeks; group IX (STZ + GL) rats were supplemented with GL (0.6mg/kg body weight) for 4 weeks after diabetes induction. For TA, forty five rats were divided into nine groups of five animals each: group I (control (C) group) rats were fed standard diet and only vehicle treated; group II, III, IV (C + TA) rats were fed standard diet and given three different doses of TA (400 mg/kg, 500 mg/kg, 600 mg/kg body weight for 4 weeks; group V (STZ group) rats were injected with STZ and then allowed 5% glucose for next 24 hour; group VI, VII, VIII (STZ + TA) rats were then injected with STZ and then
supplemented with three different doses of TA for 4 weeks; group IX (STZ + GL) rats were supplemented with GL (0.6mg/kg body weight) for 4 weeks after diabetes induction. For PYC, fifty four rats were divided into nine groups of six animals each: group I (control (C) group) rats were fed standard diet and only vehicle treated; group II, III, IV (C + PYC) rats were fed standard diet and given three different doses of PYC (5mg/kg, 10 mg/kg, 15 mg/kg body weight for 4 weeks; group V (STZ group) rats were injected with STZ and then allowed 5% glucose for next 24 hour; group VI, VII, VIII (STZ + PYC) rats were injected with STZ and then supplemented with three different doses of PYC for 4 weeks; group IX (STZ + GL) rats were supplemented with GL (0.6mg/kg body weight) for 4 weeks after diabetes induction. At the end of experiment, 8 hrs fasting rats were anesthetized by ether inhalation and blood was collected from the dorsal aorta. Blood was centrifuged at 3000 rpm for 15 min and serum was separated for analysis of fasting blood glucose.

Experiment- II: Evaluation of therapeutic dose of BM, TA and PYC against type 2 diabetes mellitus and check whether the required therapeutic dose is safe or produces some type of toxicity

Experiments were carried out to evaluate the therapeutic dose for all three herbs against T2DM. Experiments involving antihyperglycemic activity of these extract was performed. For comparative studies, glibenclamide (GL) was used as standard. Fasting blood glucose level was measured in each control, diabetic, herbal and standard treated groups. Fasting blood glucose level was measured in each control, diabetic, herbal treated control and diabetic groups. For BM, forty rats were divided into eight groups of five animals each: group I (control (C) group) rats were fed standard diet and only vehicle treated; group II, III, IV (C + BM) rats were fed standard diet and given three different doses of BM (200 mg/kg, 300 mg/kg, 400 mg/kg body weight for 4 weeks; group V (HFD/STZ group) rats were fed HFD for 2 weeks and the injected with STZ; group VI, VII, VIII (HFD/STZ + BM) rats were fed HFD for 2 weeks and then injected with STZ and then supplemented with three different doses of BM for 4 weeks; group IX (HFD/STZ + GL) rats were supplemented with GL (0.6mg/kg body weight) for 4 weeks after diabetes induction. For TA, forty rats were divided into eight groups of five animals each: group I (control (C) group) rats were fed standard diet and only vehicle treated; group II, III, IV (C + TA) rats were fed standard diet and given three different doses of TA (400 mg/kg, 500 mg/kg, 600 mg/kg body weight for 4 weeks; group V (HFD/STZ group) rats were fed HFD for 2 weeks and the injected with STZ; group VI, VII, VIII (HFD/STZ + TA) rats were fed HFD for 2 weeks and then injected with STZ and then supplemented with three different doses of TA for 4 weeks; group IX (HFD/STZ + GL) rats were supplemented with GL (0.6mg/kg body weight) for 4 weeks after diabetes induction. For PYC, fifty four rats were divided into nine groups of six animals
Section-III  Materials & Methods

each: group I (control (C) group) rats were fed standard diet and only vehicle treated; group II, III, IV (C + PYC) rats were fed standard diet and given three different doses of PYC (5mg/kg, 10 mg/kg, 15 mg/kg body weight for 4 weeks; group V (HFD/STZ group) rats were fed HFD for 2 weeks and then injected with STZ; group VI, VII, VIII (HFD/STZ + PYC) rats were fed HFD for 2 weeks and then injected with STZ and then supplemented with three different doses of PYC for 4 weeks; group IX (HFD/STZ + GL) rats were supplemented with GL (0.6mg/kg body weight) for 4 weeks after diabetes induction. At the end of experiment, 8 hrs fasting rats were anesthetized by ether inhalation and blood was collected from the dorsal aorta. Blood was centrifuged at 3000 rpm for 15 min and serum was separated for analysis of fasting blood glucose.

Experiment- III: To check the efficacy of the selected dose of Butea monosperma (BM) against type 1 diabetic rats.

