Chapter 3

Evaluation of hypoglycemic, hypolipidemic and antioxidant properties of Pithecellobium dulce pod pulp extract
INTRODUCTION

According to recent estimates, the human population worldwide appears to be in the midst of an epidemic of diabetes. Despite the great strides that have been made in the understanding and management of diabetes, the disease and disease-related complications are increasing unabated. Parallel to this, recent advancements in understanding the pathobiology of the disease process have opened up several new avenues to identify and develop novel therapies to combat the diabetic plague.

Even after sophisticated discoveries in diagnosis and treatment, the scientists of the present era are unable to control the incidence of non-communicable diseases such as diabetes, cancer and coronary heart diseases which affects a high percentage of population globally. The unprecedented economic development and rapid urbanization in Asian countries, particularly in India has led to a shift in health problems from communicable to non-communicable diseases. Many communicable diseases have been remitted, some of them have been decreased in prevalence and some have been eliminated. In the meantime, the rate of illness from chronic diseases has been growing, especially in developing and under-developing countries.

Diabetes Mellitus (DM) is one of the biggest health catastrophes which has been persistently affecting the humanity irrespective of socioeconomic profile and geographic location of the population. DM is a
group of metabolic diseases characterized by persistent hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels (American Diabetes Association, 2012).

**Terminology**

The term diabetes derived from the Greek word “diabainein” that literally means "passing through," or "siphon", a reference to one of the diabetes' major symptom (excessive-urine excretion). In 1675, Thomas Willis added the word-“mellitus” to the disease, a word from Latin meaning "honey", a reference to the sweet taste of the urine. This sweet taste had been noticed in urine by the ancient Greeks, Chinese, Egyptians, and Indians. In 1776, Matthew Dobson confirmed that the sweet taste was because of an excess of a kind of sugar in the urine and blood of individuals with diabetes (Dobson, 1776). The ancient Indians tested for diabetes by observing whether ants were attracted to an individual’s urine, and called the ailment “sweet urine disease” (Madhumeha). The Korean, Chinese, and Japanese words for diabetes are also based on the same ideographs which means “sugar urine disease”.

Diabetes usually refers to diabetes mellitus, but there are several rarer conditions also named as diabetes. Due to a similarity in the name, a misconception exists between two terms namely, diabetes mellitus and
diabetes insipidus. The latin word “insipidus” refers to “without taste” in Latin) in which the urine is not sweet; it can be caused by either kidney or pituitary gland damage. Diabetes mellitus and Diabetes insipidus are two entirely separate conditions with a separate pathogenesis. Both cause polyuria (hence the similarity in name) but whereas diabetes insipidus is a rare endocrine problem with the production of antidiuretic hormone (Cranial diabetes insipidus) or renal response to antidiuretic hormone (nephrogenic diabetes insipidus), diabetes mellitus causes polyuria via osmotic diuresis, due to the high blood sugar (over and above the threshold values) leaking into the urine, taking excess water along with it. The incidence of diabetes insipidus in the general population is three in 100,000 (Saborio et al., 2000).

PREVALENCE OF DIABETES

The prevalence of DM in adults was about 4 percent in the worldwide: this means that over 143 million persons are now affected. It is projected that disease prevalence will be 5.4 percent by the year 2025, with global diabetic population reaching to 300 million. Between 2010 and 2030, there will be a 69% increase in numbers of adults with diabetes in developing countries and a 20% increase in developed countries (Shaw et al., 2010). The highest regional prevalence for 2010 was for North America, followed by the EMME (Eastern Mediterranean and Middle-East and South Asia). The African region is expected to have the largest proportional increase in adult diabetes numbers by 2030, followed by the EMME, though North America
will continue to have the world’s highest prevalence. Every region will have an increase in numbers well in excess of adult population growth, and total numbers with diabetes are likely to increase by 50% over the 20 years (Shaw et al., 2010).

In the past, most diabetics were known to have a genetic tendency towards the disease. However, that trend has rapidly given way in the past few decades to other causes, at least from a statistical perspective. These genetically-independent trends that explain the growth in the incidence of diabetes can be summarized as follows:

- Sedentary lifestyle
- Population growth
- Increased life expectancy resulting in a higher ratio of aged population with diabetes,
- Increasing obesity trends,
- Unhealthy diets
- Stress
- Lack of exercise

Estimates of the current and future burden of diabetes are important in order to allocate community as well as health resources, to emphasize the role of lifestyle, and encourage measures to counteract trends for increasing
prevalence. The global pattern is dominated by countries with large populations, and these data highlight the extent to which demographic changes in India, China and Brazil are likely to affect the total numbers with diabetes in the future (Whiting et al., 2011).

CLASSIFICATION OF DIABETES MELLITUS

Assigning a type of diabetes to an individual often depends on the circumstances present at the time of diagnosis, and many diabetic individuals do not easily fit into a single class. The recent classification of diabetes mellitus according to American diabetes association is as follows (American diabetes association, 2012)

Etiologic Classification of Diabetes Mellitus

I. Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency).
   A. Immune mediated
   B. 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 which includes drug induced, diseases of pancreas, genetic defects in β cells.
IV. Gestational diabetes

**Type 1 diabetes**

*Type 1 diabetes* represents about 2-5% of all cases of diabetes, previously encompassed by the terms insulin dependent diabetes, or juvenile-onset diabetes, results from selective destruction of the insulin producing \( \beta \) cells in the pancreatic islets and is primarily an autoimmune response developed against one or more \( \beta \) cell antigens (Csorba et al., 2010). The markers of the immune destruction of the \( \beta \)-cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to Glutamic Acid Decarboxylase (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2b. Individuals at increased risk of developing this type of diabetes can often be identified by serological evidence of an autoimmune pathologic process occurring in the pancreatic islets and by genetic markers.

Insulin is essential for the cells of our body to metabolize glucose properly and function normally. Type I diabetes begins early in life and quickly becomes severe. It is caused by progressive loss of cells in the pancreas and the beta cell death is caused by macrophages and followed by invasion of lymphocytes (Yoon et al., 2000). The human immune system mistakenly targets non-foreign cells. When the immune system recognizes the presence of Glutamic acid decarboxylase (GAD), it acts by invading the
β cells of the pancreas. The presence of GAD autoantibodies has been shown to be a strong predictive marker for eventual onset of type 1 diabetes.

Glutamic acid decarboxylase, an enzyme that is found in relatively high concentrations in the pancreas, catalyzes the conversion of L-glutamic acid into γ-aminobutyrate (GABA) and carbon dioxide via an irreversible reaction. GABA is an inhibitory neurotransmitter that is found in pancreas and other parts of the body and considered to have a primary role as a signaling molecule in the pancreatic islets (Sills et al., 2000). When GAD catalyzes the conversion, T-cells recognize the presence of GAD as foreign antigens. The two types of T-cells, CD4 and CD8 destroy the β cells in the pancreas. When T-cells attack and destroy insulin producing cells, the severity of disease progresses.

Type 1 individuals need to receive insulin injections, as oral administration of insulin is ineffective for their survival. The negative characteristic of insulin treatment include refrigeration, very low half life, picking of the right dose at the right time, pain due to multiple sites of administration, fat pads, and immune response against exogenous insulin. Above all, most of the patients develop insulin resistance after receiving continuous insulin injection (Fleury-Milfort, 2008).

**Type 2 diabetes**
Type 2 diabetes, previously referred to as non-insulin-dependent diabetes or adult onset diabetes, is the most common type of diabetes and it accounts for 90-95% of the diagnosed diabetes and equal number of undiagnosed diabetes. Type 2 diabetes confers significant morbidity and mortality, most notably with target organ damage to the eyes, kidneys, nerves and heart (Khavandi et al., 2013). The causes of type 2 diabetes are multi-factorial and include both genetic and environmental elements that affect \( \beta \)-cell function and tissue (muscle, liver, adipose tissue, and pancreas) insulin sensitivity.

A majority of individuals suffering from type 2 diabetes are obese, with central visceral adiposity. Therefore, the adipose tissue should play a crucial role in the pathogenesis of type 2 diabetes. Glucose, one of the key physiological stimuli of \( \beta \) cells, increases the cytoplasmic free \( \text{Ca}^{2+} \) concentration and stimulates insulin secretion. However, chronic elevation of glucose concentration as a result of peripheral tissue resistance against insulin action predominantly causes \( \beta \) cell dysfunction (Choi and Kim, 2010).

The worldwide incidence of type 2 diabetes mellitus (T2DM) has increased dramatically in all age groups including children and adolescents (Alberti et al., 2004). The rising rates of obesity in youth have concurrently led to an increase in the prevalent rates of type 2 diabetes mellitus. Reducing the incidence of T2DM by preventing pediatric obesity through the
implementation of lifestyle changes in the community should be the primary objective of healthcare systems (Giampatzis and Tziomalos, 2012).

Management and treatment of type II diabetes is mainly aimed at glycaemic control and international guidelines recommend reducing glycated hemoglobin (HbA1c) to 6.5–7% (Nathan et al., 2009). Every percentage point decrease in HbA1c, reduces the risk of development of microvascular complications by 35% and every 10% reduction in HbA1c is associated with 21% reduction in cardiovascular disease (Garg and Ulrich, 2006). Insulin therapy remains as a cornerstone in the treatment of hyperglycemia. The effectiveness, cost, and lack of severe side effects apart from hypoglycemia, make insulin the ideal treatment for diabetes mellitus (Meetoo, et al. 2007).

**Gestational diabetes mellitus**

Gestational diabetes is formally defined as "any degree of glucose intolerance with onset or first recognition during pregnancy" (Jovanovic and Pettitt, 2001). This definition acknowledges the possibility that patients may have previously undiagnosed diabetes mellitus, or may have developed diabetes coincidentally with pregnancy. Whether symptoms subside after pregnancy is also irrelevant to the diagnosis. Gestational diabetes is a condition in which women without previously diagnosed diabetes exhibit persistently high blood glucose levels during pregnancy (especially during their third trimester). Gestational diabetes mellitus (GDM) complicates a
substantial number of pregnancies. Approximately 7% of all pregnancies (ranging from 1 to 14%, depending on the population studied and the diagnostic tests employed) are complicated by GDM, resulting in more than 200,000 cases annually.

Gestational diabetes generally resolves once the baby is born. The chances of developing GDM in a second pregnancy, if a woman had GDM in first pregnancy, are between 30 and 84%, depending on ethnic background. A second pregnancy within one year of the previous pregnancy has a high rate of recurrence (Kim et al., 2007). Women diagnosed with gestational diabetes have an increased risk of developing diabetes mellitus in the future. The risk is highest in women who needed insulin treatment, had antibodies associated with diabetes (such as antibodies against glutamate decarboxylase, islet cell antibodies and/or insulinoma antigen-2), women with more than two previous pregnancies, and women who were obese (in order of importance). Women requiring insulin to manage the hyperglycemia in gestational diabetes have a 50% risk of developing diabetes within the next five years (Lobner et al., 2006; Järvelä et al., 2006).

Higher dietary fat and less carbohydrate intakes during pregnancy appear to be associated with a higher risk for GDM. The most effective nutritional intervention for the control of gestational weight gain and glycemic responses could not be reached based on available studies. Weight
management through nutritional prevention strategies could be successful in reducing the risk of GDM.

**Symptoms of Diabetes Mellitus**

- Frequent urination (polyuria)
- Excessive eating (polyphagia)
- Excessive thirst (polydipsia)
- Fatigue constantly tired
- Unusual weight loss
- Impaired wound healing
- Frequent Infections
- Altered mental status
- Blurry vision

**Diagnostic criteria for diabetes mellitus**

Diabetes mellitus is characterized by persistent hyperglycemia, and is diagnosed by demonstrating any one of the following:

- Fasting plasma glucose level $\geq 7.0$ mmol/l (126 mg/dl)
- Plasma glucose $\geq 11.1$ mmol/l (200 mg/dL) two hours after a 75 g oral glucose load as in a glucose tolerance test
- Symptoms of hyperglycemia and casual plasma glucose $\geq 11.1$ mmol/l (200 mg/dl)
Glycosylated hemoglobin (Hb A1c) ≥ 6.5%

DIABETIC COMPLICATIONS

Glucose is the main fuel for energy requirement of the body. Therefore, a continuous supply of glucose is necessary to ensure proper function and survival of all organs. Hence, mammals have evolved sophisticated systems to maintain glucose levels in the blood within tight limits, despite large fluctuations in food intake and these goals are met chiefly through the hormonal modulations in the system. A dysfunction of β-cells results in diabetes characterized by an abnormally high blood glucose level and creates serious complications including blindness, kidney failure, stroke, heart and vascular complications.

Diabetes gives rise to the development of numerous complications due to hyperglycemia. The likelihood of developing complications, whether acute or chronic, is ultimately a reflection of the level of blood sugar control.

The complications of diabetes are being seen more frequently, with increase in type 2 diabetes mellitus patients. Type 2 diabetes is a very common disease, characterized by an asymptomatic phase between the actual onset of diabetic hyperglycemia and clinical diagnosis. This phase has been estimated to last at least 4–7 years, and 30–50% cases of type 2 diabetic patients remained undiagnosed. This leads to the development of chronic complications of diabetes, which remain the chief problems in diabetic care,
and which cause a lack of fitness to work, disability, and premature death (Piechowski-Jozwiak et al., 2005; Spijkerman et al., 2003).

With long duration of diabetic metabolism, diabetes-specific complications, chiefly involving small vessels (retinopathy, nephropathy and neuropathy), may ensue, and lead to serious outcomes such as visual disturbance, renal failure, and gangrene. Diabetes accelerates and exacerbates the occurrence of arteriosclerosis, increasing the risks for myocardial infarction, cerebral infarction and occlusive artery disease of the lower extremities. These complications constitute the major causes of morbidity and mortality in diabetic patients (Takeshi Kuzuya et al., 2002).
The literature traditionally divides the diverse spectrum of vasculopathy associated with diabetes into two main subtypes (Krentz et al., 2007):

1. **Diabetes-Specific Microvascular Complications:**
   - (i) Retinopathy
   - (ii) Nephropathy
   - (iii) Neuropathy

2. **Atherothrombotic Macrovascular Complications:**
   - (i) Myocardial infarction
   - (ii) Hypertension
   - (iii) Peripheral arterial disease

**DIABETIC RETINOPATHY**

Diabetic retinopathy is a leading cause of visual impairment all over the world (Sorsby, 1963; See et al., 1998). It was found that; hypertension is a risk factor for the progression of diabetic retinopathy, mostly because hyperglycemia in diabetic patients impairs the regulation of retinal perfusion, leading to increased susceptibility to injury by systemic hypertension. Blindness caused by diabetes is largely preventable by good glycemic control and timely laser treatment, early detection of retinopathy is an important preventive strategy. In developing countries, lack of health care facilities for
diabetes management is still a serious public health problem. Therefore, delayed diagnosis of diabetes and its complications, such as retinopathy, could be more common than in developed countries (Brooks et al., 1999).

Aldose reductase is the initial enzyme in the intracellular polyol pathway. This pathway involves the conversion of glucose into sorbitol. Elevated blood glucose levels increase the flux of sugar molecules through the polyol pathway, which causes sorbitol accumulation in cells. Osmotic stress from sorbitol accumulation has been postulated as an underlying mechanism in the development of diabetic microvascular complications, including diabetic retinopathy (Fong et al., 2004).

**DIABETIC NEPHROPATHY**

It has been widely accepted that long-term exposure to high blood glucose plays an important role in the development of diabetic nephropathy (Brownlee, 2005). In diabetic complications, increased glucose levels are associated with high sorbitol accumulation in the kidney, nerve, retina, and lens followed by a depletion of Myo inositol (Greene et al., 1987; Raccah et al., 1998; Del Monte et al., 1991) High glucose, together with increased flux through the polyol pathway causes myo-inositol (MI) depletion, which may have a role to play in the development of diabetic nephropathy. However, the mechanism of MI depletion is still unclear. It has been proposed that myo-inositol oxygenase plays a key role in causing the depletion of MI.
There are two enzymes in the polyol pathway, aldose reductase and sorbitol dehydrogenase. Together, they convert glucose to fructose via sorbitol. Under physiological conditions, most of the cellular glucose is phosphorylated into glucose 6-phosphate, and only a minor portion is metabolised through the polyol pathway.

**DIABETIC NEUROPATHY**

The patients with Diabetes Mellitus had various types of neuropathic complication; tarsal tunnel syndrome is one of them. In longstanding history of diabetes mellitus, individuals with diabetic neuropathy often present with distressing symptoms such as pain, burning sensation, numbness, and paraesthesia of feet and heel. The symptoms are worst with prolonged standing or on long walk. Tarsal tunnel syndrome with electrophysiological changes may be a causative or contributing factor. These features in fact could be due to compression of medial plantar nerve, a branch of tibial nerve inside the tarsal tunnel. As this nerve is analogous to the median nerve in hand, hence it will not be out of place to select the medial plantar nerve for diagnosis of tarsal tunnel entrapment. The tarsal tunnel syndrome should be kept in mind during the diagnostic workup and management of diabetes mellitus (Ashraf Husain et al., 2009).

**CARDIOVASCULAR DISEASE**
Cardiovascular disease is the most costly complication of diabetes and is the cause of about 80% of deaths in people with diabetes. There are many risk factors for cardiovascular diseases such as, smoking, diet, obesity, hypertension, physical inactivity, dyslipidemia, genetic influences, family history and diabetes. One of the potential mechanisms that could mediate the premature atherosclerosis in diabetes is oxidative stress. Oxidative stress plays a crucial role in atherogenesis probably through the oxidation of low density lipoprotein (Vega-López et al., 2004). Ox-LDL is not recognized by the LDL receptor but by the scavenger receptor pathway on macrophages, which results in unregulated cholesterol accumulation, leading to foam cell formation (Jialal et al., 2002).

**HYPERTENSION**

Hypertension usually exists as a co-morbid condition with DM and may be a part of a larger metabolic syndrome, including hyperglycemia, hyperinsulinemia, and dyslipidemia. Hypertension and diabetes, when combined, increase the risk of cognitive impairment. Studies also revealed a higher association of hypertension with diabetic group than control group, and there was significant difference in P300 trends with co-existence of DM and hypertension than DM alone.

**Diabetic foot ulcers**
Diabetic ulcers result from neuropathy or ischemia. Neuropathy is characterized by loss of protective sensation and biomechanical abnormalities. Lack of protective sensation allows ulceration in areas of high pressure. Autonomic neuropathy causes dryness of the skin by decreased sweating and therefore vulnerability of the skin to break down. Ischemia is caused by peripheral arterial disease, not by microangiopathy. Poor arterial inflow decreases blood supply to ulcer area and is associated with reduced oxygenation, nutrition and ulcer healing. Necrotic tissue is laden with bacteria apt to grow in such an environment, which also impairs general defense mechanisms against infection. Infections often complicate existing ulcers, but are seldom the cause for ulcers. Protective footwear helps to reduce ulceration in diabetic feet at risk. Relieving pressure on the ulcer area is necessary to allow healing. Blood supply needs to be improved by revascularisation whenever compromised. Systemic antibiotics are helpful in treating acute foot infections, but not uninfected ulcers (Vuorisalo et al., 2009)

OXIDATIVE STRESS AND DIABETES

Diabetes mellitus is associated with increased generation of free radicals and/or impaired antioxidant defense systems. Persistent hyperglycemia results in increased oxidative stress, which primarily contributes to the development and progression of diabetes-associated complications (Pazdro and Burgess, 2010). However, the exact mechanism by
which oxidative stress could contribute to and accelerate the development of complications in diabetic mellitus is only partly known and remains to be clarified. On the one hand, hyperglycemia induces free radicals; on the other hand, it impairs the endogenous antioxidant defense system in diabetic milieu. The superfluous generation of the free radicals is proved to initiate/aggravate most of the human ailments like arthritis, cancer, Alzheimer, Parkinson's disease, cardiovascular disease and diabetic complications (Ali et al., 2008).

ROS are neutralized by a battery of antioxidants, which can be classified into two categories: enzymatic (ex: superoxide dismutase SOD, glutathione peroxidase GPx and catalase) and non-enzymatic systems (ex: glutathione GSH, vitamins A, C and E) (Wiernsperger, 2003). $O_2^-$, the parental form of intracellular ROS, is a very reactive molecule but it can be converted to $H_2O_2$ by superoxide dismutase (SOD) isoenzymes, and then to oxygen and water by several enzymes including catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxin (Prx). Pancreatic beta-cells are equipped with $O_2^-$ inactivating SODs in the cytosol and mitochondria at levels of about 50% less of those in the liver. However, the expression levels of the $H_2O_2$-inactivating enzymes GPx and CAT are extremely low in islets, at levels of only about 1% of those in the liver (Lenzen et al., 1996; Tiedge et al., 1997). This reduced antioxidant capacity potentially makes pancreatic beta-cells sensitive to ROS mediated signal transduction and cellular response.
The balance between the rate of free radical generation and elimination is important. Excess cellular radical generation can be harmful (Rice et al., 1997). However, if there is a significant increase in radical generation, or a decrease in radical elimination from the cell, oxidative cellular stress ensues (Valko et al., 2007). There is convincing experimental and clinical evidence that the generation of reactive oxygen species (ROS) increases in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress.

Oxidative stress results from increased ROS and/or reactive nitrogen species (RNS) (Joseph et al., 2003). Examples of ROS include charged species such as superoxide and the hydroxyl radical and uncharged species such as hydrogen peroxide and singlet oxygen. The possible sources of oxidative stress in diabetes might include auto-oxidation of glucose, shifts in redox balances, decreased tissue concentrations of low molecular weight antioxidants, such as reduced glutathione (GSH) and vitamin E, and impaired activities of antioxidant defense enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Haskins et al., 2003). ROS generated by high glucose is causally linked to elevated glucose and other metabolic abnormalities important to the development of diabetic complications. However, the exact mechanism by which oxidative stress may contribute to the development of diabetic complications is undetermined (Kowluru and Chan, 2007). In the past few decades, increasing evidence has connected
oxidative stress to a variety of pathological conditions, including cancer, cardiovascular diseases (CVDs), chronic inflammatory disease, post-ischaemic organ injury, diabetes mellitus, xenobiotic/drug toxicity, and rheumatoid arthritis (El Faramawy and Rizk, 2011; Samanthi et al., 2011).

Over time, convincing evidence has established the role of free radicals and oxidative stress in the pathogenesis and development of complications from DM including retinopathy, nephropathy, neuropathy, and accelerated coronary artery disease (Phillips et al., 2004; Niedowicz and Daleke, 2005).

Several studies have shown that elevated extra- and intra-cellular glucose concentrations result in oxidative stress which was reported both in experimental diabetes in animals and in diabetic patients. The source of oxidative stress is a cascade of ROS leaking from the mitochondria. This process has been associated with the onset of type 1 diabetes (T1DM) via the apoptosis of pancreatic beta-cells, and the onset of type 2 diabetes (T2DM) chiefly via insulin resistance. The underlying mechanisms in the onset of diabetes are complex because hyperglycemia could also be due to the cause-effect relationship of increased oxidative stress. Biomarkers of increased oxidative stress, as measured by indices of lipid peroxidation and protein oxidation, increase in both T1DM, and T2DM. (Giugliano et al., 1995; West, 2000; Cederberg et al., 2001; Sayed et al., 2011; Agnieszka et al., 2011).

MANAGEMENT AND TREATMENT OF DIABETES MELLITUS
Despite significant advances in understanding the molecular mechanism involved in the pathogenesis of hyperglycemia, the ideal cure of diabetes is still beyond horizon. The success of any treatment is measured by its efficacy for prolonging life, and at the same time allowing them to carry out their usual activities.

The management of Diabetes Mellitus involves, the two ways:

(i) Non-Pharmacological Treatment

(ii) Pharmacological Treatment

Non-Pharmacological Management of Diabetes

Life style modifications are essentially important for the successful management of diabetes mellitus and include the prescription of a healthy diet, regular exercise, the management of stress and avoidance of tobacco use.

Diet

Dietary management is considered as one of the most important factors in the attainment and maintenance of good metabolic control. Diabetic diet may be used alone or else in combination with insulin injections or with oral hypoglycemic drugs. The diet plan for a diabetic is based on height, weight, age, sex, physical activity and type of diabetes. The main objective of
diabetic diet is to maintain ideal body weight, by providing adequate nutrition along with normal blood sugar levels in blood. (Salas-Salvado et al., 2011).

**Exercise**

Regular exercise may be prescribed as an adjunct to caloric restriction for weight reduction and as a means to improve insulin sensitivity in the obese insulin-resistant individual. The beneficial effects of exercise in patients with diabetes have been well documented. The beneficial role of physical exercise on glycemic control in patients with type 2 diabetes mellitus has been confirmed by several controlled trials including both aerobic and resistance exercise protocols. Exercise has been shown to increase insulin sensitivity, lower blood sugar levels, reduce body fat and improve physical fitness (Konig and Berg, 2012).

While there is still active debate regarding the most beneficial types of diet and exercise, weight loss almost always improves glycemic levels. Unfortunately, the high rate of weight regain has limited the role of lifestyle interventions as an effective means of controlling glycemia long term (Nathan et al., 2006).

**Management of streses**
Diagnosis of diabetes mellitus is a stressful situation in life of an individual and appropriate management requires a holistic approach that includes behavioral modification to develop positive attitude and healthy lifestyle. A satisfactory treatment strategy should include special attention to individual with diabetes, quality of life, coping skills, optimal family support and a healthy workplace environment. Appropriate support and counseling is an essential component of the management at the time of diagnosis and throughout life.

Pharmacological Treatment of Diabetes

The Pharmacological treatment of Diabetes Mellitus involves the two methods. Use of

(i) Conventional Medicines

(ii) Traditional Medicines

CONVENTIONAL MEDICINES

INSULIN: AN INSIGHT

Insulin is a peptide hormone that exerts a variety of effects, mostly anabolic, on different cell types, but mainly in hepatocytes, myocytes and adipocytes. The hormone stimulates cell growth and differentiation, and promotes the storage of substrates in fat, liver and muscle by stimulating lipogenesis and lipid storage, and glycogen and protein synthesis, while
inhibiting lipolysis, glycogenolysis and protein breakdown. Thus, resistance
to the hormone leads to hyperglycaemia and hyperlipidaemia, while a lack of
insulin results in protein-wasting, ketoacidosis and, ultimately, death (Saltiel,
and Kahn, 2001). Although insulin is central to all intermediary metabolic
processes, its main action is related to glucose homoeostasis.

Diabetes may be pharmacologically treated with insulin injections.
Insulin may be synthetically blended with other carrier proteins to alter its
release time, once it is in the bloodstream. For example, long-acting insulin
may be taken before a large meal to digest ingested sugar slowly but
consistently over a period of several hours, while immediate acting insulin
can metabolize a surplus of sugar within several minutes.

**Insulin Production and Action**
Role of Insulin
Insulin therapy

Insulin therapy is the treatment of diabetes by administration of exogenous insulin. Patients with Type 1 diabetes mellitus depend on external insulin (most commonly injected subcutaneously) for their survival because the hormone is no longer produced internally. Patients with Type 2 diabetes mellitus are insulin resistant, have relatively low insulin production, or both; certain patients with Type 2 diabetes may eventually require insulin if other medications fail to control blood glucose levels adequately.
There are several problems with insulin as a clinical treatment for diabetes which includes mode of administration, selecting the 'right' dose and timing, refrigeration, selecting an appropriate insulin preparation, adjusting dosage and timing to fit food intake timings quantity, adjusting dosage and timing to fit exercise undertaken, adjusting dosage, type, and timing to fit other conditions, for instance the increased stress of illness.

**Oral antidiabetic drugs**

Drugs used to treat diabetes mellitus by lowering glucose levels in the blood are called as oral antidiabetic drugs. The major aim of treatment of diabetes is to control hyperglycemia and its mediated complications. Rational therapy of diabetes requires the application of principles derived from current knowledge concerning both the nature of the particular type of diabetes and the mechanism of action, efficacy and safety of the available treatment regimens. The list of oral hypoglycemic drugs and their mechanism of action is as follows

1. Sulphonyl ureas
2. Meglitines
3. Biguanides
4. Thiazolidinediones
5. α-Glucosidase inhibitors
6. Dipeptidyl peptidase inhibitor
Side effects of oral hypoglycemic drugs

The modern drugs, insulin and other oral hypoglycemic agents such as biguanides, sulphonylureas, α-glucosidase inhibitors often elicit characteristic profile of adverse effects, which include frequent diarrhea, hypoglycemia, hepatotoxicity, lactic acidosis, dyslipidemia, hypertension, and hypercoagulability. Significantly, for effective control of diabetes, combination therapy is being considered because no single drug is able to target diabetes and its associated complications. This necessitates the identification of novel drugs which might function in a mechanistically
distinct fashion to the existing drug targets. Hence, the search for a definitive
cure for diabetes mellitus is being pursued vigorously by the scientific
community.

**Experimental induction of diabetes mellitus**

The use of chemical agent to produce diabetes permits detailed study
of biochemical, hormonal and morphological events that occur during and
after induction of diabetes.

The existence of experimental animal model of a disease aids not
only the understanding of the pathophysiology of such disease, but also the
development of drugs for its treatment. The agents that are capable of
inducing diabetes in mammals are termed as diabetogens. The diabetogens are
chemicals and the diabetes induced by diabetogen is termed as chemical
induced diabetes or experimental diabetes. The two agents that have been
comprehensively studied and have yielded majority of information pertinent
to human diabetes are alloxan and streptozotocin.

**Alloxan**

Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) was first
described by Brugnatelli in 1818. Wöhler and Liebig used the name “alloxan”
and described its synthesis by uric acid oxidation.
Biological effects

Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing β cells in the pancreas when administered to rodents and many other animal species. This causes an insulin-dependent diabetes mellitus (called "Alloxan Diabetes") in these animals, with characteristics similar to type 1 diabetes in humans. Alloxan is selectively toxic to insulin-producing pancreatic beta cells because it preferentially accumulates in beta cells through uptake via the GLUT2 glucose transporter. Alloxan, in the presence of intracellular thiols, generates reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid. The beta cell toxic action of alloxan is initiated by free radicals formed in this redox reaction (Szkudelski, 2001).
**Streptozotocin**

Streptozotocin (STZ, 2-deoxy-2-3-(methyl-3-nitrosoureido)-D-glucopyranose) is synthesized by *Streptomyces achromogenes* and is used to induce both type1 and type2, respectively.

![Streptozotocin molecule]

**Mechanism of action**

STZ is a nitrosourea analogue, enters the pancreatic beta cells via GLUT2 glucose transporter and causes DNA alkylation followed by the activation of poly ADP ribosylation leading to depletion of cytosolic concentration of NAD$^+$ and ATP. Enhanced ATP dephosphorylation after STZ administration, supplies substrate for xanthine oxidase resulting in the formation of superoxide radicals. Further, NO liberation from STZ also causes the destruction of β cells by necrosis (Szkudelski, 2001).
MEDICINAL PLANTS IN THE TREATMENT OF DIABETES

In the last few years there has been an exponential growth in the field of herbal medicine and these drugs are gaining popularity both in developing and developed countries because of their natural origin and relatively less side effects. Many traditional medicines in use are derived from medicinal plants, minerals and organic matter. A number of medicinal plants, traditionally used for over 1000 years named rasayana are present in herbal preparations of Indian traditional health care systems.

India has a rich heritage of traditional medicine and the traditional health care system have been flourishing for many centuries. In developing countries, traditional medicine is often the only accessible and affordable treatment available. Medicinal plants consist of components of therapeutic values and have been used as remedies for human diseases since antiquity. There has been resurgence in the consumption and demand for medicinal plants. These plants are finding use as pharmaceuticals, nutraceuticals,
cosmetics and food supplements. Even as traditional source of medicines and they continue to play pivotal rule.

Several medicinal plants have been used as dietary adjunct and in the treatment of numerous diseases without proper knowledge of their function. Although phytotherapy continues to be used in several countries, most of the traditional medicinal plants have not received scientific or medical scrutiny. One such medicinal plant, which lacks scientific evidence for its wide folklore use is *Pithecellobium dulce*.

*Pithecellobium dulce* Benth. (*Leguminosae*) is a small to medium sized, evergreen, spiny tree up to 18 m height, native of tropical America and cultivated throughout the plains of India and in the Andamans. Different parts of the plant such as leaves, bark, roots, flowers and fruits have been used traditionally for the treatment of various ailments. The fruits of *P. dulce* have been consumed as a dietary supplement for its high nutritive and medicinal value. The edible fruit has been widely used traditionally to combat gastric problems and found to be non-toxic in nature (Megala and Geetha, 2012). The fruit extract was found to be rich in phenolic compounds and revealed the presence of flavonoids – quercitrin, rutin, kaempferol, naringin and daidzein (Megala and Geetha, 2010).

In the light of the above folklore applications, the present study was carried out to scientifically validate the hypoglycemic, hypolipidemic and antioxidant properties of edible part of *P. dulce* fruits (Pod pulp) on streptozotocin induced experimental diabetes in rats.
EXPERIMENTAL DESIGN AND METHODOLOGY

Plant Material

Fresh, mature *P. dulce* pods (fruits) were collected manually from the trees growing under natural conditions from the village Mudivaithanendhal near the river banks of Thamirabarani in Tirunelveli district. The plant materials were identified and authenticated by a taxonomist and an exemplar specimen was deposited at the CAS in Botany, University of Madras, Chennai.

Preparation of pod pulp extract by delipidation and soxhalation

The edible part of pods (pulps) were selectively removed and dried at room temperature and powdered in an electrical grinder, which was then stored in an airtight container at 5° C until further use. The powdered edible pods were delipidated with petroleum ether (60 - 80° C) for overnight. It was then filtered and soxhalation was performed with 95% Ethanol. Ethanol was evaporated in a rotary evaporator at 40 – 50° C under reduced pressure. Extractive value (%W/W) of ethanolic extract was 17.6g. The ethanolic extract was stored at 5 C in a refrigerator.

PRELIMINARY PHYTOCHEMICAL SCREENING

The ethanolic extract of *Pithecellobium dulce* pod pulp was subjected to phytochemical screening for the qualitative analysis of various plant constituents (Harborne, 1998; Kokate, 2001).
TEST FOR ALKALOIDS

(i) Dragendorff’s Test

Dragendorff’s Reagent: Eight grams of Bi(NO$_3$)$_3$ 5H$_2$O was dissolved in 20 ml of HNO$_3$ and 2.72 g of potassium iodide in 50 ml of H$_2$O. These were mixed and allowed to stand until KNO$_3$ crystals were formed. The supernatant was decanted off and made up to 100 ml with distilled water.

Procedure: 0.5 ml of the pod pulp extract was added to 2 ml of HCl. To this acidic medium, 1 ml of dragendorff’s reagent was added. An orange or red precipitate produced immediately indicates the presence of alkaloids.

(ii) Wagner’s Test

Wagner’s Reagent: 1.2 g of iodide and 2.0 g of potassium iodide were dissolved in 5 ml of sulphuric acid and the solution was diluted to 100 ml.

Procedure: 10 ml of the pod pulp extract was acidified by adding 1.5% V/V of HCl and a few drops of wagner’s reagent. Formation of yellow or brown precipitate confirmed the presence of alkaloids.

(iii) Mayer’s Test

Mayer’s Reagent: 1.36 g of mercuric chloride was dissolved in 60 ml of distilled water and 5 g of potassium iodide in 10 ml of water. The solutions were mixed and diluted to 100 ml with distilled water.
**Procedure**

1.2 ml of the pod pulp extract was taken in a test tube, 0.2 ml of dilute HCl and 0.1 ml of Mayer’s reagent were added. Formation of yellowish buff coloured precipitate confirmed the presence of alkaloids.

**TEST FOR FLAVONOIDS**

(i) **Shinoda’s Test**

In a test tube containing 0.5 ml of pod pulp extract, 5 – 10 drops of diluted HCl and a small piece of zinc chloride or magnesium were added and the solution was boiled for few minutes. In the presence of flavonoids, reddish pink colour was produced.