This experiment was carried out to evaluate the therapeutic effect of BM extract (300 mg/kg body weight; orally; in normal saline) against STZ-induced type 1 diabetes on morphological and biochemical changes in the liver and pancreas of rats. The BM dose used in this experiment was determined from a pilot study (from experiment I) using different BM doses (200, 300 and 400 mg/kg body weight), as well as, from a published literature showing that this dose provided the maximal protective effects [Bavarva and Narasimhacharya, 2008; Sharma and Garg, 2009]. The rats were divided into four groups of 8 animals each: group I (control (C) group) control rats were fed standard diet throughout the experiment; group II (C + BM) rats were fed standard diet throughout the experiment and given BM (300 mg/kg body weight; orally; in saline) for 4 weeks; group III (STZ group) rats given a single intraperitoneal injection of STZ at a dose of 60 mg/kg body weight (fresh solution in 0.1 N citrate buffer, pH 7.5). The rats had free access to 5% of glucose water and basal diet ad libitum during the next 24 hours. Group IV (STZ + BM) rats were supplemented with BM (300 mg/kg body weight; orally; in saline) for 4 weeks after diabetes induction; group V (STZ + GL) rats were supplemented with GL (0.6mg/kg body weight) for 4 weeks after diabetes induction. In the last week of experiment, the OGTT was performed to assess the glucose tolerance. At the end of experiment, rats were anesthetized by ether inhalation and blood was collected from the dorsal aorta. Approximately 0.2 ml of whole blood was taken in EDTA containing microtubes from the total blood of each animal, and used for subsequent analysis of HbA1c. Remaining blood was centrifuged at 3000 rpm for 15 min and serum was separated for biochemical analysis of blood glucose, amylase activity, insulin, Ser, BUN, ALP, TG, TC, HDL-C, NO, TNF-α, IL-1β. Animal were sacrificed after 4 weeks for the evaluation of biochemical assays (TBARS, MDA, GSH, PC, CAT and GST) with
Histopathological changes were studied in the liver and pancreas. Hepatic glycogen was assayed to evaluate the effect on glucose metabolism.

Experiment- IV: To check the efficacy of the selected dose of *Terminalia arjuna* (TA) against type 1 diabetic rats.

This experiment was carried out to evaluate the therapeutic effect of TA extract (500 mg/kg body weight; orally, in normal saline) against STZ-induced type 1 diabetes on morphological and biochemical changes in the liver and pancreas of rats. The TA dose used in this experiment was determined from a pilot study (from experiment I) using different TA doses (400, 500 and 600 mg/kg body weight), as well as, from a published literature showing that this dose provided the maximal protective effects [Raghavan and Kumari, 2006]. Thirty two rats were divided separated into four groups of eight animals each: group I (control (C) group) rats were fed standard diet throughout the experiment; group II (C + TA) rats were fed standard diet throughout the experiment and given TA (500 mg/kg body weight; orally) for 4 weeks; group III (STZ group) rats given a single intraperitoneal injection of STZ at a dose of 60 mg/kg body weight (fresh solution in 0.1 N citrate buffer, pH 7.5). The rats had free access to 5% of glucose water and basal diet ad libitum during the next 24 hours. Group IV (STZ + TA) rats were supplemented with TA (500 mg/kg body weight; orally; in saline) for 4 weeks after diabetes induction; group V (STZ + GL) rats were supplemented with GL (0.6 mg/kg body weight) for 4 weeks after diabetes induction.

In the last week of experiment, OGTT was performed to assess the glucose tolerance. At the end of experiment, rats were anesthetized by ether inhalation and blood was collected from the dorsal aorta. Approximately 0.2 ml of whole blood was taken in EDTA containing microtubes from the total blood of each animal, and used for subsequent analysis of HbA1c. Remaining blood was centrifuged at 3000 rpm for 15 min and serum was separated for biochemical analysis of blood glucose, amylase activity, insulin, renal function markers (Scr, BUN, ALP), lipid profile (TG, TC, HDL-C) and NO, TNF-α and IL-1β. Animal were sacrificed and analysis of TBARS, MDA, GSH, PC and the activities of CAT and GST with histopathological changes were studied in the liver and pancreas. Hepatic glycogen was assayed to evaluate the effect on glucose metabolism.

Experiment- V: To check the efficacy of the selected dose of Pycnogenol (PYC) against type 1 diabetic rats.

Experiments were carried out to evaluate the effect of four weeks of PYC treatment on the markers of diabetic diagnosis, inflammation and renal injury in serum: oral glucose tolerance test (OGTT), fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), amylase activity, insulin, blood urea nitrogen (BUN), serum creatinine (Scr), alkalize phosphatase (ALP), total
triglycerides (TG), total cholesterol (TC), HDL- cholesterol (HDL-C), nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and oxidative damage: thiobarbituric reactive substances (TBARS), malonaldehyde (MDA), protein carbonyl (PC), reduced glutathione (GSH), and antioxidant enzymes activity [catalase (CAT) and glutathione-s-transferase (GST)] and histopathological changes in the liver and pancreas of diabetic rats. Furthermore immunohistochemistry of pancreas tissue was done to evaluate the possible mechanism of action of PYC. PYC (10 mg/kg body weight) in saline was administered intraperitoneally. The PYC dose used in this experiment was determined from a pilot study (from experiment I) using different PYC doses (5, 10 and 20 mg/kg body weight), as well as, from a published literature showing that this dose provided the maximal protective effects [Maritim et al., 2003; Ahn et al., 2003; Parveen et al., 2009]. Forty rats were divided separated into five groups of eight animals each: group I (control (C) group) control rats were fed standard diet throughout the experiment; group II (C + PYC) rats were fed standard diet throughout the experiment and given PYC (10 mg/kg body weight; intraperitoneally; in saline) for 4 weeks; group III (STZ group) rats given a single intraperitoneal injection of STZ at a dose of 60 mg/kg body weight (fresh solution in 0.1 N citrate buffer; pH 7.5). The rats had free access to 5% of glucose water and basal diet ad libitutri during the next 24 hours. Group IV (STZ + PYC) rats were diabetic supplemented with PYC (10 mg/kg body weight; intraperitoneally; in saline) for 4 weeks; group V (STZ + GL) rats were supplemented with GL (0.6mg/kg body weight) for 4 weeks after diabetes induction.