(ii) **Alkaline Reagent Test**

To 1 ml of the extract, a few drops of dilute sodium hydroxide were added. An intense yellow colour was produced in the pod pulp extract, which becomes colourless on the addition of a few drops of dilute acid indicates the presence of flavonoids.

**TEST FOR GLYCOSIDES**

The pod pulp extract was hydrolysed with HCl for few hours on a water bath and the hydrolysate was subjected to legal’s or borntrager’s test to detect the presence of glycosides.
(i) **Legal’s Test**

To the hydrolysate, 1 ml of pyridine and few drops of sodium nitroprusside solutions were added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour shows the presence of glycoside in the pod extract.

(ii) **Borntrager’s Test**

The hydrolysate was treated with chloroform and then the chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Ammonia layer acquires pink colour, shows the presence of glycosides.

**TEST FOR SAPONINS**

(i) The pod pulp extract was diluted with 20 ml of distilled water and it was agitated in a graduated cylinder for 15 minutes. The formation of 1 cm layer of foam shows the presence of saponins.

(ii) 1 ml of the pod pulp extract was treated with 1% lead acetate solution. Formation of white precipitate indicates the presence of saponins.
TEST FOR TANNINS

(i) **Ferric Chloride Test:** To 1 – 2 ml of pod pulp extract, a few drops of 5% aqueous ferric chloride solution was added. A violet colour formation indicates the presence of Tannins.

(ii) **Lead Acetate Test:** In a test tube containing about 5 ml of the pod pulp extract, a few drops of 1% lead acetate was added. A yellow precipitate was formed, indicates the presence of Tannins.

(iii) 5 ml of the pod pulp extract was treated with 1 ml of 10% aqueous potassium dichromate solution. Formation of yellowish brown precipitate suggests the presence of tannins.

TEST FOR PHYTOSTEROL

The pod pulp extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification takes place. The mixture was diluted and extracted with ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol.

(i) **Liebermann Burchard Test**

The pod pulp extract was dissolved in few drops of diluted acetic acid; 3 ml of acetic anhydride was added followed by few drops of concentrated $\text{H}_2\text{SO}_4$. Appearance of bluish green colour shows the presence of phytosterol.
(ii) **Salkowski’s Test**

10 mg of the pod pulp extract was dissolved in 1 ml of chloroform; 1 ml of concentrated \( \text{H}_2\text{SO}_4 \) was added carefully along the sides of the test tube. The red colour was produced indicating the presence of steroids.

**TEST FOR TRITERPENOIDS**

(i) **Libermann Burchard Test**

10 mg of the pod pulp extract was dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was added following the addition of 2 ml of concentrated \( \text{H}_2\text{SO}_4 \). Formation of reddish violet colour indicates the presence of triterpenoids.

(ii) **Noller Test**

5 mg of the pod pulp extract was dissolved in 2 ml of 0.01% anhydrous stannic chloride in pure thionyl chloride. A purple colour formed was then changed to deep red after few minutes and indicates the presence of triterpenoids.

**TEST FOR ANTHRAQUINONES**

5 ml of the pod pulp extract solution was hydrolysed with concentrated \( \text{H}_2\text{SO}_4 \) extracted with benzene. 1 ml of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones.
TEST FOR PHENOLS

(i) A small quantity of the pod pulp extract was treated with 1% aqueous or alcoholic ferric chloride solution. Formation of green, purple, blue or black colour indicates the presence of phenol.

(ii) A small quantity of the pod pulp extract was treated with aqueous mixture of 1 % ferric chloride and 1% potassium ferricyanide. Appearance of green or purple or blue colour shows the presence of phenols.

Determination of total phenolic content

Total polyphenol content in the ethanol extract of P. dulce pod pulp was determined according to the Folin-Ciocalteu colorimetric method (Singleton et al., 1999; Kumazawa et al., 2002). A standard curve was built with gallic acid reference solutions. Aliquots ranging from 2 to 10 mL of standard aqueous gallic acid solution (100 μg/mL) were pipetted in to 100 mL volumetric flasks containing 70 mL of distilled water. Folin-Ciocalteu reagent (5 mL) and 10 mL of saturated sodium bicarbonate solution were added, and the volume was made up to 100 mL with distilled water. The solution was thoroughly mixed. The blank was prepared in the same manner, but without gallic acid. After 1 h of incubation at room temperature, the absorbance was measured at 760 nm. The samples were prepared in triplicates for each
analysis and the mean value was calculated. For the determination of total phenolic content of *P. dulce* pod pulps, aqueous solutions at the final concentration of 20μg/mL were used; proceeding in the same manner described for the reference solutions and the total polyphenolic content was expressed as mg per g of gallic acid equivalents.

**Determination of total flavonoid content**

Total flavonoid content in the ethanolic extract of *P. dulce* pod pulps was determined according to the method of Quettier et al., 2000 with minor modifications. A standard curve was built with quercetin reference solutions. Aliquots ranging from 2 to 8 mL of standard quercetin ethanol extract solution (50 μg/mL) were pipetted in to 25 mL volumetric flasks containing 1 mL of 2% aluminum chloride dissolved in ethanol and the volume was made up with ethanol. The blank was prepared by diluting 1 mL of 2% aluminum chloride dissolved in ethanol in a 25 mL volumetric flask with ethanol. After 1 h at room temperature, the absorbance was measured at 420 nm. *P. dulce* pod pulps samples were evaluated at a final concentration of 20 μg/mL, proceeding in the same manner described for the reference solutions and the total flavonoid content was calculated as quercetin equivalents (mg/g) from a calibration curve. The samples were prepared in triplicate for each analysis and the mean value of absorbance was recorded.
HPLC–DAD system for analysis of phenolic compounds

HPLC analysis was performed using Shimadzu HPLC system equipped with a diode array detector. The chromatographic separations were performed on a Inertsil C18 analytical column (4.6 × 250 mm i.d., 5 μm). The composition of solvents and the gradient elution conditions used were described previously by Bengoechea et al., 1997, Schieber et al., 2001 and Butsat et al., 2009, with some modifications. The mobile phase consisted of purified water with acetic acid (pH 2.74) (solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 ml/min. Gradient elution was performed as follows: from 0 to 5 min, linear gradient from 5% to 9% solvent B; from 5 to 15 min, 9% solvent B; from 15 to 22 min, linear gradient from 9% to 11% solvent B; from 22 to 38 min, linear gradient from 11% to 18% solvent B; from 38 to 43 min, from 18% to 23% solvent B; from 43 to 44 min, from 23% to 90% solvent B; from 44 to 45 min, linear gradient from 90% to 80% solvent B; from 45 to 55 min, isocratic at 80% solvent B; from 55 to 60 min, linear gradient from 80% to 5% solvent B and a re-equilibration period of 5 min with 5% solvent B used between individual runs. Operating conditions were as follows: column temperature, 38 °C, injection volume, 20 μl, and UV-diode array detection at 280 nm (hydroxybenzoic acids), 320 nm (hydroxycinnamic acids) and 370 nm (flavonols) at a flow-rate of 0.8 ml/min. Spectra were recorded from 200 to 600 nm. Phenolic compounds in the samples were identified by comparing their relative retention times and UV
spectra with those of authentic compounds and were detected using an external standard method.

**Experimental Animals**

Male albino Wistar rats (150-180g) were purchased from Tamilnadu Veterinary and Animal Sciences University, Madavaram, Chennai. The rats were housed in polypropylene cages lined with husk and maintained in centralized Animal house Facility. The husk was renewed every 24 hours. The rats were fed with commercial pelleted rats chow (VRK Nutritional Solutions, Maharashtra, India) and had free access to water. The experimental rats were maintained in a controlled environment (12:12 hours light/dark cycle) and temperature (30 ± 2° C). The experiments were designed and conducted in accordance with the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines for the investigation of experimental pain in conscious rats. The rats were acclimatized for one week before initiating the experiments. [IAEC NO. 17/01/2012].

**Induction of Diabetes Mellitus**

Experimental diabetes was induced in overnight fasted rats by single intraperitoneal injection of streptozotocin (45 mg/kg b.w) dissolved in freshly prepared 0.1M of cold citrate buffer (pH 4.5) (Rakieten et al., 1963). Since, STZ is capable of inducing fatal hypoglycemia due to massive pancreatic
insulin release, the rats were provided with 10% glucose solution after 6 h of STZ administration for the next 24 h to overcome drug induced hypoglycemia (Fischer and Rickert, 1975). Neither death nor any other adverse effect was observed. After a week time, for the development and aggravation of diabetes, rats with moderate diabetes (i.e. fasting blood glucose concentration, >250 mg/dl) that exhibited hyperglycemia and glycosuria were selected for further experimentation.

**Experimental Design**

The rats were divided into four groups, each group comprising of a minimum of 6 rats as follows:

**Group I** : Control rats (water and food *ad libitum*).

**Group II** : Streptozotocin induced diabetic Rats.

**Group III** : Diabetic rats treated with *P. dulce* pod pulp extract (300 mg/Kg body weight/rat/day) in aqueous solution orally for 30 days.

**Group IV** : Diabetic rats treated with gliclazide (5mg/kg body weight/day) in aqueous solution orally for 30 days.

During the experimental period, body weight and blood glucose levels of all the rats were determined at regular intervals. At the end of the
experimental period, the rats were fasted over night, anaesthetized, and sacrificed by cervical decapitation. The blood was collected with and without anticoagulant for plasma and serum separation respectively.

**PREPARATION OF TISSUE HOMOGENATE**

The liver, kidney and pancreatic tissues were excised, rinsed in ice-cold saline. Known amount of the tissues were homogenized in Tris–HCl buffer (100 mM, pH 7.4) at 4°C, in a Potter–Elvehjem homogenizer with a Teflon pestle at 600 rpm for 3 min. The homogenate was then centrifuged at 12,000-×g for 30 min at 4°C. The supernatant was collected as tissue homogenate, which was used to assay various parameters.

**ORAL GLUCOSE TOLERANCE TEST (OGTT)**

At the end of the experimental period, fasting blood samples were taken from all the groups of rats to perform oral glucose tolerance test. Four more blood samples were collected at 30, 60, 90 and 120 min intervals after an oral administration of glucose solution at a dosage of 2 g kg-1 body weight. All the blood samples were collected with EDTA for the estimation of glucose by O-toluidine method. Blood samples were collected from the tail vein by tail milking at -30 just prior to drug administration and at 30, 60, 90 and 120 min. after glucose loading. The level of Glucose was measured using Glucometer.
HISTOLOGICAL STUDIES

A slice of pancreas, liver and kidney tissues were fixed in 10% formalin for 1 week at room temperature. Then the specimens were dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin wax. Tissue blocks were sectioned into 5µm thickness using a rotary microtome. Sections of liver and kidney tissues were stained with hematoxylin and eosin.

A qualified pathologist without prior knowledge of the groups viewed histological changes in the stained sections under the light microscope.

TRANSMISSION ELECTRON MICROSCOPY

A portion of pancreas, liver and kidney (about 1mm3) from control and experimental groups of rats were fixed in 3% glutaraldehyde in sodium phosphate buffer (0.2 M, pH 7.4) for 3 h at 4°C. Tissue samples were washed with the same buffer, post-fixed in 1% osmium tetroxide and sodium phosphate buffer (0.2 M, pH 7.4) for 1 h at 4°C. The samples were again washed with the same buffer for 3 h at 4°C, dehydrated with graded series of ethanol and embedded in Araldite. Thin sections were cut with LKBUM4 ultramicrotome using a diamond knife, mounted on a copper grid and stained with 2% uranyl acetate and Reynolds lead citrate (Kalender et al., 2004). The grids were examined under a Philips EM201C transmission electron microscope.
BASIC BIOCHEMICAL PARAMETERS

ESTIMATION OF BLOOD GLUCOSE

Blood glucose level was estimated by the method of glucose oxidase/peroxidase method as described by Trinder (1969) using a commercial kit (Span Diagnostic Chemicals, India).

Reagents

1. Enzyme reagent
2. Buffer solution
3. Glucose standard (100 mg/dl)

Procedure

10 µl of blood was added to 1.0 ml of working enzyme reagent, mixed well and incubated at 37°C for 15 min. The colour developed was read at 505 nm against blank containing distilled water instead of the sample. A standard was also processed similarly.

The level of glucose is expressed as mg/dl.

ESTIMATION OF PROTEIN

The protein content was estimated according to the method of Lowry et al. (1951).
**Reagents**

1. Alkaline - copper reagent

   Solution A : 2% sodium carbonate in 0.1 N NaOH solution

   Solution B : 0.5% copper sulphate in 1% sodium potassium tartrate

   50 ml of solution A was mixed with 1 ml of solution B just before use.

2. Folin's phenol reagent (commercial reagent, 1:2 dilutions)

3. Standard protein solution: 10 mg of crystalline bovine serum albumin (BSA) was dissolved in 100 ml of distilled water.

**Procedure**

To 100 µl of test sample, 1 ml of water was added and this was mixed with 4.5 ml of alkaline copper reagent and then allowed to stand at room temperature for 10 min. 0.5 ml of Folin's phenol reagent was added. The blue colour developed was read at 640 nm in a Shimadzu spectrophotometer after 20 min. A standard graph was obtained using bovine serum albumin solution in the range of 10-100 µg.

The protein content was expressed as g/dl of plasma or mg/g of tissue.
ESTIMATION OF UREA

Urea was determined by the method of Natelson et al. (1951) using diacetylmonoxime.

Reagents

1. Diacetylmonoxime reagent: 2 g of diacetylmonoxime was dissolved in 100 ml of 2 % acetic acid.

2. Sulphuric acid-Phosphoric acid reagent: 25 ml of concentrated sulphuric acid, 75 ml of 85% O-phosphoric acid and 70 ml of distilled water were mixed.

3. 10% sodium tungstate solution

4. 0.67 N sulphuric acid

5. Standard urea: 20 mg of urea dissolved in 100 ml of water.

Procedure

To 0.1 ml of blood, 3.3 ml of water, 0.3 ml each of 10% sodium tungstate and 0.67 N sulphuric acid were added. The suspensions were centrifuged. To 1.0 ml of the supernatant added 1.0 ml of water, 0.4 ml of diacetylmonoxime and 2.6 ml of sulphuric acid-phosphoric acid reagent. Standard urea (20-50 µg/ml) were also treated in a similar manner and all the tubes were heated in a boiling water bath for 30 min, cooled and the colour developed was measured at 480 nm in a Shimadzu spectrophotometer.

The values were expressed as mg of urea/dl of blood.
ESTIMATION OF URIC ACID

Uric acid was estimated in the serum according to the method of Caraway (1963).

Reagents

1. Phosphotungstic acid reagent: 30 g of sodium tungstate was dissolved in 300 ml of water. 32 ml of 85% \( O \)-phosphoric acid was added and refluxed under a low flame for 2 h. After cooling to room temperature, 18 g of lithium sulphate was dissolved and the solution was finally made up to 1 litre.

2. 14% sodium carbonate

3. 2/3 N sulfuric acid

4. 10% sodium tungstate

5. Stock uric acid standard: 100 mg of uric acid and 60 mg of lithium carbonate were taken in about 50 ml of water. This was heated to about 80° C to dissolve the uric acid completely. After cooling, the solution was finally made up to 100 ml with distilled water.

6. Working standard: 1.0 ml of the stock standard was diluted to 10 ml to get the working standard, which contained 100 µg uric acid/ml.
**Procedure**

A protein free filtrate was prepared by precipitating 1.0 ml of serum with 8.0 ml of water, 0.5 ml of 2/3 N sulphuric acid and 0.5 ml of 10% sodium tungstate. After centrifugation, 2.0 ml of supernatant was mixed with 1.0 ml of phosphotungstic acid and 1.0 ml of 14% sodium carbonate. The blue colour developed was read at 640 nm after 10 min. The standard curve prepared with standard uric acid was used to arrive at the serum uric acid levels. The levels are expressed as mg/dl serum.

**ESTIMATION OF SERUM CREATININE**

Creatinine was estimated according to the method of Brod and Sirota (1948) using Jaffe's reaction.

**Reagents**

1. Saturated picric acid
2. 0.75 N sodium hydroxide
3. 2/3 N sulphuric acid
4. 10% Sodium tungstate solution
5. Standard creatinine: 100 mg of creatinine was dissolved and made up to 100 ml in 0.1 N HCl. Working standard was prepared by dilution of 10 ml of the stock solution to 100 ml with 0.1 N HCl.
**Procedure**

A protein free filtrate was prepared by precipitating 1.0 ml of plasma with 8.0 ml of water, 0.5 ml of 2/3 N sulphuric acid and 0.5 ml of 10% sodium tungstate. After centrifugation, 5.0 ml of the clear filtrate was taken. To this, 1.5 ml of each of saturated picric acid solution and 0.75 N sodium hydroxide was added. The colour intensity was measured at 460 nm after 15 min in a Shimadzu spectrophotometer. Standard and blank were also processed similarly.

The values were expressed as mg/dl for plasma.

**ASSAY OF ASPARTATE AMINOTRANSFERASE (AST)**

The enzyme activity was assayed by the method of King (1965a).

**Reagents**

1. 0.15 M phosphate buffer, pH 7.5

2. Substrate: 300 mg of DL-aspartate and 50 mg of α-ketoglutarate were dissolved in 20-30 ml of phosphate buffer and 10% sodium hydroxide was added to bring the pH to 7.5 and made up to 100 ml with phosphate buffer.

3. Aniline-citrate reagent: 5.0 g of citric acid was dissolved in 50 ml of distilled water and to this equal volume of redistilled aniline was added.
4. DNPH reagent: 200 mg of 2,4-dinitrophenyl hydrazine (DNPH) was dissolved in 85 ml of concentrated hydrochloric acid and made up to a litre with water.

5. 0.4 N sodium hydroxide solution

6. Standard pyruvate: 12.5 mg of sodium pyruvate was dissolved in 100 ml of distilled water. 10 ml of this was diluted to 100 ml with distilled water and was used as working standard for the standard calibration curve.