In the last week of experiment, the OGTT was performed to assess the glucose tolerance. At the end of experiment, rats were anesthetized by ether inhalation and blood was collected from the dorsal aorta. Approximately 0.2 ml of whole blood was taken in EDTA containing microtubes from the total blood of each animal, and used for subsequent analysis of HbA1c. Remaining blood was centrifuged at 3000 rpm for 15 min and serum was separated for biochemical analysis of blood glucose, amylase activity, insulin, Scr, BUN, ALP, TG, TC, HDL-C, NO, tumor necrosis factor-α and interleukin-1β. Animal were sacrificed and liver and pancreas were trimmed, washed with cold saline, wiped with filter paper and used for biochemical (lipid peroxidation, reduced glutathione, and antioxidant enzymes) and histopathological and immunohistochemical analysis. Hepatic glycogen was assayed to evaluate the effect on glucose metabolism.

Experiment- VI: To check the efficacy of the selected dose of Butea monosperma against type 2 diabetic rats.

This experiment was carried out to evaluate the therapeutic effect of BM extract (300 mg/kg body weight; orally; in normal saline) against HFD/STZ-induced type 2 diabetes on morphological and biochemical changes in the liver and pancreas of rats. The BM dose used in this experiment was determined from a pilot study (from experiment II) using different BM
Section-III  Materials & Methods

doses (200, 300 and 400 mg/kg body weight), as well as, from a published literature showing that this dose provided the maximal protective effects [Bavarva and Narasimhacharya, 2008; Sharmma and Garg, 2009]. The rats were divided into four groups of 8 animals each: Thirty two rats were divided separated into four groups of eight animals each: group I (control (C) group) rats were fed standard diet (12% calories as fat) throughout the experiment; group II (C + BM) rats were fed standard diet throughout the experiment and given BM (300 mg/kg body weight; orally; in saline) for 4 weeks; group III (HFD/STZ group) rats were fed HFD (40% fat, 18% protein and 41% carbohydrate, as a percentage of total kcal) for 2 weeks and then injected with STZ (40 mg/kg body weight; intraperitoneally; in citrate buffer; pH 4.5); group IV (HFD/STZ + BM) rats were fed HFD for 2 weeks and then injected with STZ and then supplemented with BM (300 mg/kg body weight; orally; in saline ) for 4 weeks; group V (HFD/STZ + GL) rats were supplemented with GL (0.6mg/kg body weight) for 4 weeks after diabetes induction. The development of hyperglycemia in rats was confirmed by fasting blood glucose estimation after 6 days of STZ injection. In the last week of experiment, OGTT was performed to assess the changes in glucose tolerance. At the end of experiment, rats were anesthetized by ether inhalation and blood was collected from the dorsal aorta. Approximately 0.2 ml of whole blood was taken in EDTA containing microtubes from the total blood of each animal, and used for analysis of HbA1c. Remaining blood was centrifuged at 3000 rpm for 15 min and serum was separated for biochemical analysis of blood glucose, insulin, Scr, BUN, ALP, TG, TC, HDL-C, NO, TNF-α and IL-1β. Animal were sacrificed after 4 weeks for the estimating the contents of TBARS, MDA, GSH, PC and the activities of antioxidatants enzymes and further histopathological changes were studied in the liver and pancreas. Hepatic glycogen was assayed to evaluate the effect on glucose metabolism.

Experiment- VII: To check the efficacy of the selected dose of Terminalia arjuna against type 2 diabetic rats.

This experiment was carried out to evaluate the therapeutic effect of TA extract (500 mg/kg body weight; orally; in normal saline) against HFD/STZ-induced type 2 diabetes on morphological and biochemical changes in the liver and pancreas of rats. The TA dose used in this experiment was determined from a pilot study (from experiment II) using different TA doses (400, 500 and 600 mg/kg body weight), as well as, from a published literature showing that this dose provided the maximal protective effects [Raghavan and Kumari, 2006].Thirty two rats were divided separated into four groups of eight animals each: group I (control (C) group) rats were fed standard diet (12% calories as fat) throughout the experiment; group II (C + TA) rats were fed standard diet throughout the experiment and given TA (500 mg/kg body weight; orally) for 4 weeks; group III (HFD/STZ group) rats were fed HFD (40% fat, 18% protein and 41% carbohydrate, as a percentage of total kcal) for 2 weeks and then
injected with STZ (40 mg/kg body weight; intraperitoneally; in citrate buffer; pH 4.5); group IV (HFD/STZ + TA) rats were fed HFD for 2 weeks and then injected with STZ and then supplemented with TA for 4 weeks; group V (HFD/STZ + GL) rats were supplemented with GL (0.6 mg/kg body weight) for 4 weeks after diabetes induction. The development of hyperglycemia in rats was confirmed by fasting blood glucose estimation after 6 days of STZ injection.

In the last week of experiment, OGTT was performed to assess the changes in glucose tolerance. At the end of experiment, rats were anesthetized by ether inhalation and blood was collected from the dorsal aorta. Approximately 0.2 ml of whole blood was taken in EDTA containing microtubes from the total blood of each animal, and used for analysis of HbA1c. Remaining blood was centrifuged at 3000 rpm for 15 min and serum was separated for biochemical analysis of blood glucose, insulin, Scr, BUN, ALP, TG, TC, HDL-C, NO, TNF-α and IL-1β. The rats were scarified for biochemical estimation and histopathological analysis four weeks after the diabetes induction. Biochemical markers: hepatic glycogen, TBARS, MDA, PC and GSH and activity of CAT and GST were assessed with the morphological changes in the liver and pancreas.

Experiment- VIII: To check the efficacy of the selected dose of Pycnogenol against type 2 diabetic rats.

The experiment was carried out to evaluate the effect of PYC (10 mg/kg body weight) treatment for 30 days on HFD/STZ-induced biochemical and morphological alterations in diabetic rats. To evaluate the possible mechanism of action of PYC western blotting and tunnel staining techniques were used. The PYC dose used in the experiment was determined from a pilot study (from experiment II) using different PYC doses (5, 10 and 20 mg/kg body weight), as well as, from published literature showing that this dose provided the maximal protective effects [Maritim et al., 2003; Ahn et al., 2007; Parveen et al., 2009]. Thirty two rats were divided into four groups of eight animals each: group I (control (C) group) rats were fed standard diet (12% calories as fat) throughout the experiment; group II (C + PYC) rats were fed standard diet throughout the experiment and given PYC (10 mg/kg body weight; intraperitoneally; in saline) for 4 weeks; group III (HFD/STZ group) rats were fed HFD (40% fat, 18% protein and 41% carbohydrate, as a percentage of total kcal) for 2 weeks and then injected with STZ (40 mg/kg body weight; intraperitoneally; in citrate buffer; pH 4.5); group IV (HFD/STZ + PYC) rats were fed HFD for 2 weeks and then injected with STZ and then supplemented with PYC (10 mg/kg body weight; intraperitoneally; in saline) for 4 weeks; group V (HFD/STZ + GL) rats were supplemented with GL (0.6 mg/kg body weight) for 4 weeks.
after diabetes induction. The development of hyperglycemia in rats was confirmed by fasting blood glucose estimation after six days of STZ injection.

In the last week of experiment, OGTT was performed to assess the changes in glucose tolerance. At the end of experiment, rats were anesthetized by ether inhalation and blood was collected from the dorsal aorta. Approximately 0.2 ml of whole blood was taken in EDTA containing microtubes from the total blood of each animal, and used for analysis of HbA1c. Remaining blood was centrifuged at 3000 rpm for 15 min and serum was separated for biochemical analysis of blood glucose, insulin, Scr, BUN, ALP, TG, TC, HDL-C, NO, TNF-α and II-1β. Animal were sacrificed after 4 weeks for the estimating the contents of TBARS, MDA, GSH, PC and the activities of CAT and GST with histopathological changes were studied in the liver and pancreas. Hepatic glycogen was assayed to evaluate the effect on glucose metabolism. Protective role of PYC on phosphorylated Akt (pAkt) expression level and apoptotic β-cell was observed using western and TUNNEL techniques, respectively.

**Blood sampling**

Body weight was monitored at every week between 9.00 and 10.00 am for 4 weeks. At the end of experiment, rats were anesthetized by ether inhalation and blood was collected from the dorsal aorta. Approximately 0.2 ml of whole blood was taken in EDTA containing microtubes from the total blood of each animal, and used for analysis of HbA1c. Remaining blood was centrifuged at 3000 rpm for 15 min and serum was separated for biochemical analysis.

**Biochemical estimations in the serum**

(i) **Oral Glucose Tolerance Test**

In the last week of each experiment, the Oral Glucose Tolerance Test (OGTT) was performed to assess the glucose tolerance. For this purpose, overnight fasted rats were fed orally 2 gm/kg body weight glucose. Blood was collected at 0, 30, 60, and 120 min intervals from orbital sinus for glucose estimation. Animals were not anesthetized for this procedure.

(ii) **Measurement of Fasting blood glucose**

Fasting blood glucose was measured by glucose-oxidase-peroxidase (GOD/POD) method [Braham and Tinder, 1972] using a commercial diagnostic kit from Span diagnostic Limited, Surat, India.