**Procedure**

To 1.0 ml of the buffered substrate, 0.1 ml of enzyme source was added and incubated for one hour at 37° C. At the end of the incubation period, 0.07 ml of aniline-citrate reagent was added and incubated for another 20 min. Then, 1.0 ml of the dinitrophenyl hydrazine reagent was added and left for twenty min. At the end of 20 min, 10 ml of 0.4 N sodium hydroxide was added and the color developed was read at 540 nm in a Shimadzu spectrophotometer after 10 min. The standards were also treated similarly.

The enzyme activity in serum was expressed as µmoles of pyruvate/h/mg of protein and in tissues as moles of pyruvate/min/mg of protein.
ASSAY OF ALANINE AMINOTRANSFERASE

The enzyme activity was assayed by the method of King (1965a).

Reagents

1. Buffered substrate solution: 1.5 g of dipotassium hydrogen phosphate, 0.2 g of potassium dihydrogen phosphate, 0.03 g of α-ketoglutarate and 1.78 g of DL-alanine were dissolved in distilled water. The pH was adjusted to 7.4 with 1 N sodium hydroxide if necessary and made up to 100 ml with distilled water.

2. DNPH reagent: 20 mg of DNPH reagent in 100 ml of 1 N hydrochloric acid.

3. 0.4 N sodium hydroxide solution

4. Standard pyruvate: 12.5 mg of sodium pyruvate was dissolved in 100 ml of distilled water. 10 ml of this was diluted to 100 ml with distilled water and was prepared freshly before use.

Procedure

To 1.0 ml of the buffered substrate, 0.1 ml of enzyme source was added and incubated at 37 °C for 30 min. The reaction was arrested by adding 1.0 ml of dinitrophenyl hydrazine and left aside for 20 min at room temperature. The colour developed by the addition of 10 ml of 0.4 N sodium
hydroxide was read at 540 nm in a Shimadzu spectrophotometer against the reagent blank.

The enzyme activity in serum was expressed as μmoles of pyruvate/h/mg of protein and in tissues as moles of pyruvate/min/mg of protein.

**ASSAY OF ALKALINE PHOSPHATASE**

Alkaline phosphatase activity was assayed by the method as described by King (1965b).

**Reagents**

1. 0.1 M Carbonate-bicarbonate buffer, pH 10
2. 0.01 M Disodium phenyl phosphate solution
3. 0.1 M Magnesium chloride solution
4. Folin’s Phenol reagent: Diluted 1:2 (v/v) with distilled water
5. 15% Sodium carbonate solution
6. Standard phenol solution: 100 mg of pure, crystalline phenol was dissolved in 0.1 N HCl and made up to 100 ml with 0.1 N HCl

**Procedure**

The incubation mixture of 3.0 ml contained 1.5 ml of buffer, 1.0 ml of substrate and 0.5 ml of the enzyme source. The tubes were incubated at 37° C
for 15 min. The reaction was arrested by the addition of 1.0 ml of Folin’s phenol reagent. The control tubes received the enzymes after arresting the reaction. The contents were centrifuged and to 1 ml of the supernatant 1.0 ml of 15% sodium carbonate, 1 ml of substrate and 0.1 ml of MgCl were added and incubated for 10 min at 37° C. The colour developed was read at 640 nm in a Shimadzu spectrophotometer against a blank. The standard solutions of phenol of varying concentrations were also treated similarly.

The enzyme activity in serum and tissues were expressed as µmoles of phenol liberated/min/mg of protein.

**HEMATOLOGICAL PARAMETERS**

**Estimation of Hemoglobin**

Hemoglobin was estimated based on the formation of cyanmethemoglobin by the method of Drabkin and Austin (1932).

**Reagents**

1. Drabkin’s reagent: This reagent contained 50 mg of potassium cyanide, 200 mg of potassium ferricyanide and 1g of sodium bicarbonate in 1 litre of distilled water (pH 9.6). It was stored in brown bottle.

**Procedure**

To 0.2 ml of blood, 5.0 ml of reagent was added, mixed well and allowed to stand for 10 min. The solution was read at 540 nm together with the standard solution of cyanmethemoglobin against a blank containing 5.0 ml of the reagent.

The hemoglobin content was expressed as g/dl of blood.

**Estimation of Glycosylated Hemoglobin (HbA1c)**

The glycosylated hemoglobin was estimated by the method of Nayak and Pattabiraman (1981).

**Reagents**

1. Sodium chloride : 0.9%
2. Oxalic acid : 0.3 M
3. TCA : 40% solution
4. Thiobarbituric acid (TBA) : 0.05 M
5. Standard fructose solution : In the range of 10-40 μg.

**Procedure**

0.2 ml of the hemolysate was mixed with 1.8 ml of 0.3 M oxalic acid and hydrolyzed in a boiling water bath for two hours. To the cooled tubes, 1 ml of 40% TCA was added, mixed and centrifuged at 1,400 x g for 20 min.
To 1.5 ml of supernatant added 0.5 ml of 0.05 M TBA and incubated at 37°C for 40 min. The colour developed was read at 443 nm against the blank. Standard fructose in the range of 10-40 μg was also processed similarly.

The glycosylated hemoglobin content was expressed as % Hb.

**ESTIMATION OF PLASMA INSULIN**

**Estimation of Plasma Insulin**

Plasma insulin was estimated using ELISA Assay kit (for rats) supplied by crystalchem, Inc. Life technologies, India. Elisa plate reader, was used throughout the study. The contents of the kit are

The following are the kit contents

- Micro titer plate coated with mouse monoclonal anti-rat insulin antibodies.
- Adhesive plate sealer
- 10X concentrate HRP wash buffer 10X concentrate of 50mM Tris buffer.
- Saline containing tween 20.
- Standard-Rat insulin assay buffer : 0.2, 0.5, 1, 2, 5 and 10ng/ml
- Quality control 1 (QC1) and Quality control 2 (QC2) - Rat insulin QC buffer.
- Matrix solution (charcoal stripped pooled mouse serum)
- Assay buffer 0.05M Phospho saline, pH 7.4, containing 0.025M EDTA, 0.08% sodium azide and 1% BSA.
- Detection antibody-pretitered biotinylated anti-insulin antibodies
- Enzyme solution pre titered streptavidin horse radish peroxidase conjugate in buffer
- Substrate-3, 3', 5, 5'-tetramethylbenzidine in buffer
- Stop solution-0.3M HCl

All reagents were prewarmed to room temperature prior to setting up the assay.

10X concentrated TBS wash buffer was diluted 10 fold by mixing the entire content of the buffer with 450ml with de-ionized water.

- The micro-titer assay plate was washed 3 times with 300µl diluted TBS wash buffer per wash. The wash buffer was decanted and residual amount was removed from all the wells by inverting the plate and trapping it smartly onto absorbent towels several times. Antibody detection solution was transferred to a reagent reservoir and 80 µl of the solution was decanted from the plate and it was tapped to remove residual fluid.
• Wells were washed 6 times with diluted 300 µl TBS buffer per wash, decanted and tapped after each wash to remove residual buffer.

• 100 µl of substrate solution was added to each well covered with sealer and shaken well in plate shaker for 15min. Blue color was formed in wells of insulin standards with intensity proportional to increasing concentrations of insulin. Sealer was removed and 100 µl of stop solution was added. The plate was shaken manually to ensure complete mixing of solution in all the wells. The blue color turned to yellow after acidification. The absorbance was read at 450nm and 590nm in plate reader within 5 minutes and ensured that there were no air bubbles in any well. Difference of absorbance units was recorded.

The plasma insulin levels were determined and expressed as ng/ml.

CARBOHYDRATE METABOLIZING ENZYMES

Assay of glucokinase

A fresh sample of liver was homogenized (dilution 1:10) in ice-cold buffer (80mM Tris, 5mM EDTA, 2mM 1,4-dithiothreitol, 1mM benzamidine, 1 4-(2-aminoethyl)benzenesulfonyl fluoride, at pH 7.6. The homogenate was centrifuged for 5 min at 900 g. Enzyme activities were measured at 37°C by coupling ribulose-5-phosphate formation from glucose-6-phosphate to the reduction of NADP using purified glucose-6-phosphate dehydrogenase and
6-phosphogluconate dehydrogenase as coupling enzymes. One unit of enzyme activity was defined as the amount that phosphorylates 1 µmol glucose/min. The GK activity of the crude homogenate was estimated by the standard method subtracting the rate of NADPH formation (at 340 nm) in the presence of 1 mM glucose (scoring low-Km HK activities) from that at 100 mM glucose (scoring total HK activities) (Panserat et al., 2001).

Assay of Pyruvate Kinase

Pyruvate kinase activity was assayed by the method of Pogson and Denton (1967).

Reagents

1. Tris-HCl buffer: 0.1 M, pH 7.4
2. NADH-PEP solution: Dissolved 10 mg of NADH, 45 mg of phosphoenol pyruvate (PEP) and 10 mg of sodium bicarbonate in 3 ml of water
3. Lactate dehydrogenase (LDH): 0.5 mg/ml.
4. ADP solution: 162 mg of ADP and 30 mg of sodium bicarbonate were dissolved in 3 ml water.
5. Magnesium chloride (Mg Cl₂): 0.05 M solution
6. Potassium chloride (KCl): 0.1 M solution
Procedure

The incubation mixture containing 100 μl of Tris-HCl buffer, 240 μl of KCl, 50 μl of MgCl2, 10 μl of PEP, 2 μl of ADP, 0.5 μl of NADH, 100 μl of LDH and 20-40 μl of the enzyme source was incubated at 25°C for 5 min. The colour developed was read at 340 nm.

The enzyme activity was expressed as μmoles of pyruvate formed/min/mg of protein for tissue homogenates under incubation conditions.

Assay of Glucose-6-Phosphatase

Glucose-6-phosphatase was assayed according to the method of Koide and Oda (1959).

Reagents

1. Citrate buffer : 0.1 M, pH 6.5
2. Substrate : 0.01 M Glucose-6-phosphate
3. Trichloroacetic acid : 10% solution

Procedure

The incubation mixture in a total volume of 1.0 ml contained 0.3 ml of buffer, 0.5 ml of substrate and 0.2 ml of enzyme extract. Incubation was carried out at 37°C for 60 min. The reaction was terminated by the addition of 1.0 ml of 10% TCA. The suspension was centrifuged and the phosphorus content of the supernatant was estimated according to the method described
by Fiske and Subbarow (1925). To an aliquot of the supernatant, 4.1 ml of distilled water and 0.5 ml of ammonium molybdate were added. After 10 min 0.2 ml of ANSA was added. The tubes were shaken well, kept aside for 20 min and the blue colour developed was read at 620 nm against water blank in a Shimadzu spectrophotometer.

The enzyme activity was expressed as μmoles of phosphate liberated/h/mg of protein under the incubation conditions.

**Assay of Fructose 1, 6-Diphosphatase**

Fructose-1, 6-diphosphatase was assayed by the method of Gancedo and Gancedo (1971).

**Reagents**

1. Tris-HCl buffer : 0.1 M, pH 7.0
2. Substrate : 0.05 M Fructose-1, 6-diphosphate.
3. Magnesium chloride : 0.1 M solution
4. Potassium chloride : 0.1 M solution
5. EDTA solution : 0.001 M solution
6. Trichloroacetic acid : 10% solution
7. Molybdic acid : 2.5% ammonium molybdate in 3 N sulphuric acid.
1. ANSA reagent: 500 mg of aminonaphthol sulphonic acid (ANSA) was dissolved in 195 ml of 15% sodium bisulphite solution and 5 ml of 20% sodium sulphite solution was added for complete solubilisation. The solution was filtered and stored at 4°C in a brown bottle.

2. Standard phosphorus: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 ml of distilled water (80 μg/ml).

**Procedure**

The assay medium in a final volume of 2.0 ml contained 1.2 ml of buffer, 0.1 ml of substrate, 0.25 ml of magnesium chloride, 0.1 ml of potassium chloride, 0.25 ml of EDTA and 0.1 ml of the enzyme source. This mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 1.0 ml of 10% TCA. The suspension was then centrifuged and the phosphorus content of the supernatant was estimated according to the method described by Fiske and Subbarow (1925). To an aliquot of the supernatant, 4.1 ml of distilled water and 0.5 ml of ammonium molybdate were added. After 10 min 0.2 ml of ANSA was added. The tubes were shaken well, kept aside for 20 min and the blue colour developed was read at 620 nm against water blank in a Shimadzu UV spectrophotometer.

The enzyme activity was expressed as μmoles of phosphate liberated/h/mg of protein.
Assay of Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase was assayed by the method of Ells and Kirkman (1961).

Reagents

1. Tris-HCl buffer : 0.5 M, pH 7.4
2. Magnesium chloride : 1 M solution
3. NADP+ : 0.01 M solution
4. Phenazine methosulphate : 0.005% solution in water
5. 2,6-dichlorophenol indophenol : 0.01% in water
6. Glucose-6-phosphate : 0.02 M

Procedure

The incubation mixture in a total volume of 3.5 ml contained: 1.0 ml of Tris-HCl buffer, 0.1 ml of magnesium chloride, 0.1 ml of NADP+, 0.5 ml of phenazine methosulphate, 0.4 ml of 2,6-dichlorophenol indophenol dye and 1.4 ml of test solution. The mixture was allowed to stand at room temperature for 10 min. The reaction was initiated by the addition of 0.5 ml of glucose-6-phosphate. The optical density was read at intervals of one min for 3-5 min at 640 nm against a water blank in a Shimadzu spectrophotometer.

The activity was obtained by multiplying the change in O.D/min by 6/17.6, which is the molar extinction co-efficient of the reduced coenzyme.
The activity was expressed as μmoles of NADPH formed/min/g of tissues under the incubation conditions.

**GLYCOGEN METABOLISM**

**ESTIMATION OF GLYCOGEN**

For the estimation of glycogen, the extraction was carried out by the method of Morales et al. (1973).

Glycogen was precipitated from the alkali extract of the tissues by adding 1:3 volume of 95% ethanol and a drop of 1 M ammonium acetate. The tubes were kept in a boiling water bath for 5 min. After cooling, the tubes were shaken well and placed in a freezer overnight. The precipitated glycogen was then collected by centrifugation at 3,000 x g for 40 min. The precipitate was dissolved in water, precipitated with alcohol and centrifuged again. The final precipitate was dissolved in 3.0 ml of water and heated for 5 min in a boiling water bath and this extract was used for the estimation of glycogen.

**Procedure**

0.5 ml glycogen extract was made up to 1.0 ml with water. A set of standard glucose solutions (25-100 µg) and blank containing water alone were set up. All the tubes were cooled in an ice-bath and 4.0 ml of anthrone reagent was added. The contents of the tubes were mixed well. All the tubes were covered with glass marbles and heated for 20 min in a boiling water bath. The
tubes were cooled and the green colour developed was read at 640 nm in a Shimadzu spectrophotometer.

Values were expressed as mg of glucose/g of wet liver tissue.

ASSAY OF GLYCOGEN SYNTHASE

Glycogen synthase was assayed by the method of Leloir and Goldenberg (1979).

Reagents

1. Glycogen solution : 40 mg/ml
2. Glycine-EDTA buffer : 0.75 M, pH 8.5
3. Glucose-6-phosphate : 0.05 M
4. Cysteine : 0.03 M solution
5. UDP glucose : 25 μM of UDPG/ml of H₂O
6. Phosphoenol pyruvate : 0.01 M in 0.14 M KCl
7. Pyruvate kinase : Freshly diluted in 0.1 M magnesium sulphate solution.
8. Dinitrophenyl hydrazine : 0.1% in 2 N HCl
10. 95% ethanol.
**Procedure**

The following components were mixed: 0.09 ml each of the glycogen, buffer, glucose-6-phosphate solution, 0.015 ml of cysteine hydrochloride solution and 0.5 ml of enzyme source. The reaction was started by the addition of 0.03 ml of UDPG and the incubation was carried out at 37°C for 5-10 min. The tubes were then heated in a boiling water bath for one min. A blank in which UDPG was added after incubation was run at the same time along with UDP standards (10-60 μg).

To estimate the UDP formed in the reaction, 0.075 ml each of phosphoenol pyruvate solution and pyruvate kinase were added. The tubes were incubated for 15 min at 37°C and 0.45 ml of dinitrophenyl hydrazine solution was then added. After 10 min, 0.60 ml of 10 N sodium hydroxide solution and 1.1 ml of ethanol were added, the contents were mixed well and centrifuged. The optical density of the supernatant fluid was read at 530 nm in a Shimadzu spectrophotometer.

The enzyme activity was expressed as μmoles of UDP formed/h/mg of protein.

**ASSAY OF GLYGOGEN PHOSPHORYLASE**

Glycogen phosphorylase was assayed by the method of Cornblath et al. (1963).
Reagents

1. Substrate : 0.05 M glucose-1-phosphate solution containing 5.7 mg glycogen/ml and 0.05 M sodium fluoride was prepared in distilled water. The pH was adjusted to 6.1 using dilute hydrochloric acid.

2. 5′ AMP : 0.025 M

3. TCA : 10% solution

4. Sodium fluoride : 0.1 N

Enzyme source was diluted with ice cold 0.1 M sodium fluoride solution just before use.

Procedure

The reaction was started by the addition of 1.0 ml of substrate, 0.1 ml of 5-AMP, 0.2 ml of sodium fluoride and 0.2 ml of enzyme. An aliquot (0.5 ml) was taken from the reaction mixture at zero time and after 10 min of incubation at 37°C, 1.0 ml of 10% TCA solution was added to arrest reaction. The contents were mixed well, centrifuged and the liberated phosphorus in the supernatant was estimated by the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as μmoles of phosphate liberated/h/mg of protein.
ESTIMATION OF LIPID PEROXIDATION

Estimation of Tissue Lipid Peroxides (TBARS)

Lipid peroxide content in tissues was determined by thiobarbituric acid reactive substances (TBARS) as described by Ohkawa et al. (1979).

Reagents

1. Glacial acetic acid: 20% solution, pH 3.5.
2. Thiobarbituric acid (TBA): A mixture of equal volume of 0.67% TBA in aqueous solution and glacial acetic acid.
3. n-butanol - pyridine mixture: 15:1 (v/v)
4. Standard solution: 16 mg of 1, 1’, 3, 3’ tetramethoxy propane was accurately weighed and dissolved in 100 ml of distilled water. Further dilution was made so that the working standard solution contains 100 μM/ml.
5. Sodium dodecyl sulphate (SDS): 8.1% solution.

Procedure

To 0.5 ml of tissue homogenate added 1.5 ml of 20% acetic acid 0.2 ml of SDS and 1.5 ml of TBA. The mixture was made up to 4.0 ml with distilled water and then heated for 60 min at 95°C using glass ball as condenser. After cooling, 4 ml of butanol-pyridine mixture was added and
shaken well. After centrifugation at 4,000 rpm for 10 min, the organic layer was taken and its absorbance was read at 532 nm. 1, 1’, 3, 3’-tetramethoxy propane was used as standard and was treated similarly as test.

The lipid peroxide concentration was expressed as μmoles of TBA reactants/100 g of tissue homogenate.

Determination of Plasma Lipid Peroxides

Plasma lipid peroxides content was estimated by the method of Yagi (1976).

Reagents

1. Sulphuric acid : 0.85 N

2. Phosphotungstic acid : 10% solution

3. TBA reagent : A mixture of equal volume of 0.67% TBA in aqueous solution and glacial acetic acid.

4. n-Butanol

5. Standard : 1,1', 3,3'-tetra methoxy propane.

Procedure

1.0 ml of plasma was mixed with 4.0 ml of 0.85 N sulphuric acid and shaken gently. To this 0.5 ml of 10% phosphotungstic acid was added and
stirred well. The contents were centrifuged for 10 min. The supernatant was discarded and the pellet was suspended in 2.0 ml of N/12 sulphuric acid and 0.3 ml of 10% phosphotungstic acid. The mixture was centrifuged for 10 min. The pellet obtained was suspended in 4.0 ml of distilled water and added 1.0 ml of TBA reagent. The tubes were kept in a boiling water bath for one hour. After cooling, 5.0 ml of n-butanol was added to each tube and the colour obtained in the butanol phase was read at 532 nm in a Shimadzu UV spectrophotometer.

The plasma lipid peroxide content was expressed as nmoles/dl of plasma.

**Estimation of Lipid Hydroperoxides**

Hydroperoxides formed due to lipid peroxidation were determined by the method of Jiang et al. (1992).

**Reagents**

1. Fox Reagent: 88 mg of butylated hydroxytoluene (BHT), 7.6 mg of xylenol orange and 9.8 mg of ammonium ion sulphate were added to 90 ml of methanol and 10 ml of 250 mM sulphuric acid.
Procedure

To 0.2 ml of tissue sample 1.8 ml of Fox reagent was added and incubated at 37°C for 30 min and the color developed was read at 560 nm spectrophotometrically.

Results were expressed as μmoles of hydroperoxides/100 g of tissue and plasma -X 10^-5 μmoles/dl.

Determination of protein carbonyl content

Protein carbonyl content was determined by the method of Uchida and Stadtman (1993).

Reagents

1. 2 M HCl

2. 10 mM 2,4-Dinitrophenylhydrazine (DNPH) in 2 M HCl

3. 20% Trichloroacetic acid (TCA)

4. 6 M Guanidine hydrochloride with 20 mM potassium phosphate adjusted to pH 2-3 with trifluoroacetic acid

5. Ethanol : Ethyl acetate (1:1 v/v)
Procedure

An aliquot (500 μl) of mixture A was treated with an equal volume of 10 mM 2,4-DNPH in 2 M HCl and incubated for 1 h at room temperature with vortexing every 10-15 min. This mixture was treated with 500 μl of 20% trichloroacetic acid (w/v, final concentration) and after centrifugation 11,000Xg for 3 min, the precipitate was extracted three times with 1 ml of ethanol/ethyl acetate (l:l, v/v). Then protein sample was dissolved with 1 ml of 6 M guanidine solution. Proteins usually redissolve within 15 min at 37°C, any insoluble material is removed by centrifugation and UV absorbance is measured at 365 nm. The carbonyl content is calculated from the maximum absorbance using a molar absorption coefficient of 22,000 M⁻¹ cm⁻¹. The level of protein carbonyls was expressed as nmoles/mg of protein.

ASSAY OF ANTIOXIDANT ENZYMES

Assay of Superoxide Dismutase

Superoxide dismutase was assayed according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme.

Reagents

1. Carbonate-bicarbonate buffer : 0.1 M, pH 10.2
2. EDTA solution : 0.6 Mm
3. Epinephrine : 1.8 mM (Prepared freshly)

4. Absolute ethanol

5. Chloroform

**Procedure**

0.1 ml of tissue homogenate was added to the tubes containing 0.75 ml of ethanol and 0.15 ml of chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant, added 0.5 ml of EDTA solution and 1 ml of buffer. The reaction was initiated by the addition of 0.5 ml of epinephrine and the increase in absorbance at 30 sec interval for 3 min was measured at 480 nm in a Shimadzu UV spectrophotometer.

One unit of superoxide dismutase activity is the amount of protein required for 50% of inhibition of epinephrine autoxidation/min.

**Assay of Catalase**

Catalase was assayed according to the method of Takahara et al. (1960).

**Reagents**

1. Phosphate buffer : 0.01 M, pH 7.0

2. Hydrogen peroxide : 0.2 mM solution in phosphate buffer
**Procedure**

To 1.2 ml of phosphate buffer, 0.5 ml of tissue homogenate was added. The enzyme reaction was started by the addition of 1.0 ml of hydrogen peroxide solution. The decrease in absorbance was measured at 240 nm for every 30 seconds up to 3 min. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide.

The enzyme activity was expressed as μmoles of hydrogen peroxide decomposed/min/mg of protein.

**Assay of Glutathione Peroxidase**

The activity of glutathione peroxidase was assayed by the method of Rotruck et al. (1973)

**Reagents**

1. Phosphate buffer : 0.4 M, pH 7.0
2. EDTA : 0.8 mM
3. Sodium azide : 10 mM
4. Reduced glutathione : 4 mM
5. H₂O₂ : 2.5 mM
6. TCA : 10% solution
7. Disodium hydrogen phosphate : 0.3 M
8. DTNB solution : 40 mg of 5, 5’-dithiobis (2-nitro benzoic acid) (DTNB) in 100 ml of 1% sodium citrate.

**Procedure**

The reaction mixture consisting of 0.2 ml of EDTA, 0.1 ml of sodium azide, 0.1 ml of H_{2}O_{2}, 0.2 ml of GSH, 0.4 ml of phosphate buffer, and 0.2 ml of homogenate was incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged at 2000 rpm. To the supernatant 3 ml of disodium hydrogen phosphate and 1.0 ml of DTNB were added and the colour developed was read at 420 nm immediately.

The activity of GPx was expressed as μmoles of glutathione oxidized/min/mg of protein.

**Assay of Glutathione-S-transferase**

Glutathione-S-transferase was assayed by the method of Habig et al. (1974).
Reagents

1. Phosphate buffer : 0.3 M, pH 6.5
2. 1 chloro-2, 4-dinitrobenzene (CDNB) : 30 mM
3. Reduced glutathione (GSH) : 30 mM

Procedure

The reaction mixture containing 1.0 ml buffer, 0.1 ml of CDNB and 0.1 ml of tissue homogenate was made up to 2.5 ml with water. The reaction mixture was preincubated at 37°C for 5 min. 0.1 ml of GSH was added and the change in OD was measured at 340 nm for 3 min at 30 seconds interval.

Activity of glutathione S-transferase was expressed as nmoles of CDNB conjugate formed/min/mg of protein.

NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) AND ACTIVITY STAINING OF SUPEROXIDE DISMUTASE (SOD) & CATALASE

Reagents

1. Stock Acrylamide Solution (30%):

   Acrylamide: 30.0 g

   Bis-Acrylamide: 0.8 g

   Made up to 100 ml with distilled water.
2. **Tris Separating Buffer (1.5 M – pH 8.8):**

   Tris base: 18.16 g in 100 ml distilled water.

3. **Tris Stacking Buffer (1.0 M – pH 6.8):**

   Tris base: 12.1 g in 100 ml distilled water.

4. **Electrophoresis Buffers:**

   (i) **Pre-Electrophoresis Running Buffer (pH 8.8):**

   Tris base: 22.76 g

   Disodium EDTA: 0.38 g

   Made upto 1000 ml with distilled water.

   (ii) **Electrophoresis Running Buffer (pH 8.3):**

   Tris base: 6.06 g

   Glycine: 22.5 g

   Disodium EDTA: 0.68 g

   Made upto 1000 ml with distilled water.

5. **10% Ammonium Per Sulphate (APS)**

6. **N,N,N′,N′ – Tetra Methyl Ethylene Diamine (TEMED)**

7. **Sample Loading Buffer:**

   Tris stacking buffer (1.0 M - pH 6.8): 10 ml

   Glycerol: 10 ml

   5% Bromo Phenol Blue: 220 µl
8. **Catalase Native Gel Stain:**

2% Ferric Chloride

2% Potassium Ferricyanide

Dissolved in distilled water each separately and mixed just before use.

9. **SOD Native Gel Stain:**

NBT: 16 mg

28 mM TEMED: 34 µl

0.14 M Stock Riboflavin 5’ Phosphate: 1.6 µl

Dissolved in 10 ml of 50 mM Potassium Phosphate Buffer (pH 7.8)

**Procedure**

**Activity Staining Of SOD**

SOD activity was detected in Liver, Kidney and Pancreatic tissues of Control, Diabetic and Treated rats. They were subjected to Non-denaturing Native PAGE in 12% gel using the buffer system of Laemmli (1970), without Sodium dodecyl sulphate (SDS) and β-mercapto ethanol at 4° C. According to Beauchamp and Fridovich (1971), negative staining with Nitro Blue Tetrazolium (NBT) localized the enzyme activity.

The gel was allowed to run in the pre-electrophoresis buffer for 1 hour at 40 mA (4° C). After 1 hour, the samples were loaded and allowed to run in electrophoresis buffer for 3 hours at 40 mA (4° C). Once the dye front
reaches the bottom, the gel was removed from the sandwich and washed with distilled water. Then the gel was placed in a staining solution for 20 minutes in dark at room temperature. Later the gel was washed with distilled water and placed under UV-Transilluminator (UV-light/Fluorescence light) until colorless zone develops. The development of achromatic bands, indicating SOD activity was visible against a uniformly developed purple background. Illumination was discontinued when maximum contrast between the achromatic zones and the background was achieved. The bandwidth indicates SOD activity.

**Activity Staining of Catalase**

Catalase activity was detected in Liver and Kidney tissues of Control, Diabetic and Treated rats. They were subjected to Non-denaturing Native PAGE in 8% gel using the buffer system of Laemmli (1970), without Sodium dodecyl sulphate (SDS) and β–mercapto ethanol at 4° C.

The gel was allowed to run in the pre-electrophoresis buffer for 1 hour at 40 mA (4° C). After 1 hour, the samples were loaded and allowed to run in electrophoresis buffer for 3 hours at 40 mA (4° C). After completion of the run, the gel was removed from the sandwich and washed with distilled water for 3 times to remove the electrode buffer completely. The gel was soaked in 0.003% Hydrogen Peroxide (H₂O₂) for 10 minutes in dark at room temperature and washed twice with distilled water for 5 minutes. The gel was then placed in staining solution for 10 minutes in dark. When achromatic
bands begin to form, the staining solution was poured off and rinsed extensively with distilled water. The catalase activity zones were visualized as faint yellow band in a dark green background.

**ESTIMATION OF NON-ENZYMATIC ANTIOXIDANTS**

**DETERMINATION OF ASCORBIC ACID**

The ascorbic acid content was determined by the method of Omaye et al. (1979).

**Reagents**

1. 5% TCA solution

2. DTC reagent: 3g of 2,4-dinitrophenyl hydrazine, 0.4 g of thiourea and 0.05 g of copper sulphate were dissolved in 100 ml of 9 N sulphuric acid

3. 65% Sulphuric acid solution (ice cold)

4. Standard ascorbic acid: To 0.5 ml of test sample, 0.5 ml of water and 1 ml of TCA were added, mixed thoroughly and centrifuged.

**Procedure**

To 1 ml of the supernatant, 0.2 ml of DTC reagent was added and incubated at 37°C for 3 h. Then 1.5 ml of sulphuric acid was added, mixed
well and the solution was allowed to stand at room temperature for another 30 min. The colour developed was read at 520 nm.

The level of ascorbic acid in plasma was expressed as mg/dl and in tissues as μg/mg of protein.

**DETERMINATION OF α-TOCOPHEROL**

α -Tocopherol content was estimated by the method of Desai (1984).

**Reagents**

1. Absolute ethanol: Redistilled after addition of potassium hydroxide and potassium permanganate.
2. Petroleum ether
3. Bathophenanthroline reagent: 0.2% solution of 4,7-diphenyl1,10-phenanthroline in purified absolute ethanol.
4. Ferric chloride reagent: 0.001 M ferric chloride solution in purified absolute ethanol. This reagent was prepared freshly before use.
5. Orthophosphoric acid reagent: 0.001 M orthophosphoric acid solution in absolute ethanol.
6. Standard vitamin E: 1 mg in 100 ml of absolute ethanol.
Procedure

To 1.0 ml of test sample added 1.0 ml of ethanol and mixed thoroughly. Then to this mixture, 3.0 ml of petroleum ether was added, shaken rapidly and centrifuged. 2 ml of supernatant was taken and evaporated to dryness. To this 0.2 ml of bathophenanthroline reagent was added. Tubes containing α-tocopherol standards were also treated exactly in the same way as the test samples. Care was taken to reduce unnecessary exposure to direct light. Then added 0.2 ml of ferric chloride reagent and vortex mixed. After 1 min, 0.2 ml of orthophosphoric acid reagent was added and mixed thoroughly and the total volume of all the tubes was made up to 3 ml with ethanol. The absorbance was read at 536 nm against the reagent blank containing ethanol.

The level of α-tocopherol in plasma was expressed as mg/dl and in tissues as µg/mg of protein.

ESTIMATION OF TOTAL REDUCED GLUTATHIONE

Total reduced glutathione was determined by the method of Sedlak and Lindsay (1968) modified according to the method of Moron et al. (1979), which is based on the reaction with 5, 5'-dithio-bis (2-nitro benzoic acid) (DTNB or Ellman's reagent) to give a yellow coloured compound that absorbs at 412 nm.
Reagents

1. 0.2 M Phosphate buffer, pH 8.0
2. 0.6 mM DTNB in 0.2 M Phosphate buffer, pH 8.0
3. 5% TCA solution
4. Standard GSH: 10 mg of GSH was dissolved in 100 ml distilled water.

0.1 ml of the test sample was precipitated with 5% TCA. The precipitate was removed by centrifugation.

Procedure

To 2 ml of the supernatant 2 ml of DTNB in 0.2 M phosphate buffer was added. The absorbance was read at 412 nm against a blank containing TCA instead of sample. A series of standards treated in a similar manner were also run to determine the glutathione content.

The amount of glutathione was expressed as mg/dl for plasma and mg/100 g of tissues.

ESTIMATION OF PLASMA CERULOPLASMIN

Plasma ceruloplasmin was estimated following the method of Ravin (1961).
Reagents

1. 0.1% \( p \)-Phenylene diamine hydrochloride solution
2. 0.1 M Acetate buffer, pH 5.2
3. 0.02% Sodium azide solution

Procedure

0.1 ml of plasma was taken into three 15 ml test tubes, one for control and two for tests respectively. 1.0 ml of 0.02% sodium azide was added to the control tube only. Then 8.0 ml of acetate buffer was added to all the tubes, followed by the addition of 1 ml of 0.1% \( p \)-phenylene diamine. The solution was mixed thoroughly and placed in a water bath at 37° C for one hour. After incubation the tubes were removed and 1 ml of sodium azide was added to each of the tubes containing test solution. The contents were shaken well and incubated at 4° C for 30 min. The intensity of the colour developed was measured at 530 nm in Shimadzu UV spectrophotometer against a reagent blank.

The ceruloplasmin activity was expressed as mg/dl of plasma.

LIPID PROFILE

Extraction of Lipids

The lipids were extracted from liver and kidney tissues by the method of Folch et al. (1957).
Reagents

1. Chloroform - methanol mixture : 2:1 (v/v)
2. Saline : 0.9% solution

Procedure

Kidney and liver tissues were dried, weighed and a known weight was homogenized with 10 ml of chloroform-methanol mixture. The homogenate was filtered through Whatman filter paper (No.42) into a separating funnel. The filtrate was mixed with 0.2 ml of physiological saline and the mixture was kept overnight undisturbed. The lower phase containing the lipid was drained off into pre-weighed beakers. The upper phase was re-extracted with more of chloroform-methanol mixture and the extracts were pooled and evaporated under vacuum at room temperature. The lipid extract was re-dissolved in 3 ml of chloroform-methanol (2:1) mixture and aliquots were taken for the estimation of cholesterol and phospholipids. The total lipid contents were calculated and expressed as mg/g of fresh tissue.

Estimation of Total Cholesterol

Cholesterol content was estimated by the method of Parekh and Jung (1970).
Reagents

1. Ferric chloride - Uranyl acetate reagent: 500 mg of ferric chloride was dissolved in 10 ml of water followed by the addition of 3 ml of concentrated ammonia and centrifuged. The precipitate was washed several times with distilled water and dissolved in one litre of glacial acetic acid. 100 mg of uranyl acetate was added to the mixture and the contents were shaken well and kept over night. The reagent was stable for six months.

2. Sulphuric acid - ferrous sulphate reagent: 100 mg of ferrous sulphate was dissolved in 100 ml of glacial acetic acid and 100 ml of sulphuric acid. After cooling to room temperature the volume was made up to 1 litre with concentrated sulphuric acid. The reagent was stable for six months.