**Principle:**

Glucose is oxidised to gluconic acid and hydrogen peroxide in the presence of glucose oxidase. Hydrogen peroxide further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. The red color so developed is measured at 505 nm and is directly proportional to glucose concentration.
Section-III  Materials & Methods

Glucose oxidase

\[
\text{Glucose} + \text{O}_2 + \text{H}_2 \rightarrow \text{Gluconate} + \text{H}_2\text{O}_2
\]

Peroxidase

\[
\text{H}_2\text{O}_2 + 4 \text{Aminoantipyrine} + \text{Phenol} \rightarrow \text{Red Quinoneimine dye} + \text{H}_2\text{O}
\]

The preparation and procedure were followed as per guidance and direction given by kit.

Calculation:

Total Glucose in mg/dl = \( \frac{\text{O.D.of Test}}{\text{O.D.of Standard}} \times 100^* \)

* Dilution factor

(iii) Determination of serum insulin content

Serum insulin content was determined by ELISA kit using rat insulin as standard (Ultra sensitive Rat Insulin ELISA kit, Crystal Chem INC. USA). Preparation and procedure were followed as per guidance and directions provided by kit. Calculations were done by using standard curve. Insulin concentrations in the samples were interpolated using the standard curve and mean absorbance values for each sample.

(iv) Determination of HbA1c level

HbA1c was assayed by cation-exchange method [Nathan et al., 1984] using a diagnostic kit from Crest Biosystem, Goa, India.

Principle:

A hemolysed preparation of whole blood is mixed continuously for 5 minutes with a weakly binding cation-exchange resin. The labile fraction is eliminated during the hemolysate preparation and during the binding. During this mixing, non-glycosylated hemoglobin binds to the ion exchange resin leaving GHB free in the supernatant. After the mixing period, a filter separator is used to remove the resin from the supernatant. The percent glycosylated hemoglobin is determined by measuring absorbances of the glycosylated hemoglobin (GHB) & the total hemoglobin (THb) fraction at 415 nm. The ratio of the absorbances of the glycosylated hemoglobin & the total hemoglobin fraction of the Control and the Test is used to calculate the percent glycosylated hemoglobin of the sample.

Procedure was followed as per the instructions given by the manufacturer’s kit.
Calculations:

\[
\text{Ratio of Control (RC)} = \frac{\text{Abs. Control GHB}}{\text{Abs. Control THb}}
\]

\[
\text{Ratio of Test (RT)} = \frac{\text{Abs. Test GHB}}{\text{Abs. Test THb}}
\]

\[
\text{GHB in \%} = \frac{\text{Ratio of Test (RT)}}{\text{Ratio of Control (RC)}} \times 10 \text{ (Value of Control)}
\]

(v) Assay for amylase activity

Amylase activity was measured by street and close method [Street and Close, 1956] using a commercial diagnostic kit from Span diagnostic Limited, Surat, India.

Principle:

The enzyme amylase degrades starch into reducing dextrins and smaller saccharides. The amylase is allowed to react under defined conditions. Iodine solution is added and the resulting blue color of the test is compared with a control. The relative decrease in blue color of test is measured at 620 nm as amylase activity.

Calculations:

Amylase activity in serum:

\[
\text{Street-close units/100 ml} = \frac{\text{O.D. of Control - O.D. of Test}}{\text{O.D. of Control}} \times 100
\]

(vi) Assay for lipid profile

Lipid Profile (total-cholesterol, triglycerides (TG), and HDL cholesterol (HDL-C)) were estimated by using enzymatic kits procured from SPAN Diagnostics India, Ltd. (Surat, India).

Total cholesterol and HDL-cholesterol estimation

Total cholesterol and HDL-cholesterol were estimated by the one step method of Wybenga et al. [1970].

Principle:

Cholesterol reacts with hot solution of ferric per chlorate, ethyl acetate & sulphuric acid (Cholesterol Reagent) and gives a lavender colored complex which is measured at 560 nm. High density lipoproteins (HDL) was obtained in the supernatant after centrifugation. The Cholesterol in the HDL fraction is also estimated by this method.
**Calculations:**

1) Total Cholesterol

\[
\text{Serum Cholesterol (mg/dl) = } \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times 200
\]

2) HDL Cholesterol

\[
\text{Serum HDL Cholesterol (mg/dl) = } \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times 50
\]

**Serum Triglyceride Estimation**

Serum Triglyceride was estimated by GPO-PAP, end point assay [Stein and Mayer, 1995] using a commercial diagnostic kit.