3. Cholesterol Standard: The stock standard was prepared by dissolving 100 mg of cholesterol in 100 ml of cholesterol grade acetic acid. 10 ml of this stock standard was diluted to 100 ml with acetic acid and was used as working standard.

Procedure

About 0.1 ml of test sample was made up to 10 ml with ferric acetate - uranyl acetate reagent. 0.1 ml of the aliquot of the total lipid extract was taken and it was evaporated to dryness. The dried extract and standards were
made up to 3 ml with ferric chloride - uranyl acetate reagent. Then 2 ml of sulphuric acid - ferrous sulphate reagent was added to all the tubes and the contents were mixed well. After 20 min the colour developed was read at 540 nm using a Shimadzu UV spectrophotometer.

Total cholesterol level was expressed as mg/dl for plasma and tissue cholesterol as mg/g of fresh tissue.

**Estimation of Free Fatty Acids**

Free fatty acid content was determined by the method by Hron and Menahan (1981) with the colour reagent of Itaya (1977).

**Reagents**

1. Chloroform-heptane-methanol solvent mixture: It was prepared by mixing chloroform, heptane and methanol in the ratio of 200:150:7 (v/v).

2. Copper Triethanolamine reagent: 50 ml of 0.1 M copper nitrate and 50 ml of triethanolamine (0.2 M) were mixed with 33 g of sodium chloride. The pH of the solution was adjusted exactly to 8.1.

3. Activated silicic acid.
4. Colour reagent: 100 mg of diethyl-dithiocarbamate was diluted to 1 in 10 dilution with chloroform to give concentration of 200 μg/ml.

Procedure

To 0.1 ml of the test sample added 5.9 ml of chloroform-heptane-methanol mixture and 200 mg of activated silicic acid. The contents were shaken well and centrifuged. Standard solutions in the range of 25 to 100 μg were also pipetted out and made up to 6 ml with chloroform-heptane-methanol mixture. The blank comprised of 6.0 ml of chloroform solvent mixture only. 2.0 ml of Cu TEA reagent were added to all these samples and mixed well on a mechanical shaker for 20 min. The tubes were centrifuged to separate the two phases and 2.0 ml of the upper phase from each tube was transferred to another set of tubes. To all these tubes 1.0 ml of colour reagent was added and mixed well. The colour intensity was measured at 430 nm in a Shimadzu UV spectrophotometer.

Plasma free fatty acids were expressed as mg/dl and tissue free fatty acids as mg/g of fresh tissue.

Estimation of Triglycerides

Triacylglycerol was estimated by the method of Rice (1970). Lipids were extracted with chloroform: methanol mixture (2:1 v/v). Phospholipids present in the lipid extract were adsorbed onto silicic acid and the
triacylglycerol remaining in solution was saponified with alcoholic potassium hydroxide. The liberated glycerol were oxidized by periodate to formaldehyde and the excess oxidizing power was destroyed by reaction with sodium arsenite. The formaldehyde formed was determined by the chromotropic colour reaction.

**Reagents**

1. Chloroform - methanol mixture : 2:1 v/v
2. Saturated Sodium chloride
3. Activated silicic acid
4. Alcoholic potassium hydroxide : 400 mg of potassium hydroxide was dissolved in 100 ml of 95% ethanol.
5. Sulphuric acid : 0.2 N
6. Sodium arsenite : 0.5 M
7. Sodium metaperiodate : 0.5%
8. Chromotropic acid reagent : 1.14 g of disodium salt of chromotropic acid was dissolved in 100 ml of distilled water and 450 ml of dilute acid solution
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(prepared by mixing concentrated sulphuric acid and distilled water in the ratio of 2:1 v/v) was added and stirred well.

9. **Tripalmitin standard**: 10 mg of tripalmitin was dissolved in 100 ml of chloroform.

**Procedure**

4.0 ml of the lipid extract was added to tubes containing 8.0 ml of saturated sodium chloride and shaken vigorously. The contents were allowed to settle for one hour and then centrifuged. The supernatant saline-methanol phase was discarded. The washed chloroform phase was filtered into a dry tube, 200 mg of activated silicic acid was added to chloroform phase, shaken vigorously and allowed to stand for 30 min. After centrifugation, 0.5 ml of the supernatant as well as tripalmitin standards was evaporated to dryness. Then to the test, standard and blank tubes, 0.5 ml of alcoholic potassium hydroxide solution was added and the mixture were saponified in a 60°-70°C water bath for 20 min. 0.5 ml of 0.2 N sulphuric acid was added and heated in a boiling water bath for 10 min. After cooling the tubes, 0.1 ml of sodium metaperiodate was added and allowed to stand for 10 min. The excess periodate was reduced by the addition of 0.1 ml of sodium arsenite. Then 5.0 ml of chromotropic acid reagent was added, mixed thoroughly and kept in a boiling water bath for 30 min. After cooling, 0.5 ml of thiourea solution was
added. The colour developed was read at 540 nm against a blank in a Shimadzu spectrophotometer.

Triacylglycerol content was expressed as mg/dl in serum and mg/g in fresh tissue.

**Estimation of Phospholipids**

Phospholipid concentration was estimated by the method of Bartlett (1959) by digestion with perchloric acid and the phosphate liberated was estimated by the method of Fiske and Subbarow (1925).

**Reagents**

1. Perchloric acid

2. Ammonium molybdate reagent: 2.5% of ammonium molybdate in 3 N sulphuric acid.

3. ANSA reagent: 500 mg of ANSA was dissolved in a mixture of 195 ml of 15% sodium bisulphate. 5 ml of 20% sodium sulphite solution was added for complete solubilization. The solution was filtered and stored at 4°C in a brown bottle.

4. Standard phosphorous: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 ml of distilled water. 10 ml of this solution was made up to 100 ml with distilled water, which gives a working standard containing 8 μg of phosphorous/ml.
**Procedure**

Various aliquots of the test sample (each 0.2 ml) were digested with 0.5 ml of perchloric acid over a sandbath until the sample became colourless and clear. Standard solution of phosphorous in the range of 8 to 32 μg and a blank was mixed with perchloric acid and treated in a similar manner. The volume in all the tubes was made upto 4.3 ml with double distilled water. 0.5 ml of ammonium molybdate was added followed by 0.2 ml of ANSA reagent. The blue colour developed was read after 20 min at 640 nm.

Phosphorus content was multiplied by a factor of 25, which gave the amount of phospholipids. The values were expressed as mg/dl in plasma and mg/g in fresh tissue.

**Cholesterol in the Lipoprotein fractions**

**High Density Lipoprotein-Cholesterol (HDL-C)**

HDL-Cholesterol fraction was separated by the precipitation techniques of Burstein and Scholnick (1972) and the cholesterol content was determined by method of Parekh and Jung (1970).

**Reagents**

1. Heparin-manganese chloride - 3.167 g of manganese chloride was added to 1 ml solution of heparin containing 20,000 units. The mixture was made up to 8 ml with distilled water.
Procedure

To 1 ml of serum added 0.18 ml of heparin-manganese chloride reagent and mixed. The solution was allowed to stand at 4°C for 30 min and then centrifuged in a refrigerated centrifuge at 1800 x g for 30 min. The supernatant represented the HDL-C fraction. An aliquot of supernatant was used for cholesterol estimation.

The values were expressed as mg/dl.

Very Low Density Lipoprotein-Cholesterol (VLDL-C)

VLDL-cholesterol was calculated using the following equation (Friedewald et al., 1972).

\[
\text{VLDL-C} = \frac{\text{Triglycerides}}{5}
\]

The values were expressed as mg/dl.

Low Density Lipoprotein-Cholesterol (LDL-C)

LDL-C was calculated using the following equation:

\[
\text{LDL-C} = \text{Total cholesterol} - (\text{HDL-C} + \text{VLDL-C})
\]

The values were expressed as mg/dl.
STATISTICAL ANALYSIS

The values were expressed as mean ± S.E.M for six rats in each group. All data were analyzed with SPSS/16.0 student software. Hypothesis testing method included one way analysis of variance (ANOVA) followed by post hoc testing performed with least significant difference (LSD) test. A Value of P < 0.05, was considered as significant.
RESULTS AND DISCUSSION

Dosage fixation studies

Acute toxicity studies using graded doses of the *P. dulce* pod pulp extract revealed no signs and symptoms such as restlessness, respiratory distress, diarrhoea, convulsions, and coma. The pod pulp extract showed optimum activity at 300 mg/kg b.w and further increase in pulp extract dose did not result in a further significant decrease in blood glucose levels, thus it appears that unlike insulin and other oral hypoglycemic drugs, overdose of the pod pulp extract may not cause hypoglycemia.

The activities of pathophysiological enzymes such as AST, ALT, ALP, and LDH were assessed in the control and experimental groups of rats to validate the non-toxic nature of the pod pulp. Significant decrease in the activities of AST, ALT, ALP and LDH were observed in diabetic group of rats treated with pod pulp extract at a dosage of 300 mg/kg body weight for 30 days.

Phytochemical analysis of *pod pulp* extract

Diabetes is a metabolic disorder that afflicts a major proportion of the population globally. The treatment of diabetes mellitus is based on the variable use and combination of diet, antidiabetic oral agents (metformin, sulphonylureas, acarbose and thiazolidinediones) and insulin or its analogs, depending on the type and severity of diabetes. The conventional therapies for diabetes have many shortcomings like undesirable side effects and high rate
of secondary complications. In general, there is very little scientific knowledge on the specific modes of action in the treatment of diabetes, but most of the plants have been found to contain active ingredients such as flavonoids, alkaloids, glycosides, terpenoids, etc., which possess antidiabetic effects (Loew and Kaszkin, 2002; Jaiswal et al., 2009).

Phytochemical analysis of the pod pulp extract revealed the presence of flavonoids, alkaloids, glycosides, polyphenols, tannins, saponins, phytosterols and triterpenes in the pulp extract (Table 1). Phytoingredients based strategies play a pivotal role in the prevention and treatment of diabetes (Chang et al., 2013). Polyphenolic compounds such as flavonoids contribute to increased plasma antioxidant capacity, decreased oxidative stress markers and reduced total and LDL cholesterol (Hanhineva et al., 2010). Growing evidence indicates that various dietary polyphenols may influence carbohydrate metabolism in many levels (Soory, 2012).

Phytochemicals include compounds with various biological properties which allow plants to cope up with environmental challenges including exposure to radiation and toxins (Huffman, 2003). The bioactive compounds (secondary metabolites) found in plants works with nutrients and dietary fibers synergistically to protect against diseases (Craig and Beck, 1999). Thus the presence of the biologically active ingredients in the pod pulp extract may account for the observed pharmacological as well as beneficial actions.
The total phenolic and flavonoid content were found to be 2.52 ± 0.09 mg/g equivalents of gallic acid and 5.13 ± 0.11 mg/g equivalents of quercitin respectively. Recent studies indicate that appreciable amounts of total phenolic, flavonoid content was present in *P. dulce* pod pulp peel, seeds and leaves (Sukantha et al., 2011; Nagmoti and Juvekar 2013; Katekhaye and Kale, 2012). From the data obtained through HPLC analysis, it is evident that *P. dulce* pod pulp extract was found to contain biologically important flavonoids such as Rutin, Quercetin, Myricetin, Luteolin, Apigenin and Kaempferol (Fig 1 and 2).

**STZ induced diabetes mellitus**

Animal models of diabetes such as genetically derived, nutrition induced, and chemically induced have been used extensively in diabetes research. Alloxan and streptozotocin are widely used chemicals in the diabetic research to induce diabetes in experimental animals. However, STZ induced diabetes exhibits many characteristic features similar to human diabetes (Srinivasan and Ramarao, 2005). Streptozotocin is specifically cytotoxic to β-cells of the pancreas. The mechanism behind its action is that STZ is preferentially up taken by pancreatic beta cell via GLUT2 transporter and causes DNA alkylation followed by the activation of poly ADP ribose polymerase leading to depletion of NAD$^+$ and ATP. Enhanced ATP dephosphorylation after STZ treatment supplies substrate for xanthine oxidase resulting in the formation of superoxide radicals and also nitric oxide moiety is liberated. As a net result, destruction of β-cells occurs by necrosis.
(Szkudelski, 2001). However, the dose of streptozotocin required for inducing diabetes depends on the animal species, route of administration, dosage, duration and nutritional status.

According to the administered dose of streptozotocin, features similar to either type 1, or type 2 diabetes can be induced (Mythili et al., 2004). The intraperitoneal administration of single low dose of STZ (45mg/kg) induces moderate diabetes by selectively destroying the pancreatic beta cells (Yamabe et al., 2010). Hence, streptozotocin-induced experimental diabetes in rats was chosen in the present study.

**Changes in body weight gain in control and experimental groups of rats**

Figure 3 shows the changes in body weight gain in control and experimental groups of rats. The body weight of control group of rats was gradually increased throughout the experimental period. The body weight was significantly (p < 0.05) decreased in STZ-induced diabetic rats when compared to control rats. Oral administration of pod pulp extract as well as gliclazide to STZ-induced diabetic rats significantly (p < 0.05) increased the body weight to near normalcy.

STZ-induced diabetes is characterized by a severe loss of body weight, which is due to increased catabolism of tissue proteins and due to unavailability of carbohydrate as energy source as a result of deficient insulin secretion leading to significant reduction in the body weight gain of diabetic rats, which was observed in the present study (Pepato et al., 1991). A
significant increase in the body weight observed in diabetic rats treated with pod pulp extract indicates the beneficial effect of the extract in controlling muscle wasting (Pradeepa et al., 2013).

**Effect of *P. dulce* pod pulp extract on experimental rats after receiving oral glucose challenge**

Figure 4 shows the changes on glucose tolerance curve in control and experimental groups of rats. The blood glucose level in the control rats was increased to a peak value at 60 min after oral glucose load and decreased to near normal level at 120 min. In STZ-induced diabetic rats the peak increase in blood glucose concentration was observed after 60 min and remained high over the next 60 min. Oral administration of pod pulp extract as well as gliclazide on STZ-induced diabetic rats showed significant \((p < 0.05)\) decrease in blood glucose concentration at 60 and 120 min when compared with diabetic control suggesting the glucose lowering properties of the pod pulp extract as well as gliclazide.

The oral glucose tolerance test (OGTT) measures the body's ability to utilize glucose, the body's main source of energy. An oral glucose tolerance test is a perceptive measure of early abnormalities in glucose regulation than fasting plasma glucose (Singleton et al., 2003). Impaired glucose tolerance due to pancreatic dysfunction results in the defective utilization of glucose by the tissues and increased hepatic gluconeogenesis (Robertson, 2007). The impaired glucose tolerance observed in STZ induced diabetic group of rats
were altered to near normal upon treatment with pod pulp extract which indicates improved glucose homeostasis (Pradeepa et al., 2013).

**Effect of *P.dulce* pod pulp extract on basic biochemical parameters in experimental diabetes in rats**

The levels of fasting blood glucose, hemoglobin, glycosylated hemoglobin, plasma insulin, and urine sugar of control and experimental groups of rats are shown in Table 2. Control rats did not show any significant variation in the blood glucose throughout the experimental period. Administration of STZ led to hyperglycemia, which was maintained over the experimental period. In experimental rats, the levels of blood glucose, glycosylated hemoglobin was increased and the levels of hemoglobin, plasma insulin were decreased when compared with the control group of rats. Upon oral administration of pod pulp extract as well as gliclazide, these levels were found to be similar to those of normal rats and the effect was more distinct in the group of rats treated with pod pulp extract. Urine sugar present in diabetic rats was found to be absent in the rats treated with pod pulp extract as well as gliclazide.

Persistent hyperglycemia is considered as a main factor in the development and progression of the complications of diabetes mellitus (Luzi, 1998). STZ induced diabetes leads to beta cell necrosis resulting in insulin deficiency and decreased utilization of glucose by the peripheral tissues contributes to hyperglycemia (Giugliano et al., 1996). Also, hyperglycemia and renal glycosuria are the most critical abnormalities in diabetes. Therefore,
the hypoglycemic effect and consequent decrease in urine sugar excretion have been considered as one of the essential characteristics of anti-diabetic agents (Ojewole, 2002). In the present study, oral administration of pod pulp extract to diabetic rats showed the absence of urine sugar due to the normalization of blood glucose levels. The reduction in blood glucose level accompanied with the absence of glucose in urine (glycosuria) indicates the hypoglycemic nature of the pod pulp extract.

Glycated hemoglobin or βN-1-deoxyfructosyl-hemoglobin (HbA1c) is the product of a nonenzymatic reaction of glycation, namely a condensation between the aldehyde group of glucose and the amino group of the terminal valine in the β-chain of hemoglobin A₀. The amount of HbA1c is strictly related to blood glucose concentration. Considering the average life span of red cells, the HbA1c value should mimic the mean glycemic value of the previous 2–3 months. The American Diabetes Association (ADA) recommends HbA1c determination in patients with diabetes mellitus on therapy in order to monitor the glyco-metabolic status in the medium–long term and thus reduce the risk of vascular complications (ADA, 2010). During diabetes, the excess of glucose present in the blood reacts irreversibly with amino groups of lysine residues in Hb to form HbA1c. Diabetic rats showed higher levels of HbA1c indicating their poor glycemic control. Oral administration of pod pulp extract to diabetic rats decreased the levels of glycosylated hemoglobin by virtue of its hypoglycemic activity. This
normalization of HbA1c indicates decreased glycation of proteins and confirms the anti-diabetic potential of pod pulp extract (Pradeepa et al., 2013).

Chemically induced diabetes causes a notable reduction in insulin release by the destruction of the pancreatic β-cells and thereby induces hyperglycemia (Schein, 1973). In the present study, the decreased plasma insulin content was elevated upon treatment with the pod pulp extract. The possible mechanism by which *P. dulce* pod pulp extract brings about its antidiabetic action may be by potentiating the insulin effect through the stimulation of insulin release from remnant pancreatic β cells or its release from the bound form. In the above context a number of other plants have also shown similar mechanism (Arulselvan et al., 2006; Mahadeva Rao and Subramanian, 2009; Subramanian et al., 2011).

Table 3 shows the levels of total proteins, blood urea, serum uric acid and serum creatinine of control and experimental groups of rats. The level of total protein was found to be decreased in STZ induced diabetic rats. The levels of blood urea, serum uric acid and serum creatinine were found to be elevated in STZ induced diabetic rats. These biochemical markers were reverted back to near normalcy upon the oral administration of the pod pulp extract.

Defect in insulin action/secretion leads to defective amino acid/protein metabolism, which may be a more crucial factor than hyperglycemia in the etiology of some diabetic complications (Rosenlund,
Experimentally induced diabetes in rat model indicates several alterations of amino acid metabolism, which may be attributed to increased muscle proteolysis, reduced protein synthesis, an energy-dependent process in the liver, and stimulated hepatic gluconeogenesis utilizing gluconeogenic amino acids (Fando et al., 1985). This readily accounts for observed decrease in the total protein content in STZ-induced rats. Administration of pod pulp extract to diabetic rats significantly inhibits proteolysis caused by insulin deficiency and improves total protein level.

The amount of urea in the blood is increased with concomitant decrease in plasma protein levels in experimental diabetes as a result of increased breakdown of plasma and tissue proteins due to negative nitrogen balance. Further, the supraphysiological concentration of glucose in diabetic state causes severe derangement in protein metabolism that result in the development of negative nitrogen balance. This in turn elevates urea and creatinine levels (Asayama et al., 1994) which acts as biochemical diagnostic markers for assessing renal impairment and drug-induced toxicity (Braunlich et al., 1997). Serum creatinine concentration is often used as a variable not only to assess impairment of kidney function but also as clinical end point to detect treatment related toxic effects of compounds on the kidney in experimental animals (Perrone, 1992). The observed alteration in the levels of blood urea and serum creatinine in group of diabetic rats reverted to near normalcy by treatment with pod pulp extract, indicating renal protective nature of the extract during glucose toxicity.
The level of purines is elevated due to accelerated muscle wasting. These accumulated purines are the main source for the production of uric acid by the activity of xanthine oxidase (Anwar and Meki, 2003). This accumulated purines evidence the increased oxidative stress which is closely related to diabetes and its vascular complications. In the present study, the increased levels of serum uric acid observed in diabetic rats were restored to near normalcy by the administration of pod pulp extract indicating the free radical scavenging activity of pod pulp.

**Effect of *P. dulce* pod pulp extract on the activities of pathophysiological enzymes in experimental groups of rats**

The activities of AST, ALT and ALP in the serum of control and experimental groups were presented in Table 4. A significant (p<0.05) elevation in the levels of AST and ALT were noted in serum of STZ induced diabetic rats. Oral administration of pod pulp extract brought down the activity of AST, ALT and ALP to near normal in serum of diabetic rats.

Transaminases such as ALT and AST are the intracellular cytosolic enzymes that have leaked into the circulation and serve as a marker of tissue injury chiefly hepatocyte as well as renal injury. ALP acts as a marker of biliary function and cholestasis. It is hypothesized that elevation in the levels of serum ALT, AST and ALP are considered as predictors of diabetes. The cytosolic enzymes AST, ALT and membrane bound ALP are the physiological markers normally present in low levels in serum and their activities elevated during tissue damage. A rise in ALT activity indicates the
hepatocellular damage followed by cardiac tissue damage and is usually accompanied by a rise in AST activity. Further, ALP is a marker of biliary function and cholestasis. The observed increase in activities of these enzymes in the serum of diabetic rats may be due to the leakage of these enzymes from the liver cytosol into blood stream as a consequence of the hepatic tissue damage (El-Demerdash et al., 2005). The reversal of AST, ALT and ALP activities in the pod pulp extract treated diabetic rats towards near normalcy indicate the non toxic as well as hepatoprotective nature of the pod pulp (Pradeepa et al., 2013).

**Modulatory effects of pod pulp extract on the activities of key enzymes of carbohydrate metabolism**

The level of plasma glucose is maintained in a narrow range during periods of fasting and feeding in normal subjects. This homeostasis is the net balance between glucose absorption from the intestine, production from the liver and kidney and glucose uptake and utilization/storage by the tissues of the body. Indeed, the liver has been shown to play a central role in the maintenance of glucose homeostasis (Nordlie et al., 1999) with a minor contribution to kidney.

Tables 5 depict the effect of pod pulp extract on the activities of glucokinase and pyruvate kinase in liver tissues of control and experimental groups of rats. The activities of glucokinase and pyruvate kinase were significantly diminished in liver tissues of streptozotocin induced diabetic rats. Oral administration of pod pulp extract to diabetic rats altered the
activities of these to near normalcy in liver tissues similar to gliclazide treated rats.

Glucokinase (GK) is the isoenzyme in the liver and it plays a key role in glucose homeostasis. GK is a potential target for new treatment strategies for the management of type 2 diabetes, as has recently been reviewed (Coghlan and Leighton, 2008; Matschinsky, 2009). The observed increased in the activity of GK in pod pulp extract treated diabetic rats indicates the effective utilization of glucose for oxidation. The activation of GK in mice with high-fat diet-induced insulin resistance improves islet function and normalizes glucose tolerance in mice (Winzell et al., 2011).

Pyruvate kinase (PK) is a ubiquitously expressed, rate-controlling, terminal, key glycolytic enzyme that catalyzes the conversion of phosphoenolpyruvate to pyruvate with the generation of ATP. The altered activity during diabetic conditions could be expected to diminish the metabolism of glucose and ATP production. Hence, the observed decline in the activity of PK in the liver tissues of diabetic rats promptly responsible for the reduced glycolysis and amplified gluconeogenesis signifying that these two pathways are distorted in diabetes.

Table 6 depicts the activities of glucose-6-phosphatase, fructose-1, 6-bisphosphatase and glucose-6-phosphate dehydrogenase in liver tissues of control and experimental groups of rats. The liver tissues of diabetic rats showed a significant (p < 0.05) elevation in the activities of glucose-6-
phosphatase and fructose-1, 6-bisphosphatase and a significant (p < 0.05) decrease in the activity of glucose-6-phosphate dehydrogenase. The altered activities of these enzymes were reverted to near normalcy by treatment with pod pulp extract as well as gliclazide in diabetic groups of rats.

Glucose-6-phosphatase, a key enzyme in the homeostatic regulation of blood glucose catalyzes the dephosphorylation of glucose-6-phosphate to free glucose as the final step in gluconeogenesis and glycogenolysis (Roden and Bernroider, 2003). The activity of glucose-6-phosphatase is stimulated by cAMP and repressed by insulin.

Fructose-1, 6-bisphosphatase is another gluconeogenic enzyme which catalyzes the dephosphorylation of fructose-1, 6- bisphosphate to fructose-6-phosphate in the gluconeogenic pathway (Pilkis and Claus 1991). The increased activity of fructose-1, 6-bisphosphatase has been observed in experimental animal models of diabetes, insulin resistance and obesity and suggests a principal role for fructose-1, 6-bisphosphatase in the gluconeogenic flux. In the present study, the reduced activities of both glucose-6-phosphatase and fructose-1, 6-bisphosphatase in hepatic tissues of diabetic rats upon oral administration of pod pulp extract reveal the reduced endogenous glucose production there by plays a crucial role in maintaining the fasting blood glucose level.

Glucose-6-phosphate dehydrogenase catalyzes the rate-limiting step of the hexose monophosphate shunt and produces NADPH required for the
maintenance of the levels of reduced glutathione, a non enzymatic antioxidant (Xu et al., 2005). The observed decrease in the activity of glucose- 6-phosphate dehydrogenase in the hepatic tissues of diabetic rats illustrate a decreased metabolism via the phosphogluconate oxidation pathway.

The glycogen content in the liver and muscle tissues is presented in Figure 5. There was a significant decrease in glycogen content in both the liver and muscle tissues of STZ-diabetic rats compared with control rats. Oral administration of pod pulp extract as well as gliclazide to diabetic rats for 30 days significantly improved glycogen content in both the liver and muscle tissues compared with untreated diabetic rats.

Glycogen is a storage form of carbohydrates in vertebrates. The excess glucose is converted into glycogen and stored as an energy fuel in tissues, predominantly in the liver and skeletal muscle (Thoburn et al., 1991). During diabetes, there is a decrease in liver weight due to enhanced catabolic processes such as glycogenolysis, lipolysis and proteolysis (Naresha et al., 2012) and therefore the quantification of glycogen, the primary intracellular storage form of glucose in liver can be considered as an important indicator of diabetes mellitus. A significant decline in the glycogen level was observed in diabetic group of rats. Oral treatment with pod pulp extract as well as gliclazide to diabetic rats restored the level of glycogen indicating the improved glucose homeostasis.
Table 7 represents the activities of glycogen synthase and glycogen phosphorylase in liver of control and experimental groups of rats. A significant (P < 0.05) decline in the glycogen level as well as in the glycogen synthase activity and a concomitant increase in the activity of glycogen phosphorylase were noted in the liver of diabetic group of rats. Oral treatment with pod pulp extract as well as gliclazide to diabetic rats restored the activities of glycogen synthase, glycogen phosphorylase to near normalcy when compared to control group of rats.

Glycogen synthase is a crucial and rate-limiting enzyme which catalyzes the transfer of glucose from UDP-glucose to glycogen. Glycogen phosphorylase is a rate-limiting enzyme of glycogenolysis and is regulated by phosphorylation and by allosteric binding of AMP, ATP, glucose-6-phosphate and glucose (Greenberg et al., 2006). During diabetic conditions, the glycogen levels, glycogen synthase activity and responsiveness to insulin signaling are diminished and glycogen phosphorylase activity is significantly increased. Oral administration of pod pulp extract to diabetic rats restored the glycogen content and the activities of glycogen metabolizing enzymes demonstrating the possible role of pod pulp in the regulation of glycogen metabolism.

Glycogen phosphorylase, a rate-limiting enzyme of glycogenolysis, cleaves α (1→4) linkage to remove glucose molecules from the glycogen. During diabetic conditions, the glycogen levels, glycogen synthase activity and responsiveness to insulin signaling are diminished and glycogen phosphorylase activity is significantly increased (Parker et al., 2004). Oral
administration of pod pulp to diabetic rats regulated the activity of glycogen metabolizing enzymes and thereby normalized the altered glycogen content.

Thus, the oral administration of pod pulp extract to STZ induced diabetic rats brings about the normoglycemia by regulating the key enzymes of carbohydrate as well as glycogen metabolism.

Effect of *P. dulce* pod pulp extract on lipid peroxidation status in streptozotocin- induced diabetes in rats

Oxidative stress occurs as a result of increased ROS generation and/or declined antioxidant system. Oxidative stress is a biological entity quoted as accountable for several pathological conditions including diabetes mellitus. Chronic hyperglycemia in diabetes is associated with oxidative stress mediated tissue damage.

Tables 8, 9, 10 and 11 exemplify the levels of lipid peroxides, hydroperoxides and protein carbonyls in plasma, pancreatic, hepatic and kidney tissues of control and experimental groups of rats. The significant increase noted on the levels of lipid peroxides, hydroperoxides and protein carbonyls in plasma, pancreatic, hepatic and kidney tissues of diabetic group of rats were declined significantly to near normalcy by the treatment of *P. dulce* pod pulp extract as well as gliclazide to diabetic groups of rats.

Streptozotocin induction uniformly results in an increase in lipid peroxidation (TBARS), an indirect evidence of intensified free radical production (Maritim et al., 2003). Most of the tissue damage is considered to
be mediated by these free radicals by attacking membranes through peroxidation of unsaturated fatty acids (Stringer et al., 1989). Lipid peroxidation is a critical biomarker of free radical-mediated oxidative stress. It is also an important pathological indicator in many diseases such as cancer and diabetes. Consequently, mechanisms in the formation of lipid hydroperoxides and biologically active metabolites, together with their effect on cellular structure and function, are of increasing importance to the study of diabetogenesis (Matough et al., 2012). Lipid peroxides, hydroperoxides and protein carbonyls are the secondary products of oxidative stress and are unleashed as a result of the toxic effect of reactive oxygen species produced during lipid peroxidation in diabetes (Evans et al., 2002). The concentration of lipid peroxidation products may reflect the degree of oxidative stress in diabetes (Baynes, 1991). In the present study, the increased levels of lipid peroxides, hydroperoxides and protein carbonyls in plasma and tissues of diabetic rats were observed. Oral administration of *P. dulce* pod pulp extract to diabetic group of rats significantly normalized these levels indicating the *P. dulce* pod pulp extract possess anti-lipid peroxidative property in diabetic state. The observed normalization substantiates the antioxidant and free radical quenching nature of *P. dulce* pod pulp.

**Effect of *P. dulce* pod pulp extract on antioxidant status of experimental groups of rats**

Persistent hyperglycemia causes increased oxidative stress, which contributes to the initiation and progression of most of the diabetes-associated...
complications. During oxidative stress, endogenous immunomodulatory mechanisms, enzymes and antioxidant molecules are deployed to destroy reactive oxygen species and prevent the harmful effects of oxidants. In normal physiological conditions, these mechanisms are sufficient to counteract free radical production, but in diabetes, they are overwhelmed because of an increased oxidative stress.

Hyperglycemia induces free radicals and it impairs the endogenous antioxidant defense system in patients with diabetes. The natural antioxidant system consists of numerous antioxidants and several antioxidant enzymes such as SOD, CAT and GPX. The primary ROS produced in the aerobic organisms is superoxide radical that is a highly reactive cytotoxic agent. Superoxide radical is converted to \( \text{H}_2\text{O}_2 \) by SOD. \( \text{H}_2\text{O}_2 \), in turn, is converted to molecular oxygen and \( \text{H}_2\text{O} \) by either CAT or GPX. Additionally, GPX can reduce lipid peroxides and other organic hydroperoxides that are highly cytotoxic products. Thus SOD, CAT and GPX constitute the principal components of the antioxidant system and their deficiencies can cause oxidative stress.

**Pancreatic tissues**

Table 12 depicts the activities of enzymatic antioxidants such as Superoxide Dismutase (SOD), catalase, GPx and GST in pancreatic tissues of control and experimental groups of rats. The activities were significantly diminished in the pancreatic tissues of diabetic group of rats. Oral treatment
of *P. dulce* pod pulp extract significantly attenuated the altered activities of these enzymatic antioxidants to near normalcy in pancreatic tissues of diabetic rats.

The reduced antioxidant capacity potentially makes pancreatic β-cells sensitive to ROS mediated signal transduction and cellular response. The sensitivity of pancreatic β-cells to oxidative stress has been attributed to their low content of antioxidants compared with other tissues. Thus, the protection of β-cell against oxidative damage might delay the onset of diabetes as well as the progression of its complications. Oral administration of *P. dulce* pod pulp extract to diabetic group rats significantly recuperates the activities of enzymatic antioxidants signifying the antioxidant property of *P. dulce* pod pulp.

Table 13 and 14 represent the effect of *P. dulce* pod pulp extract on the activities of enzymatic antioxidants such as SOD, catalase, GPx, GST and GR in hepatic and renal tissues of control and experimental groups of rats. The activities were significantly diminished in the hepatic and renal tissues of diabetic group of rats. Oral treatment of *P. dulce* pod pulp extract similar to that of gliclazide, significantly attenuated the altered activities of these enzymatic antioxidants to near normalcy in hepatic and renal tissues of diabetic rats.

Liver is the pivotal organ of oxidative and detoxifying processes as well as free radical reactions and the biomarkers of oxidative stress are
elevated in the liver at an early stage in many diseases, including diabetes mellitus (Stadler et al., 2003). In experimental diabetes, streptozotocin exerts its toxic effects on liver and other organs in addition to pancreatic β cells. The insulin insufficiency and hyperglycemia that result from β cell necrosis further augment liver damage through reactive free radicals mediated lipid peroxidation of hepatocellular membrane (Kume et al., 2004).

The endogenous antioxidant enzymes such as SOD, CAT and GPx are responsible for the detoxification of deleterious oxygen radicals. SOD has been postulated as one of the most important enzymes in the enzymatic antioxidant defense system which catalyses the dismutation of superoxide radicals to produce H$_2$O$_2$ and molecular oxygen hence diminishing the toxic effects caused by these radicals. The reduction in SOD and catalase activities in diabetic condition may be due to direct glycation of enzyme protein (Yan and Harding, 1999).

Catalase is a hemeprotein which catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals. The superoxide anion has been known to inactivate CAT, which is involved in the detoxification of hydrogen peroxide. Hydrogen peroxide toxicity is traced to the formation of reactive hydroxyl radical upon capture of an electron from Fe (II) or Cu (I). The hydroxyl radical reacts instantly with all cellular components resulting in the modifications of proteins and nucleic acids. In diabetic conditions, the elevated production of hydrogen peroxide due to the
autoxidation of glucose, protein glycation and LPO leads to a marked decline in the catalase activity (Mate’s and Sanchez- Jimenez, 1999).

GPx, a selenium-containing peroxidase is concerned in the detoxification of hydrogen peroxide and lipid peroxide by using GSH as a hydrogen donor and acts as a peroxynitrite reductase (Sies et al., 1997). Hyperglycemia is known to greatly increase oxidative stress through diverse mechanisms; the defective antioxidant function of GPx is a hallmark in the diabetic state. GST belongs to a group of multifunctional detoxification enzymes, which protect cells against a wide assortment of toxic insults from chemicals, metabolites, and oxidative stress (Wang et al., 2006). The GST catalyzes the conjugation of glutathione to a wide range of electrophiles and support a protective mechanism against hyperglycemia mediated oxidative stress. The GST is critical in the protection of cells from reactive species because they utilize a wide variety of products of oxidative stress as substrates (Bekris et al., 2005).

The levels of activities of enzymatic antioxidants such as SOD, CAT, GPx and GST in the pancreatic, hepatic and renal tissues were significantly improved in the podpulp extract treated diabetic rats indicating the antioxidant potential of P. dulce pod pulp.

The activity of these antioxidant enzymes (SOD and catalase) were also observed by means of activity staining using native PAGE, where a similar results were obtained in which the activity was determined by
observing the band width [Plate 3 A-B and Plate 4 A-B]. In diabetic rats, the width of the band is less compared to normal; whereas in treated rats, it showed a normal band width as that of normal rats. Thus, the pod pulp extract treated rats showed an increased activity of SOD and catalase, preventing the accumulation of free radicals.