**Principle:**

Lipoprotein lipase hydrolyses triglycerides to glycerol and free fatty acids. The glycerol reacts with ATP in the presence of glycerol kinase to form glycerol-3-phosphate, which is oxidised by the enzyme glycerol-3-phosphate oxidase (GPO) to form hydrogen peroxide. The hydrogen peroxide further reacts with phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. Intensity of the color formed is measured at 505 nm and is directly proportional to the amount of triglycerides present in the sample.

\[
\begin{align*}
\text{Lipoprotein Lipase} & \quad \text{Glycerol + Free Fatty Acids} \\
\text{Glycerol + ATP} & \quad \rightarrow \text{Glycerol 3 Phosphate + ADP} \\
\text{GPO} & \quad \rightarrow \text{Dihydroxyacetone phos. + H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{Phenol} & \rightarrow \text{Red Quinoneimine dye + 4H}_2\text{O}
\end{align*}
\]

Procedure for triglyceride estimation was followed as per the direction given by kit.

**Calculations:**

\[
\text{Triglycerides in mg/dl = } \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times 200
\]
Calculations for LDL cholesterol and VLDL cholesterol [Friedewald et al., 1972]

LDL cholesterol (LDL-C) and VLDL cholesterol (VLDL-C) were calculated by using Friedewald’s equation.

\[
\text{LDL cholesterol} = \text{Total Cholesterol} - \left(\frac{\text{Triglyceride}}{5} - \text{HDL Cholesterol}\right)
\]

\[
\text{VLDL cholesterol} = \frac{\text{Triglycerides}}{5}
\]

(vii) Free fatty acids (FFAs) determination

The method of Falhot et al. [1973] 15 was used for FFAs determination. 50μl serum was added with 1.0 ml phosphate buffer and 6.0 ml chloroform-heptane-methanol (CHM) solution in a test tube. This was shaken vigorously for 50 set, left to stand for 15 min and then centrifuged (4000 x g, 10 min). The buffer was removed carefully by suction and 5.0 ml of the CHM phase was shaken with 2.0 ml Cu-TEA in 5 min on a Vortex mixer. After centrifugation (4000 x g, 5 min) 3.0 ml of the upper phase was transferred to a test tube containing 0.5 ml DPC solution and mixed carefully. Absorbance was taken after 15 min at 550 nm.

(viii) Renal function markers in serum

Kidney damage during diabetes was evaluated by the following markers in serum: serum creatinine (Scr) concentration, blood urea nitrogen (BUN) level and alkaline phosphatase (ALP) activity. Scr and BUN were estimated by using enzymatic kits procured from SPAN Diagnostics India, Ltd. (Surat, India).

Blood urea nitrogen estimation

Blood urea was estimated by the Diacetylmonoxime (DAM) method [Wybenga et al., 1971] using a commercial diagnostic kit.

Principle:

Urea reacts with hot acidic diacetylmonoxime in presence of thiourea carbonic acid and produces a rose-purple colored complex, which is measured at 525 nm spectrophotometrically. The preparation and procedure were followed as per guidance and direction given by kit.

**Calculations:**

\[
\text{Serum Urea in mg/100 ml, (A)} = \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times 30
\]

\[
\text{Blood Urea Nitrogen in mg/100ml} = (A) \times 0.467
\]

Serum creatinine estimation:

Serum creatinine was estimated by the alkaline picrate method [Bonses and Taussky, 1945].
Principle:
Creatinine in a protein free solution reacts with alkaline picrate and produces a red colored complex, which is measured at 520 nm spectrophotometrically. The preparation and procedure were followed as per guidance and direction given by kit.

Calculations:
Serum creatinine in mg/100 ml = \[ \frac{\text{O.D. of Test} - \text{O.D. of Blank}}{\text{O.D. of Std.} - \text{O.D. of Blank}} \times 3 \]

Estimation of Alkaline phosphatase activity
The activity of ALP was determined using p-nitrophenyl phosphate (PNPP) as a substrate [Bessey et al., 1946]. The assay contained equal volumes of 0.1M glycine and 0.4% PNPP. The mixture was incubated for 30 min at 25 °C after adding 0.1ml serum. The absorbance was read at 410 nm. ALP activity was expressed as equiunits/dl in serum.

(ix) Assessment of serum TNF-α and IL-β and nitric oxide (NO) levels
Serum TNF-α and IL-β levels were measured by commercially available rat enzyme-linked immunosorbent assay (ELISA) kits from Hysel India Pvt. Ltd, India. Procedure and calculation were followed as per the instructions given by the manufacturer's kit. The NO level is most often measured by the determined stable end products of NO, nitrite and nitrate using the Griess reagent (sulfanilamide and n-naphyl-ethylenediamine) [Cortas and Wakis, 1990]. Serum was clarified by zinc sulphate solution and NO was then reduced to NO by cadmium overnight at 20°C under shaking. Samples were added to the Griess reagent and incubated for 20 min at room temperature. Absorbance was measured at 540nm. Standard curves were prepared with known concentrations (1 to 100 μmol/l) of sodium nitrite.

Tissue preparation for oxidative markers
After four weeks treatment rats were sacrificed, and their liver and pancreas tissues were excised immediately and perfused with ice-cold saline. For hepatic glycogen assay, immediately after excision from the animal, approximately 1 g of liver tissue is dropped in 3ml of 30% KOH. For biochemical estimations, liver and pancreas tissues were homogenized at 4 °C with 10 times [w/v] 0.1 M phosphate-buffer [PB, pH 7.4] containing protease inhibitors in a polytron homogenizer (Kinematica A.G.). The homogenate was centrifuged at 800 x g for 5 min at 4 °C to separate the nuclear debris and was used for estimation of thiobarbituric reactive substances (TBARS) and malonaldehyde (MDA). The supernatant was further centrifuged at 10,000 x g for 20 min at 4 °C to get the post-mitochondrial supernatant (PMS), which was used for GSH, PC, CAT and GST assays.

Estimation of liver glycogen content
Liver glycogen content was determined using the anthrone reagent method [Seifter et al., 1950]. Approximately 1 g of liver tissue was dropped in 3 ml of 30% KOH. This tube was
placed in a boiling water bath for 30 min, following cooling, this digest was transferred to a 50 ml volumetric flask and diluted with water. Slowly, 5 ml of solution from the previous step was added to a test tube containing 10 ml of anthrone reagent, which was placed in cold water to prevent excessive heating. After thorough mixing, the tube was placed in a boiling water bath for exactly 10 min for the development of color and cooled with running tap water. The optical density was read within 2 h in a spectrophotometer at 620 nm against a blank that was prepared by subjecting 5 ml of distilled water instead of sample to the same procedure.

Oxidative stress markers in liver and pancreas tissues

(i) TBARS content
The method of Utley et al. [1967] with some modification was used to estimate the rate of LPO. Homogenate (0.25 ml) was pipetted into 15×100 mm test tubes and incubated at 37 °C in a metabolic shaker for 1 h. An equal volume of homogenate was pipetted into a centrifuge tube, placed at 0 °C and marked at 0 h incubation. After 1 h of incubation, 0.5 ml of 5% (w/v) chilled trichloroacetic acid (TCA), followed by 0.5 ml of 0.67% TBA (w/v) was added to each test tube and centrifuge tube, and centrifuged at 1000×g for 15 min. Thereafter, the supernatant was transferred to other test tubes and was placed in a boiling water bath for 10 min. The absorbance of pink color produced was measured at 535 nm in a spectrophotometer (Shimadzu-1601, Japan). The TBARS content was calculated by using a molar extinction coefficient of 1.56 × 10^{5} M^{-1} cm^{-1} and expressed as nmol of TBARS formed/min/mg protein.

(ii) Assay for MDA
MDA which is a measure of the end product of lipid peroxidation was measured Ohkawa et al. [1979]. Briefly, the reagents acetic acid 1.5 ml [20%] pH 3.5, 1.5 ml TBA [0.8%], and 0.2 ml sodium dodecyl sulfate [8.1%] were added to 0.1 ml of processed tissue sample. The mixture was then heated at 100 °C for 1 h. The mixture was cooled with tap water and 5 ml of n -butanol: pyridine [15:1% v/v] 1 ml of distilled water was added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the organic layer was withdrawn, and absorbance was measured at 532 nm using a spectrophotometer (Shimadzu-1601, Japan). The amounts of MDA formed in each of the samples were expressed as the nmol MDA formed/mg protein by using a molar extinction coefficient of 1.56 × 10^{5} M^{-1} cm^{-1}.

(iii) Assay for protein carbonyl (PC)
PC level was measured by the method of Levine et al. [1990]. The PMS (0.5 ml) was treated with an equal volume of 20% TCA for protein precipitation. After centrifugation, the pellet was resuspended in 0.5 ml of 10 mM DNPH in 2 M HCl and vortexed repeatedly at 10 min intervals for 1 h in dark. This mixture was treated with 0.5 ml of 20% TCA. After
centrifugation at 10,000 × g at 4 °C for 3 min, the precipitate was extracted three times with 0.5 ml of 10% TCA and dissolved in 2.0 ml of NaOH at 37 °C. Absorbance was recorded at 360 nm in a spectrophotometer (Shimadzu-1601, Japan). PC level was expressed as nmol carbonyl/mg protein, using a molar extinction coefficient of 22 × 10^4 M⁻¹ cm⁻¹.

(iv) Assay for reduced glutathione (GSH)
Reduced GSH content was determined by the method of Jollow et al. [1974] with slight modification. PMS was mixed with 4.0% sulfosalicylic acid (w/v) in a 1:1 ratio (v/v). The samples were incubated at 4 °C for 1 h, and later centrifuged at 1200 × g for 15 min at 4 °C. The assay mixture contained 0.1 ml of supernatant, 1.0 mM DTNB, and 0.1M PB (pH 7.4) in a total volume of 1.0 ml. The yellow color that developed was read immediately at 412 nm in a spectrophotometer (Shimadzu-1601, Japan). The GSH content was calculated as nmol DTNB/mg of protein, using a molar extinction coefficient of 13.6 × 10^3 M⁻¹ cm⁻¹.

(v) Assay for glutathione-s-transferase (GST)
The activity of GST was measured by the method of Habig et al. [1974]. The reaction mixture consisted of 1.0 mM GSH, 1.0 mM CDNB, 0.1 M phosphate buffer (pH 7.4) and 0.1 ml of PMS in a total volume of 3.0 ml. The change in absorbance was recorded at 340 nm by using Shimadzu spectrophotometer UV-1601 and enzyme activity was calculated as nmol of CDNB conjugate formed/min/mg protein using molar extinction coefficient of 9.6 × 10^3 M⁻¹ cm⁻¹.