**Non enzymatic antioxidants**

The levels of plasma non-enzymatic antioxidants such as vitamin C, vitamin E and ceruloplasmin, GSH are represented in Table 15. Diabetic group of rats showed a significant decrease in these levels when compared with control group of rats. Conversely, administration of *P. dulce* pod pulp extract as well as gliclazide to diabetic group of rats significantly increased the levels to near control values.

Vitamin C, a hydrophilic antioxidant, has the ability to sequester the singlet oxygen radical, stabilize the hydroxyl radical and regenerate reduced vitamin E back to the active state. Vitamin E, a lipophilic antioxidant, transfers its phenolic hydrogen to a peroxyl free radical of peroxidized polyunsaturated fatty acids, thereby breaking the radical chain reaction and averting the peroxidation of membrane lipids (Opara, 2002). GSH is a chief intracellular redox buffer that functions as a direct free radical scavenger, cosubstrate for GPx activity and cofactor for many enzymes. It is also involved in the maintenance of exogenous antioxidants such as vitamins E and vitamin C in their active states. The maintenance of normal ratio of GSH
to oxidized glutathione requires NADPH. The cellular demand for NAPDH is obviously increased, when the level of GSH is decreased and the level of oxidized glutathione is increased. This necessitates an increase in glucose oxidation via the pentose phosphate cycle. During insulin deficiency the level of intracellular NADPH is declined because of defective glucose oxidation thereby the level of GSH is decreased (Maritim, 2003). The notable decline in the key cellular non-enzymatic antioxidant defense system extensively provokes the hepatocytes susceptibility to oxidative stress (Hasanain and Mooradian, 2002).

The declined levels of the non enzymatic antioxidants due to the increased production of free radicals during hyperglycemia mediated oxidative stress. Oral administration of *P.dulce* pod pulp extract to diabetic group of rats showed improved status of non-enzymatic antioxidants suggesting the free radical scavenging potential of the pod pulp. Recently it has been reported that the antioxidant activity of *P. dulce* may be endorsed to its high phenolic content (Dyaneshwar et al., 2012). *P.dulce* possess significant amounts of phenols and flavonoids (Pradeepa et al., 2014).

**Pancreatic tissue protective nature of *P.dulce* pod pulp extract**

Plate 5 (A–D) represents the photomicrographs of hematoxylin eosin staining of pancreatic tissues of control and experimental groups of rats. Plate 5A shows the section of pancreatic tissue of control rats showing normal islets. Plate 5B shows the section pancreatic tissues of diabetic group of rats
showing the extensive degenerative changes of islets. It is characterized by reduction in the number and size of islets. The central areas of most pancreatic islets are completely empty when compared to those of control groups. Plate 5C reveal the section of pancreatic tissues of diabetic group of rats treated with *P. dulce* pod pulp extract presenting with less marked β-cells degeneration than that of diabetic group of rats. Several β-cells are well granulated and increased in islets when compared to the pancreatic tissues of diabetic rats. Similarly, the pancreatic tissues of diabetic rats treated with gliclazide shows the improved number of granulated cells in islets because of β-cell proliferation (Plate 5D).

The ultrastructural observations in the pancreatic β-cells of control and experimental groups of rats is depicted in Plate 6A-D. Plate 6A represents the electron micrograph of pancreatic β-cell of control group of rats showing normal cellular organelles such as mitochondria, endoplasmic reticulum, Golgi complex and large number of secretory granules. The granules were diffusely distributed in the cytoplasm. The electron micrograph of pancreatic β-cell of diabetic group of rats (Plate 6 B) revealed the destruction of β-cell with loss of nuclear envelope and mitochondrial cristae, vacuolization with ballooning appearance of mitochondria as well as dilation of the rough endoplasmic reticulum. A decrease in the secretory granules of β-cell of the diabetic group of rats is also observed (Plate 6B). The electron micrograph (Plate 6C) apparently shows the pancreatic β-cell protective nature of *P. dulce* pod pulp extract in diabetic group of rats by means of moderate increase in
secretory granules, minimal nuclear membrane damage, and minimal loss in the cristae with weak swelling of mitochondria. The electron micrograph of β-cell of diabetic group of rats treated with gliclazide shows similar pattern of β-cell protection (Plate 6D). Thus, pancreatic tissue protective nature of *P. dulce* pod pulp extract was evidenced by histopathological as well as ultrastructural studies, respectively.

**Hepatoprotective nature of *P. dulce* pod pulp extract**

Plate 7 A–D represents the histological observations of hematoxylin–eosin staining of hepatic tissues of control and experimental groups of rats. Plate 7A shows the section of hepatic tissue of control rats exhibiting a concentric arrangement of the hepatocytes with sinusoidal cards around the central vein and portal tracts. The portal tracts show portal triad with portal vein, hepatic artery and bile duct. Plate 7B portrays the section of hepatic tissues of diabetic group of rats exhibiting distortion in the arrangement of hepatocytes around the central vein, fatty infiltration, congestion of sinusoids around central vein regions, granular degeneration, microvesicular vacuolization, hyperemia in the sinusoids and portal tract inflammation. Plate 7C demonstrates the section of hepatic tissues of diabetic group of rats treated with *P. dulce* pod pulp extract presenting the normal hepatocytes arrangement around the central vein with abridged necrosis, declined fat accumulation and mild sinusoidal dilatation with decreased number of Kupffer cells. Similarly, the hepatic tissues of diabetic rats treated with gliclazide shows normal appearance of hepatocytes arrangement (Plate 7D).
The ultrastructural observations in hepatocytes of control and experimental groups of rats are shown in Plate 8A-D. Plate 8A represents the electron micrograph of hepatocyte of control group of rats showing the normal cellular organelles, mitochondria, rough endoplasmic reticulum, golgi complex, nucleus with intact nuclear membrane and nuclear chromatin. The electron micrograph of hepatocyte of STZ induced diabetic rats (Plate 8B) revealed the decrease of organelles regeneration, swelling in the cisternae of the rough endoplasmic reticulum and mitochondrial cristae, fusion or disappearance of mitochondrial crests, degranulation of rough endoplasmic reticulum, damaged nuclear membrane, increased smooth endoplasmic reticulum and fat accumulation. The electron micrograph (Plate 8C) illustrates the hepato-protective nature of *P. dulce* pod pulp extract in diabetic group of rats which is evident from normal appearance of nuclear membrane and chromatin, significant reduction in the swelling in the cisternae of the rough endoplasmic reticulum and mitochondrial cristae and reduction of smooth endoplasmic reticulum. The electron micrograph of hepatocyte of diabetic group of rats treated with gliclazide shows similar pattern of hepatocyte protection (Plate 8D).

The hepato protective nature of *P. dulce* pod pulp extract in experimental groups of rats was further evidenced from histological as well as ultrastructural studies, respectively. The major pathological alterations observed in the diabetic liver were the swelling of the capillaries and the thickening of the capillary walls. The diabetic liver sections revealed the
augmented fibrosis with plasmacytic infiltrate triggering distortion of typical
concentric arrangement of hepatocytes around the central vein. However,
P. dulce pod pulp extract treatment to diabetic rats revealed the normalization
of the mitochondrial size, regeneration of rough endoplasmic reticulum,
which substantiates its hepatoprotective nature.

**P. dulce pod pulp extract protects renal tissues**

Plate 9 A-D represents the photomicrographs of hematoxylin-eosin
stained renal tissues of control and experimental groups of rats. Plate 9A
shows the section of renal tissue of control rats demonstrating normal
architecture with normal glomeruli and tubules. Plate 9B portrays the section
of renal tissues of diabetic group of rats exhibiting tubular cell necrosis,
tubular lumen dilation, foci of denuded basement membrane, intraluminal
casts, swelling or flattening of proximal tubular cells with brush border loss,
diffuse interstitial edema, interstitial inflammatory cell infiltrates
vacuolization, pyknotic nuclei, medullary congestion, epical blebbing and
decreased cellularity of the glomeruli. Plate 9C demonstrates the section of
renal tissues of diabetic group of rats treated with P. dulce pod pulp extract
exhibiting a normal glomerular, renal tubule and interstitial tissue appearance.
Likewise, the renal tissues of diabetic rats treated with gliclazide shows
similar pattern of renal histological architecture (Plate 9 D).

The ultrastructural changes in the kidney of control and experimental
groups of rats are shown in Plate 10A-D. Plate 10A represents the electron
micrograph of kidney of control group of rats showing the normal glomeruli, renal tubules, podocytes and mesangial cells. The electron micrograph of kidney of diabetic group of rats (Plate 10B) revealed the glomerular filtration barrier i.e. thickened basement membrane, decreased number of podocytes, loss of podocytes foot processes, reduction of mesangial cells around the filtration barrier, capillary with fenestrated endothelial layer, disruption of basal infoldings of proximal and distal convoluted tubules, decreased number of mitochondria and fibrosis of proximal convoluted tubules. The electron micrograph (Plate 10 C) apparently shows the renal tissue protective nature of *P. dulce* pod pulp extract in diabetic group of rats by virtue of a clear appearance of normal architecture. Likewise, the electron micrograph of kidney of diabetic group of rats treated with gliclazide shows similar pattern of renal tissue protection (Plate 10 D). Thus, it is evident that *P. dulce* pod pulp extract protects renal tissues.

The results of the present study indicate that *P. dulce* pod pulp extract possess antioxidant properties. The decline oxidant status and improved antioxidant status indicate the antioxidant potential of the extract which is attributed to the presence of biologically active ingredients of the extract. Also, the increased activity of SOD and catalase is evident from the native gel. The histological and ultra structural studies exemplify the tissue protective role of the extract in ameliorating the hyperglycemia induced oxidative stress in pancreatic, hepatic and renal tissues (Pradeepa et al., 2014).
Effect of *P. dulce* pod pulp extract on lipid metabolism

Diabetic dyslipidaemia is a cluster of plasma lipid and lipoprotein abnormalities that are metabolically interrelated. Dyslipidemia is associated with insulin resistance, visceral obesity and liver fat content. It is a major factor contributing to an increased risk of cardiovascular complications found in diabetic individuals. It is well known that the complications of atherosclerotic macrovascular disease account for the majority of mortalities and a disproportionate amount of morbidity in people with diabetes. Diabetes is associated with profound alterations in the plasma lipid and lipoprotein profile and with an increased risk of premature atherosclerosis, coronary insufficiency and myocardial infarction (Reasner, 2008).

Hyperglycemia ultimately impairs the removal of triglyceride-rich lipoproteins, the accumulation of which highlights hypertriglyceridemia. Hyperlipidaemia is characterized by elevated serum total cholesterol, low-density lipoprotein cholesterol, very low density lipoprotein cholesterol and decreased high-density lipoprotein cholesterol levels (Javed et al., 2009). The major pathogenesis of lipid abnormalities in diabetes is the increased mobilization of fatty acids from adipose tissue and secondary elevation of free fatty acid level in the blood, leading to the production of ketone bodies in the liver. The liver and other tissues participate in the uptake, oxidation and metabolic conversion of free fatty acids, synthesis of cholesterol and phospholipids and secretion of specific classes of plasma lipoproteins. As an
insulin dependent tissue, liver plays a pivotal role in glucose and lipid homeostasis and it is severely affected during diabetes.

The characteristic features of diabetic dyslipidemia are a high plasma triglyceride concentration, low HDL cholesterol concentration and increased concentration of small dense LDL-cholesterol particles (Muacevic-Katanec and Reiner, 2011).

Figure 6 represents the effect of *P. dulce* pod pulp extract on the serum levels of cholesterol, triglycerides, phospholipids and free fatty acids in the experimental groups of rats. The levels were significantly elevated in the diabetic group of rats. Oral treatment of *P. dulce* pod pulp extract restores the altered levels to near normalcy in the diabetic group of rats.

Tables 16 and 17 depict the effects of *P. dulce* pod pulp extract on the levels of cholesterol, triglycerides, phospholipids and free fatty acids in the liver and kidney tissues of experimental groups of rats. The levels were significantly elevated in the diabetic group of rats when compared with control group of rats. However, the oral administration of *P. dulce* pod pulp extract as well as gliclazide to diabetic groups of rats significantly lowered the levels to near normalcy.

Hypercholesterolemia and hypertriglyceridemia are independent key risk factors that can accelerate the development of coronary artery diseases (McKenney, 2001). The cause of hyperlipidemia has been related to increased lipid synthesis, decreased lipid clearance from the blood or a combination of
these two processes. Generally, hepatic and muscle tissues lose their sensitivity to the action of insulin (Porte and Kahn, 2001). High levels of TC and TG are major risk factors for atherosclerosis and coronary heart disease.

In the early stages of the disease, the β-cells of the pancreatic islets compensate for decreased insulin sensitivity by increasing the insulin secretion. As the disease progresses, diabetes ensues when the β-cell is no longer able to compensate for insulin resistance. It has been suggested that raised lipid levels play a key role in the development of T2DM (Boden and Shulman, 2002).

An increase in the levels of total lipids in liver and kidney of STZ-induced diabetic rats may indicate an increased mobilization of lipids from these tissues and storage capacity, which may have caused an increase in serum triglycerides and phospholipids.

The elevated level of serum lipids in diabetic individuals is essentially due to increase in the mobilization of free fatty acids from the peripheral depots, since insulin inhibits the activity of hormone sensitive lipase. On the other hand, glucagon, catecholamines and other hormones enhance the lipolysis. The marked hyperlipemia that characterizes the diabetic state may therefore be regarded as a consequence of the uninhibited actions of lipolytic hormones on the fat depot (Al-Shamaony et al., 1994). Treatment with the *P. dulce* pod pulp extract to diabetic rats resulted in the correction of hyperlipidemia and this may be attributed to the enhanced glucose utilization. Increased lipid accumulation in the diabetic group and its decline levels in the treated groups show a parallelism with the insulin effect on lipid metabolism.
Diabetes is also known to be associated with an increase in the synthesis of cholesterol, which may be due to the increased activity of HMG CoA reductase (Li et al., 2009). Cholesterol is a powerful risk factor for many of the coronary heart diseases. The degree of hypercholesterolemia is directly proportional to the severity of diabetes. An increase in the hepatic cholesterol levels might be due to an increase in the transport of chylomicron cholesterol to the liver (Quarfordt et al., 1994). In the present study, we have observed higher levels of cholesterol in the serum, liver and kidney of diabetic rats. The increased levels of cholesterol in the plasma, liver and kidney are due to the decreased level of HDL-cholesterol. This in turn results in decreased removal of cholesterol from the extrahepatic tissues by the HDL-cholesterol (Loh and Tan, 1996). An increase in HDL-C is associated with a decrease in atherosclerotic and coronary risk (Chait and Brunzell, 1996). The results of the present study indicate that the HDL-Cholesterol level was significantly increased upon treatment with the *pod pulp* extract.

Phospholipids are vital part of biomembrane rich in polyunsaturated fatty acids, which are susceptible substrates for free radicals. The elevated serum phospholipid levels result in elevation of lipoproteins (Jain et al., 2000). Phospholipids, cholesterol and triglycerides are formed as lipoproteins in the liver (Bopanna et al., 1997). The restoration of phospholipids level by *P. dulce* pod pulp extract may be due to the restricted mobilization of plasma triglycerides, regulating the tissue metabolism and improving the level of insulin secretion and action presumably mediate cholesterol and phospholipids.
Fatty acids exhibit a wide array of functions that are necessary for normal physiological health. Saturated fatty acids are nonessential fatty acids and are harmful if ingested excessively in food. They favor excess weight gain, insulin resistance, increased LDL-cholesterol and are atherogenic. On the contrary, polyunsaturated fatty acids have a beneficial effect on cholesterol metabolism and a protective role against cardiovascular diseases (De LaCruz et al., 2000). Polyunsaturated fatty acids have important effects on the structure and physical properties of localized membrane domains. They modulate enzyme activities, carriers and membrane receptors.

The elevated levels of free fatty acids was observed in the serum, liver and kidney tissues of diabetic group of rats in the present study which may be attributed to the increased transport of fatty acids as a result of excessive mobilization of fatty acids. The increased levels of free fatty acids may promote synthesis of phospholipids and cholesteryl esters by the liver and kidney. Oral administration of *P. dulce* pod pulp extract to diabetic group of rats reduces the level of liver and kidney free fatty acids and thereby alleviates the diabetic dyslipidemia. Recent studies indicate that the fatty acid compositions of various tissues are altered in both experimental and human diabetes (Chen et al., 2010; Han et al., 2007).

The effect of *P. dulce* pod pulp extract on the levels of plasma lipoproteins such as HDL, LDL and VLDL in experimental groups of rats were illustrated in Table 18. The levels of LDL and VLDL were elevated significantly with concomitant decline in the levels of HDL in the diabetic group of rats than that of control group of rats. Further, the altered levels of
plasma lipoproteins in the diabetic group of rats were significantly normalized by the oral administration of *P. dulce* pod pulp extract.

Lipid disorders, most often encountered in diabetic patients, include increased levels of VLDL cholesterol, LDL cholesterol and low levels of HDL cholesterol (McKenney 1993). The raised plasma total cholesterol and LDL concentrations are having negative correlation with plasma HDL (Mayne, 1996). These abnormalities appear to result from increased hepatic secretion of VLDL particles due to increased concentration of free fatty acids and glucose, reduced VLDL clearance due to reduced activity of lipoprotein lipase and reduced LDL clearance due to glycation of ligand proteins. LDL-cholesterol may arise from glycosylation of the lysyl residues of apoprotein B as well as from decreasing affinity for the LDL receptor. Thus, *P. dulce* pod pulp extract may normalize lipoproteins through the enhancement of lipoprotein lipase activity and decreased glycation of lipoproteins.

Thus, the alterations in lipids, lipoproteins cholesterol, and fatty acid levels in streptozotocin-induced diabetic rats were restored to near normalcy upon oral administration of *P. dulce* pod pulp extract, which indicates the hypolipidemic activity of the extract.