(vi) Assay for catalase (CAT)
Catalase activity was assayed by the method of Claiborne [1985]. Briefly, the assay mixture consisted of 0.05 M phosphate buffer (pH 7.00), 0.019 M hydrogen peroxide (H₂O₂), and 0.05 ml PMS in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was expressed as nmol H₂O₂ consumed/min/mg protein.

(s) Protein content
Protein content was determined by the method of Lowry et al. [1951], using bovine serum albumin (BSA) as a standard.

(i) Histopathological Examination
For histological examinations, liver and pancreas tissues from different groups were stained with hematoxylin and eosin (H and E). Haematoxylin stains the nuclei blue and eosin stains the cytoplasm in shades of blue. This method is used for identification of tumors, recognition of inflammatory cells and certain inclusion bodies. Briefly, at end of experiment, the rats were anesthetized with ether and perfused transcardially with saline followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB; 0.1 M, pH 7.2). Liver and Pancreas were
removed quickly and postfixed in buffered formalin (10%) for 48 h. After fixation was completed, slices (3-4 mm) of these tissues were dehydrated and embedded in paraffin. At least four cross-sections were taken from each tissue in 5-μm thickness and stained with H and E. Following two changes xylene washes of 2 min each tissue sections were mounted with DPX mountant. The slides were observed for histopathological changes and microphotographs were taken using an Olympus BX50 microscope system (Olympus, Japan).

(ii) Immunohistochemistry

For Immunohistochemical study, the rats were anesthetized with ether and perfused with saline followed by buffered formalin (10%). Pancreas was removed quickly and postfixed in buffered formalin (10%) for 48 h. After fixation was completed, slices (3-4 mm) of these tissues were dehydrated and embedded in paraffin. At least four cross-sections were taken from each tissue in 5-μm thickness and used for immunostaining procedure. The sections were preincubated for 30 min with 10% bovine serum albumin (BSA) in a PBS solution containing 0.25% TritonX-100 (PBS/TX100). Sections were then incubated with rabbit anti-insulin antibody (1:50, SC-9168, Santa Cruz Biotechnology, Inc., CA, U.S.A) containing 3% BSA in TBS/TX100 for 4 h at room temperature. Sections were rinsed in PBS (3 times) before being incubated 1 h at room temperature in 0.1M TBS containing 1:500 goat anti-rabbit Alexa fluor IgG 488 (Invitrogen, Carlsbad, CA). Sections were then rinsed and mounted with ant-fading compound on glass slides and cover slipped. The slides were then used for microscopic analysis.

Western blotting

Tissues were homogenized in T-per (Pierce, Rockford, IL) containing protease inhibitor cocktail (P8340, Sigma). Homogenates were centrifuged for 20 min at 10,000g. A bicinchoninic acid protein assay (Pierce, 23225) was performed for protein equalization. Using BSA as standard the protein content of each cytosolic fraction was evaluated. 40μg of total protein was separated at 200V for 1 h on 8-14% SDS gel and transferred onto PVDF membrane at 100 V for 30 min. After blocking with 5% milk, membranes were probed with primary antibodies followed by incubation with peroxidase-conjugated secondary antibodies (1:5,000; KPL, Gaithersburg, MD). The peroxidase reaction was developed with an ECL-plus detection kit (Amersham BioSciences, Piscataway, NJ). The following primary antibodies were used: anti-Akt, pAkt, (1:1000, Cell Signaling Denver, MA). All blots were stripped and re-incubated with β-actin antibodies (1:10,000, Sigma Aldrich) as a loading control. Intensity of the bands was measured by densitometry and quantified using Quantity One software (BioRad Laboratories, Hercules, CA).
**Tunnel Staining**

For TUNEL staining, rats were overdosed and anesthetized with Nembutal at 24h post-pMCAO and then perfused transcardially with cold PBS and 4% paraformaldehyde in PBS. Pancreas were removed and postfixed in the same fixative overnight at 4°C and then with 30% sucrose in PBS for 72h. After cryopreservation, pancreas was cut into two slices at 2 mm posterior from bregma (bregma -2 mm). Each slice was cut on a cryostat to obtain five sections (12 μm). TUNEL-staining was performed using an *In Situ* Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. Briefly, sections were permeabilized with 0.01% Triton X-100 (Sigma) in 0.1% sodium citrate (2 min on ice), and then incubated (60 min, 37°C) with the TUNEL reaction mixture in a humidified atmosphere. Every fifth section of each slice was collected. We then counted TUNEL-positive cells in using a light microscope with 200 x magnification.

**Statistical analysis**

Results are expressed as mean ±SE (n = 5 or 8). Statistical analysis of the data was done by applying the analysis of variance (ANOVA), followed by Tukey’s test for biochemical parameters. The P-value < 0.05 was considered statistically significant.